PROTEIN PURIFICATION
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Published by John Wiley & Sons, Inc., Hoboken, New Jersey
Published simultaneously in Canada

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Library of Congress Cataloging-in-Publication Data:
Protein purification : principles, high resolution methods, and applications / edited by Jan-Christer Janson. — 3rd ed.
p. cm.
Includes index.
QP551.P69754 2011
572.6—dc22
2010033316

Printed in the United States of America
10 9 8 7 6 5 4 3 2 1
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Most will agree that the major achievement in bioscience since 1998, when the second edition of this book was published, is sequencing of the human genome. Rather than diminishing interest in proteins, this has led to a revival in protein exploration and an intensive search for better understanding of molecular processes in health and disease. During this time, industrial exploitation of proteins in healthcare has hardly declined. The application of monoclonal antibodies targeted against rheumatoid arthritis and cancer has been booming, many second- and third-generation biopharmaceuticals have been approved, and modern technologies for vaccine production based on protein engineering and cell culture are being developed on a wide front.

There are approximately 21,000 protein-encoding genes, and the human proteome is much larger than this. Although mapping the genome revealed what was in the box, the jigsaw puzzle is far from complete. Several major research projects exemplify the revitalized interest in proteins. One is the Protein Atlas initiative (www.protematlas.org), aimed at providing a comprehensive database of high resolution microscopic images identifying proteins in normal and cancer tissues. Others involve an ever-widening range of refined tools exploiting protein profiling micro arrays, surface plasmon resonance, mass spectrometry, ELISA, quantitative 2D electrophoresis, and so on. Many technologies are aimed at parallel processing of thousands of targets, and this is profoundly changing the way structural biology projects are managed. Streamlined, miniaturized, automated high throughput (HTP) protocols are becoming the standard, but there is still a fundamental need for protein expression and purification, not least for X-ray structural studies. Many “proteomic” projects exploit high throughput purification of tagged proteins or antibodies.

On the industrial side, particular in healthcare, protein production is rapidly maturing. Platform technologies are being applied both upstream and downstream, allowing faster and leaner implementation as well as better control. Expression of monoclonal antibodies in mammalian cells is at the multi-gram per liter level, with cell densities of more than twenty million per milliliter, specific productivity over 20 picograms per cell per day, in bioreactors with capacities up to 20,000 liters. This several-hundred-fold increase in productivity has changed the pressures on downstream purification, resulting in the development of very high capacity chromatography media for product capture and highly selective media (frequently “multimodal”) for polishing. Downstream purification of biopharmaceuticals uses platform modules for assuring virus safety and for removal of host cell proteins, aggregates and critical contaminants. Regulatory agencies are encouraging greater understanding and control of production processes, a quality by design (QbD) doctrine, and the use of modern risk management techniques and experimental design—all of which is impacting the development of purification methods.

Compared to the second edition of this book, four chapters have been deleted (Chromatofocusing, Affinity Partitioning, Immunoelectrophoresis, and Large-Scale Electrophoresis). Three chapters have been totally rewritten by new authors: Chapter 5 (High Resolution Reversed-Phase Liquid Chromatography of Proteins), Chapter 15 (Electrophoresis in Gels), Chapter 16 (Conventional Isoelectric Focusing in Gel Slabs and Capillaries and Immobilized pH Gradients). Six new chapters have been added: Chapter 10 (Affinity Ligands from Chemical Combinatorial Libraries), Chapter 11 (Affinity Ligands from Biological Combinatorial Libraries), Chapter 12 (Membrane Separations), Chapter 13 (Refolding of Inclusion Body Proteins from E. coli), Chapter 14 (Purification of PEGylated Proteins), and Chapter 20 (High Throughput Screening Techniques in Protein Purification). These new chapters have been written by leading experts in their respective fields. All other chapters have been thoroughly revised and updated regarding recent
applications. A new section on the history of protein chromatography has been added to Chapter 2 (Introduction to Chromatography).

It is my hope that the third edition will receive the same overwhelmingly positive response as the first and second editions, and I would like to express my appreciation to all contributing authors and to Ms Anita Lekhwani and her staff at John Wiley & Sons, Inc., Hoboken, New Jersey, for their patience and never-failing support of this project.

JAN-CHRISTER JANSON
Since 1989, when the first edition of this book was launched, the development of biosciences has meant a revival of protein chemistry in the wake of the molecular biology revolution and the HUGO project. The total genome of baker’s yeast is now sequenced, that of E. coli is not far behind, and within a not too distant future the feat of the total mapping of the human genome, which at the beginning seemed fictitious, is now within reach. This means that the attention of the world’s bioscientific community will again, as in the 1960s and most of the 1970s, focus on the structure and function of the proteins. The PROTEOME era has thus begun, and with it follows the need of more efficient and more selective tools for the separation, isolation, and purification of the gene products, the proteins.

The development of new chromatographic separation media since 1989 has mainly been focused toward improvements demanded primarily by process development engineers in the biopharmaceutical industry. This has resulted in media with higher efficiencies, leading to shorter cycle times, primarily based on suspension polymerized styrene-divinylbenzene polymers with optimized internal pore size distributions, some allowing partial convective flow through the particles. This trend has received its ultimate solution in totally perfusive systems based on stacked membranes, or continuous “monolithic” columns made of cross-linked polymers, derivatized with various kinds of protein adsorptive groups. New composite media have been introduced primarily to increase the industrial applicability of size exclusion chromatography of proteins but also to increase binding capacity in, for example, ion exchange chromatography. The concept of “solid diffusion” in highly ionic group substituted composite media is still awaiting its physicochemical explanation.

The demand for systems allowing direct capture of target proteins directly from whole cultures or cell homogenates, resulting in fewer process steps and concomitantly higher yields, has led to a revival of the fluidized bed concept. However, now optimized with regard to the design of both media and columns by the introduction of the more efficient one cycle technique called expanded bed adsorption.

As long as scientists have been engaged in the isolation and purification of proteins from crude extracts, there has been a demand for media with higher adsorptive selectivities. The extremely high variability in protein surface structure as well as their wide range of functional stabilities, makes it necessary for every protein chemist to have a stock of several different separation media, ion exchangers, hydrophobic interaction media, and a variety of general affinity media. Literature survey data presented in some of the chapters of this book reveal that on average somewhere between three and four steps are required to purify a protein to homogeneity. The hope for one-step purifications raised by the introduction of immobilized monoclonal antibodies has not yet been fulfilled. However, there is a renewed opportunity at hand to increase the selectivity of immobilized ligands in affinity chromatography and thus decrease the number of steps in the purification process. This opportunity has been raised by the recent rapid development in the design of a large variety of chemical and biological combinatorial libraries and high-speed screening technologies. It is easy to predict that over the next few years there will be an unprecedented number of new highly selective ligands, monospecific as well as group specific, introduced for the synthesis of new protein separation media.

Compared to the first edition of this book, there exists one additional chapter (Chapter 18) on large-scale electrophoretic processes. Three chapters (Chapters 15, 16, and 17) have been totally rewritten. Chapters 15 and 16 by new authors. Most other chapters have been thoroughly revised, and all have been updated regarding recent applications.

It is our hope that this new edition will receive the same overwhelmingly positive response as the first edition, and we would like to express our appreciation to Dr. Edmund H. Immergut and the staff of VCH Publishers, now John Wiley & Sons, Inc., for their patience and never-failing support of this project.

Jan-Christen Janson
Lars Rydén
Over the last two decades the scientific community has witnessed an unprecedented expansion within the biosciences and biotechnology. This expansion has been to a large extent driven by advances in several key areas, most notably recombinant DNA technology, hybridoma and cell culture techniques and, finally, in biochemical separation methods. This book is a description of the current status of one of these areas: modern techniques for protein purification and analysis.

The research on which the progress in separation techniques is based has been conducted both in university departments, devoted to basic research, and in industrial laboratories whose main concern is the development of new equipment and tools. In many cases the two communities have cooperated to their mutual benefit. In fact, a great number of the products now available for the separation and purification of proteins, such as chromatographic media with a wide range of selectivities and efficiencies, as well as equipment for electrophoretic separation and analysis, were originally developed in a university setting. This book is also the result of a joint effort between university researchers, in particular at Uppsala University, and the research staff of a company, Pharmacia LKB Biotechnology. Although it is thus a product of this condition of mutual benefit, the ambition has not been to give a selective description of methods or materials from a single commercial source, but rather to give an unbiased account of all key techniques in the field.

Today it is to a great extent possible to base the separation of proteins on knowledge of their molecular properties, structural as well as functional. Suggestions on how to solve a separation problem can best be made if data on protein structure and function, including particular structural details, is available. Conversely, results from the application of a particular separation method can often be interpreted in terms of molecular properties of the protein under study. Throughout the text of this book, separation results are related to protein properties, often in a detailed manner. We are the first generation to be on the verge of rational protein management.

Starting with this general concept, we have aimed at providing students, teachers and research workers in biomedicine, bioscience and biotechnology with a concise and practical treatise covering, in a single volume, all important chromatographic and electrophoretic techniques used in preparative and analytical protein chemistry. The book contains a general introductory chapter on protein preparative work, Chapter 1, where the key concepts are introduced. Similarly, a general introduction to chromatography is given in Chapter 2 and an introduction to analytical electrophoresis in Chapter 12. The major chromatographic and electrophoretic techniques are presented in individual chapters, including one chapter on affinity partitioning in aqueous polymer two-phase systems.

No single person can today be even close to acquiring the amount of experience necessary to describe with confidence the wealth of techniques and methods which makes up the arsenal for protein separations. We have thus chosen to produce a multi-author volume recruiting expertise from the entire field. All chapters have, however, been thoroughly worked through by the editors to achieve a reasonable uniformity of style and organization. Each chapter deals first with the theory and underlying principles of each separation technique, followed by a section on methodology, and ends with a number of representative application examples described in detail.

The preparation of this book has been a matter of several years. We would like to thank the authors for their cooperation, from the first planning stage to the last phase of updating and addition. We would also like to thank our editors at VCH Publishers in New York, in particular Dr. Edmund H. Immergut who took the first initiative and who followed the project up to its realization. The management and staff of Pharmacia LKB Biotechnology are thanked for their cooperation and support which allowed the selling price to be considerably reduced. Many staff members have made invaluable contributions to the final result, which are
gratefully acknowledged. We also thank Elizabeth Hill and Ursula Snow for their contributions in the early phase of the project; Inger Galvér, Gull-Maj Hedén, Inga Johansson and Madeleine de Sharengrad for secretarial work; Bengt Westerlund for handling the computer programmes for the chemical structures; Uno Skatt and Lilian Forsberg for producing a number of the illustrations; and David Eaker and John Brewer for keeping our freedom with the English language within limits. Finally, we would like to add that we are well aware that much of our own efforts, occasional achievements and sometimes hardwon experience, as well as that of several of the other authors of this book, spring from the tree planted long ago by The Svedberg and Arne Tiselius, and later kept alive by Jerker Porath and Stellan Hjertén and many of their colleagues and pupils through fifty years of separation science at Uppsala University. We offer this book as the latest fruit of this tree, hopefully to be enjoyed by many.

Jan-Christen Janson
Lars Rydén
Uppsala, Sweden, June 21, 1989
CONTRIBUTORS

Francisco Batista-Viera, Cátedra de Bioquímica, Dpto. de Biociencias, Facultad de Química Gral. Flores 2124. Casilla de Correo 1157, Montevideo, Uruguay

Herbert Baumann, GE Healthcare Bio-Sciences AB, SE-751 84 Uppsala, Sweden

Makonnen Belew, GE Healthcare Bio-Sciences AB, SE-751 84 Uppsala, Sweden

Eggert Brekkan, GE Healthcare Bio-Sciences AB, SE-751 84 Uppsala, Sweden

Jan Carlsson, Department of Physical & Analytical Chemistry, Uppsala University, Box 579, S-751 23 Uppsala, Sweden

Enrique Carredano, GE Healthcare Bio-Sciences AB, SE-751 84 Uppsala, Sweden

Kjell-Ove Eriksson, GE Healthcare Bio-Sciences AB, SE-751 84 Uppsala, Sweden

Bo Ersson, Medicago AB, Danmark-Berga 13, SE-755 98 Uppsala, Sweden

Elisa Fasoli, Department of Chemistry, Materials and Chemical Engineering, “Giulio Natta,” Politecnico di Milano, Via Mancinelli 7, 20131 Milano, Italy

Conan J. Fee, Biomolecular Interaction Centre and Department of Chemical and Process Engineering, University of Canterbury, Private Bag 4800, Christchurch 8020, New Zealand

Angelika Görg, Department of Proteomics, Technische Universität München, D-85350 Freising-Weihenstephan, Germany

Uwe Gottschalk, Sartorius Stedim Biotech GmbH, August-Spindler-Straße 11, D-37079 Göttingen, Germany

Lars Hagel, GE Healthcare Bio-Sciences AB, SE-751 84 Uppsala, Sweden

Irwin Hirsh, Novo Nordisk AS, Nybrovej 80, 2820 Gentofte, Denmark

Jan-Christer Janson, Department of Physical and Analytical Chemistry, Uppsala University, Box 579, S-751 23 Uppsala, Sweden

Zuwei Jin, GE Healthcare Life Sciences, Building 1, No 1 Huatuo Road, Zhangjiang Hi-Tech Park, Pudong New Area, Shanghai 201203, China

Maik W. Jornitz, Sartorius Stedim North America Inc., 5 Orville Drive, Bohemia, New York 11716, USA

Jan Åke Jönsson, Center for Analysis and Synthesis, Department of Chemistry, Lund University, Box 124, S-22100 Lund, Sweden

Lennart Kågedal, GE Healthcare Bio-Sciences AB, SE-751 82 Uppsala, Sweden

Evert Karlsson, Department of Biochemistry and Organic Chemistry, Uppsala University, Box 576, SE-751 23 Uppsala, Sweden

Karol M. Lacki, GE Healthcare Bio-Sciences AB, SE-751 84 Uppsala, Sweden

Zheng Liu, Department of Chemical Engineering, Tsinghua University, Beijing 100084, China

Diannan Lu, Department of Chemical Engineering, Tsinghua University, Beijing 100084, China

Per-Åke Nygren, Division of Molecular Biotechnology, School of Biotechnology, Royal Institute of Technology (KTH), SE-106 91 Stockholm, Sweden

Sylvia Winkel Pettersson, Eka Chemicals AB/Akzo Nobel, Bohus, Sweden

Pier Giorgio Righetti, Department of Chemistry, Materials and Chemical Engineering, “Giulio Natta,” Politecnico di Milano, Via Mancinelli 7, 20131 Milano, Italy
Sabina Carla Righetti, Department of Chemistry, Materials and Chemical Engineering, “Giulio Natta,” Politecnico di Milano, Via Mancinelli 7, 20131 Milano, Italy

Lars Rydén, Centre for Sustainable Development (CSD) Uppsala, Uppsala University, Villavägen 16, SE-752 36 Uppsala, Sweden

Zhiguo Su, State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100080, China

Wolfgang Thormann, Department of Clinical Pharmacology, University of Bern, Murtenstraße 35, CH-3010 Bern, Switzerland

James M. Van Alstine, GE Healthcare Bio-Sciences AB, 751 84 Uppsala, Sweden

Joachim K. Walter, InnoBiologics Sdn Bhd, Lot 1, Persiaran Negeri BBN, 71800 Nilai, Malaysia

Reiner Westermeier, SERVA Electrophoresis GmbH, Carl-Benz-Strasse 7, D-69115 Heidelberg, Germany
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Figure 20.4  Schematic of the workflow of a batch uptake experiment occurring in the wells of a microtiter plate, showing the same steps as in a column experiment: equilibration, sample addition, wash, and elution. Work from GE Healthcare Life Sciences, reproduced with permission.

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Figure 20.9  Binding capacity maps showing effect of pH and ionic strength on adsorption of amyloglucosidase on Capto™ DEAE measured in PreDictor™ plates filled with 2 μL resin per well after (a) 2 min; (b) 60 min; and (c) 20 h of incubation. Work from GE Healthcare Life Sciences, reproduced with permission.

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Figure 20.12  Apparent adsorption isotherm generated from data presented in Figure 20.11. Work from GE Healthcare Life Sciences, reproduced with permission.
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Figure 20.16 Example of results from a screening of a multimodal anion exchange library: capacity of carbonic anhydrase (arbitrary units) on different ligands coupled to Sepharose™ FF base matrix. Screening performed using PreDictor™ microtiter plates. Work from GE Healthcare Life Sciences, reproduced with permission.
PART I

INTRODUCTION
INTRODUCTION TO PROTEIN PURIFICATION

BO ERSSON
Medicago AB, Danmark-Berga 13, SE-755 98 Uppsala, Sweden

LARS RYDEN
Centre for Sustainable Development (CSD) Uppsala, Uppsala University, Villavägen 16, SE-752 36 Uppsala, Sweden

JAN-CHRISTER JANSON
Department of Physical and Analytical Chemistry, Uppsala University, Box 579, S-751 23 Uppsala, Sweden

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1.1 INTRODUCTION

The development of techniques and methods for the separation and purification of biological macromolecules such as proteins has been an important prerequisite for many of the advancements made in bioscience and biotechnology over the past five decades. Improvements in materials, utilization of computerized instruments, and an increased use of in vivo tagging have made protein separations more predictable and controllable, although many still consider purification of non-tagged proteins more an art than a science. However, gone are the days when an investigator had to spend months in search of an efficient route to purify an enzyme or hormone from a cell extract. This is a consequence of the development of new generations of chromatographic media with increased efficiency and selectivity as well as of new automated chromatographic systems supplied with sophisticated interactive software packages and data bases. New electrophoresis techniques and systems for fast analysis of protein composition and purity have also contributed to increasing the efficiency of the evaluation phase of the purification process.

In the field of chromatography, the development of new porous resin supports, new crosslinked beaded agaroses, and new bonded porous silicas has enabled rapid growth in high resolution techniques (high performance liquid chromatography, HPLC; fast protein liquid chromatography, FPLC), both on an analytical and laboratory preparative scale as well as for industrial chromatography in columns with bed volumes of several hundred liters. Expanded bed adsorption enables rapid isolation of target proteins, directly from whole cell cultures or cell homogenates. Another field of increasing importance is micropreparative chromatography, a consequence of modern methods for amino acid and sequence analysis requiring submicrogram samples. The data obtained are efficiently exploited by recombinant DNA technology, and biological activities previously not amenable to proper biochemical study can now be ascribed to identifiable proteins and peptides.

A wide variety of chromatographic column packing materials such as gel-filtration media, ion exchangers, reversed phase packings, hydrophobic interaction adsorbents, and affinity chromatography adsorbents are today commercially available. These are identified as large diameter media (90–100 μm), medium diameter media (30–50 μm) and small diameter media (5–10 μm) in order to satisfy the different requirements of efficiency, capacity, and cost.

However, not all problems in protein purification are solved by the acquisition of sophisticated laboratory equipment and column packings that give high selectivity and efficiency. Difficulties still remain in finding optimum conditions for protein extraction and sample pretreatment, as well as in choosing suitable methods for monitoring protein concentration and biological activity. These problems will be discussed in this introductory chapter. There will also be an overview of different protein separation techniques and their principles of operation. In subsequent chapters, each individual technique will be discussed in more detail.

Finally, some basic equipment necessary for efficient protein purification work will be described in this chapter.

Several useful books covering protein separation and purification from different points of view are available on the market or in libraries (1–3). In “Methods of Enzymology,” for example, in older volumes 22, 34, 104, and 182 (4–7), but particularly in the most recent volume, 463 (8), a number of very useful reviews and detailed application reports will be found. The booklets available from manufacturers regarding their separation equipment and media can also be helpful by providing detailed information regarding their products.

1.2 THE PROTEIN EXTRACT

1.2.1 Choice of Raw Material

In most cases, interest is focused on one particular biological activity, such as that of an enzyme, and the origin of this activity is often of little importance. Great care should therefore be taken in the selection of a suitable source. Among different sources there might be considerable variation with respect to the concentration of the enzyme, the availability and cost of the raw material, the stability of the enzyme, the presence of interfering activities and proteins, and difficulties in handling a particular raw material. Very often it is compelling to choose a particular source because it has been described previously in the literature. However, sometimes it is advantageous to consider an alternative choice.

Traditional animal or microbial sources have today, to a large degree, been replaced by genetically engineered microorganisms or cultured eukaryotic cells. Protein products of eukaryotic origin, cloned and expressed in bacteria such as *Escherichia coli*, may either be located in the cytoplasm or secreted through the cell membrane. In the latter case they are either collected inside the periplasmic space or they are truly extracellular, secreted to the culture medium. Proteins that accumulate inside the periplasmic space may be selectively released either into the growth medium by changing the growth conditions (9), or following cell harvesting and washing of the resuspended cell paste. At this stage, a considerable degree of purification has already been achieved by choosing a secreting strain as illustrated in Figure 1.1. In connection with the cloning, the recombinant protein may be equipped with an “affinity handle” such as a His-tag or a fusion protein such as Protein A, glutathione-S-transferase, or maltose binding protein in order to facilitate purification. The handle is often designed such that it can be cleaved off using highly specific proteolytic enzymes. Proteins of eukaryotic origin, and some virus surface proteins are often
glycosylated why eukaryotic host cells have to be chosen for their production.

1.2.2 Extraction Methods

Some biological materials themselves constitute a clear or nearly clear protein solution suitable for direct application to chromatography columns after centrifugation or filtration. Examples include blood serum, urine, milk, snake venoms, and—perhaps most importantly—the extracellular medium after cultivation of microorganisms and mammalian cells, as mentioned above. It is normally an advantage to choose such a starting material because of the limited number of components and also because extracellular proteins are comparatively stable. Some samples, such as urine or cell culture supernatants, are normally concentrated before purification begins.

In most cases, however, it is necessary to extract the activity from a tissue or a cell paste. This means that a considerable number of contaminating molecular species are set free, and proteolytic activity will make the preparation work more difficult. The extraction of a particular protein from a solid source often involves a compromise between recovery and purity. Optimization of extraction conditions should favor the release of the desired protein and leave difficult-to-remove contaminants behind. Of particular concern is to find conditions under which the already extracted protein is not degraded or denatured while more is being released.

Various methods are available for the homogenization of cells or tissues. For further details and discussions the reader is referred to the paper by Kula and Schütte (10). The extraction conditions are optimized by systematic variation of parameters such as the composition of the extraction medium (see below), time, temperature, and type of equipment used.

The proper design of an extraction method thus requires preliminary experiments in which aliquots are taken at various time intervals and analyzed for activity and protein content. The number of parameters can be very large, so this part of the work has to be kept within limits by applying proper judgment. However, it is not recommended to accept a single successful experiment. Further investigations of the required extraction time, in particular, often pays in the long run. The number of optimization experiments can be reduced considerably by using chemometrics (multivariate analysis), for which there are computer programs available (www.chemometrics.com/software).

The major problems confronted when preparing a protein are in general denaturation, proteolysis, and contamination with pyrogens, nucleic acids, bacteria, and viruses. These can be limited by appropriate choice of the extraction medium, as we shall show. However, we can already state that many of the above problems can be reduced by short preparation times and low temperatures. It is therefore good biochemical practice to carry out the first preparation steps as fast as possible and at the lowest possible temperature. However, low temperatures are not always necessary and are sometimes inconvenient. The working temperature is therefore one of the parameters that should be optimized carefully, especially if a preparation is to be done routinely in the laboratory or if it is going to be scaled up to pilot or production scale.

The extract must be clarified by centrifugation and/or filtration before submission to column chromatography. A preparative laboratory centrifuge is normally sufficient for this step.

A common phenomenon when working with intracellularly expressed recombinant proteins is their tendency to accumulate as insoluble aggregates known as inclusion bodies, which have to be solubilized and refolded to recover their native state. At first glance, the formation of insoluble aggregates in the cytoplasm might be considered a major problem. However, as the inclusion bodies seem to be fairly well defined with regard to both particle size and density (11), they should provide a unique means for rapid and efficient enrichment of the desired protein simply by low speed fractional centrifugation and washing of the resuspended sediment. The critical step is solubilization and refolding, often combined with chromatographic purification under denaturing conditions in the presence of high concentrations of urea or guanidine hydrochloride. This area is treated in more detail in Chapter 13 and has recently been reviewed by Burgess (12).

1.2.3 Extraction Medium

To arrive at a suitable composition for the extraction medium it is necessary initially to study the conditions at which the
INTRODUCTION TO PROTEIN PURIFICATION

protein of interest is stable and secondly, where it is most efficiently released from the cells or tissue. The final choice is usually a compromise between maximum recovery and maximum purity. The following factors have to be taken into consideration: pH, buffer salts, detergents/chaotropic agents, reducing agents, chelators or metal ions, proteolytic inhibitors, and bacteriostatics.

1.2.3.1 pH Normally, the pH value is chosen such that the activity of the protein is at a maximum. However, it should be noted that this is not always the pH that gives the most efficient extraction, nor is it necessarily the pH of maximum stability. For example, trypsin has an activity optimum at pH 8–9, but is much more stable at pH 3, where autolysis is avoided. The use of extreme pH values, for example, for the extraction of yeast enzymes in 0.5 M ammonia, is sometimes very efficient and is acceptable for some proteins without causing too much denaturation.

1.2.3.2 Buffer Salts Most proteins are maximally soluble at moderate ionic strengths, 0.05–0.1, and these values are chosen if the buffer capacity is sufficient. Suitable buffer salts are given in Table 1.1.

An acceptable buffer capacity is obtained within one pH unit from the pK_a values given. The proteins as such also act as buffers, and the pH should be checked after addition of large amounts of proteins to a weakly buffered solution. Some extractions do not give rise to acids and bases and thus do not need a high buffer capacity. In other cases this might be necessary, and occasional control of the pH value of an extract is recommended.

1.2.3.3 Detergents and Chaotropic Agents In many extractions the desired protein is bound to membranes or particles, or is aggregated due to its hydrophobic character. In these cases one should reduce the hydrophobic interactions by using either detergents or chaotropic agents (not both!). Some of the commonly used detergents are listed in Table 1.2. Several of them do not denature globular proteins or interfere with their biological activity. Others, such as sodium dodecyl sulfate (SDS), will do that. Quite often it is not necessary to continue using a detergent in the buffer after the first step(s) in the purification, so its use is restricted to the extraction medium. In other cases it might be necessary to use a detergent throughout the whole preparation process, leading to the final purification of a protein–detergent complex. More information about detergents, including their chemical structures, can be found in Reference 13.

Detergents are amphipathic molecules. When their concentration increases they will eventually aggregate; that is, they will form micelles at the so-called critical micelle concentration (CMC). Because micelles often complicate purification procedures, in particular column chromatography, detergent concentrations below the CMC should be used.

Instead of using detergents to dissolve aggregates, chaotropic agents such as urea or guanidine hydrochloride, or moderately hydrophobic organic compounds such as ethylene glycol, can be tried. Urea and guanidine hydrochloride have proven particularly useful for the extraction and solubilization of inclusion bodies (12).

1.2.3.4 Reducing Agents The redox potential of the cytosol is lower than that of the surrounding medium where atmospheric oxygen is present. Intracellular proteins often have exposed thiol groups and these might become oxidized in the purification process. Thiol groups can be protected by reducing agents such as 1,4-dithioerythritol (DTE), dithiothreitol (DTT) or mercaptoethanol (Table 1.3). Normally, 10–25 mM concentrations are sufficient to protect thiols without reducing internal disulfides. In other cases a higher concentration might be needed (14). Ascorbic acid is sometimes added to polyphenol containing crude plant extracts in order to avoid oxidation and miscoloration.

1.2.3.5 Chelators or Metal Ions The presence of heavy metal ions can be detrimental to a biologically active protein,
for two main reasons. They can enhance the oxidation of thiols by molecular oxygen and can form complexes with specific groups, which may cause problems. Heavy metals can be trapped by chelating agents. The most commonly used is ethylenediamine tetraacetic acid (EDTA) in the concentration range 10–25 mM. An alternative is ethylene glycol tetraacetic acid (EGTA), which is more specific for calcium. It should be noted that EDTA is a buffer. It is therefore best to add EDTA before final pH adjustment. The chelating capacity of EDTA increases with increasing pH.

In other cases, stabilizing metal ions are needed. Many proteins are stabilized by calcium ions. However, the divalent ions calcium and magnesium are trapped by EDTA and cannot be used in combination with this chelator.

1.2.3.6 Proteolytic Inhibitors The most serious threats to protein stability are the omnipresent proteases. The simplest safeguard against proteolytic degradation is normally to work quickly at low temperatures. An alternative, or added, precaution is to make use of protease inhibitors (Table 1.4), especially in connection with the extraction step. Often there is a need for a combination of inhibitors, for example, for both serine proteases and metalloproteases. In general, protein inhibitors are expensive, which may limit their use in large-scale applications. Proteolysis can also be reduced by rapid extraction of the fresh homogenate in an aqueous polymer two-phase system (15) or by adsorption of the proteases to hydrophobic interaction adsorbents (16). Sometimes it is sufficient to adjust the pH to a value at which the proteases are inactive, but where the stability of the protein to be purified is maintained. A classical example is the purification of insulin from the pancreas.

1.2.3.7 Bacteriostatics It is wise to take precautions to avoid bacterial growth in protein solutions. The simplest remedy here is to use sterile filtered buffer solutions as routine in the laboratory. This will also reduce the risk of bacterial growth in columns. A common practice for avoiding bacterial growth in chromatographic columns is to allow the column to flow at a reduced rate, even when it is not in operation. Some buffers are more likely than others to support bacterial growth, such as phosphate, acetate, and carbonate buffers at neutral pH values. Buffers at pH 3 and below or at pH 9 and above usually prevent bacterial growth, but may occasionally allow growth of molds.

Whenever possible it is recommended to add an antimicrobial agent to the buffer solutions. Often used are sodium azide at 0.001 M or merthiolate at 0.005%, or alcohols such as n-butanol at 1%. Sodium azide has the drawback that it is a nucleophilic substance and binds metals. In cases where these substances may interfere with activity measurements or the chromatography itself, it is always possible to add the substances to solutions of the protein to be stored.

1.3 AN OVERVIEW OF FRACTIONATION TECHNIQUES

In early work, complex protein mixtures were fractionated mainly by adsorption and precipitation methods. These methods are still used today as preliminary steps for initial fractionation or for concentration of sample solutions. Preparative electrophoretic and chromatographic techniques developed during the 1950s and 1960s made possible rational purification protocols and laid the foundation for the situation we have today. The following sections give a short overview of the various techniques normally used in preparative biochemical work. Chapter 2 contains an introduction to chromatography, including a historical review, and Chapter 15 gives an introduction to electrophoresis. Each individual chromatographic and electrophoretic separation technique is then treated in detail in subsequent chapters.

<table>
<thead>
<tr>
<th>Table 1.3 Reducing Agents</th>
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<tbody>
<tr>
<td>Agent</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
</tr>
<tr>
<td>1,4-Dithioerythitol (DTE)</td>
</tr>
<tr>
<td>1,4-Dithiothreitol (DTT)</td>
</tr>
<tr>
<td>Ascorbic acid</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 1.4 Proteolytic Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor</td>
</tr>
<tr>
<td>Diisopropyl fluorophosphate (DFP)</td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride (PMSF)</td>
</tr>
<tr>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>Cysteine reagents</td>
</tr>
<tr>
<td>Pepstatin A</td>
</tr>
<tr>
<td>Leupeptin</td>
</tr>
</tbody>
</table>
1.3.1 Precipitation

Precipitation of a protein in an extract may be achieved by adding salts, organic solvents, or organic polymers, or by varying the pH or temperature of the solution. The most commonly used precipitation agents are listed in Table 1.5. The strength of a particular ion as a precipitation agent is shown by its position in the so-called Hofmeister series:

Anions: $\text{PO}_4^{3-}, \text{SO}_4^{2-}, \text{CH}_3\text{COO}^-, \text{Cl}^-, \text{Br}^-, \text{NO}_3^-, \text{ClO}_3^-$

Cations: $\text{NH}_4^+, \text{K}^+, \text{Na}^+, \text{guanidine} \text{C(NH}_2)_3^+$

The so-called antichaotropic ions to the left are the most efficient salting out agents. They are efficient water molecule binders, thus increasing the hydrophobic effect in the solution and promoting protein aggregation by facilitating the association of hydrophobic surfaces. The chaotropic salts on the right-hand side in the series decrease the hydrophobic effect, and thus help maintain the proteins in solution.

Polar organic solvents such as ethanol promote the precipitation of proteins due to the decrease in water activity in the solution as the water is replaced by organic solvent. They have been widely used as precipitation agents, especially in the fractionation of serum proteins. The following five variables are usually kept under control: concentration of organic solvent, protein concentration, pH, ionic strength, and temperature (17). Low temperature during the precipitation operations is often necessary to avoid protein denaturation; the addition of an organic solvent decreases the freezing point of the solution and temperatures below 0°C can be used. In reversed phase chromatography, some proteins can be chromatographed in solutions that contain up to ~50% organic solvent, with retention of their biological activity.

Organic polymers function in a way similar to that of organic solvents. The most widely used polymer is polyethylene glycol (PEG), with an average molecular weight of either 6000 or 20,000. The main advantage of PEG over organic solvents is that it is more easily handled. It is unflammmable, not poisonous, uncharged, and relatively inexpensive. Rather low concentrations are required (often less than 25%) to precipitate most proteins. One disadvantage is that high concentration solutions of PEG are viscous. PEG can also be difficult to remove from protein solutions. However, after dilution with buffer the viscosity decreases, and because the substance is uncharged, the solution may be applied directly to an ion exchange column to further separate the proteins, simultaneously removing the polymer.

pH adjustment has been used as a simple and cheap way to precipitate proteins. Proteins have their lowest solubility at their isoelectric point. This is sometimes used in serum fractionation and also in the purification of insulin.

Besides pH, another parameter that influences precipitation of proteins in salt solutions is temperature (see below). Keeping the salt concentration constant and varying the temperature is another way of fractionating a protein solution.

The salting out of a protein can be described by the equation

$$\log S = B - Kc$$

where $S$ is the solubility of the protein in g/L of solution, $B$ is an intercept constant, $K$ is the salting out constant, and $c$ is salt concentration in mol/L.

The value of $B$ depends on the salt used, the pH, the temperature, and the protein itself; $K$ depends on the salt used and the protein. It should be stressed that addition of a salt or another precipitating agent to a protein solution only decreases the solubility of the proteins. This is why a very dilute protein solution for precipitation may lead to low recovery, because a major part of the protein simply remains in solution. Reproducible results can only be achieved if all the parameters mentioned above, including the protein concentration, are kept constant.

Centrifugation is used routinely in the protein purification laboratory to recover precipitates. It can also be used to separate two immiscible liquid phases. Another application is density gradient centrifugation. Today this is used predominantly for the fractionation of subcellular particles and nucleic acids. An alternative is the use of liquid–liquid phase extraction, which seems to offer several advantages over the more classical methods.

### Table 1.5 Precipitation Agents

<table>
<thead>
<tr>
<th>Agent</th>
<th>Type</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulfate</td>
<td>Salt</td>
<td>Easily soluble, stabilizing</td>
</tr>
<tr>
<td>Sodium sulfate</td>
<td>Salt</td>
<td>Flammable, risk of denaturation</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Solvent</td>
<td>Flammable, risk of denaturation</td>
</tr>
<tr>
<td>Acetone</td>
<td>Solvent</td>
<td>Flammable, risk of denaturation</td>
</tr>
<tr>
<td>Polyethylene glycol (PEG)</td>
<td>Polymer</td>
<td>Uncharged, unflammmable</td>
</tr>
</tbody>
</table>

1.3.2 Electrophoresis

Electrophoresis in free solution or in macroporous gels such as 1–2% agarose separates proteins mainly according to their net electric charge. Electrophoresis in gels such as polyacrylamide separates mainly according to the molecular size of the proteins.

Today, analytical gel electrophoresis requiring microgram amounts of proteins is an important tool in bioscience and biotechnology (see Chapter 15). Convenient methods for the extraction of proteins after electrophoresis have been developed, in particular protein blotting (see Chapter 18), making the technique micropreparative. There are also many instances where a very small amount of protein is
sufficient for the analysis of size and composition as well as the primary structure. Finally, there are cases where the starting material is extremely limited, such as protein extracts from small amounts of tissue (biopsies, etc.). In these cases, the protein “extract” might be just large enough for gel electrophoretic analysis.

Larger scale (milligrams to grams of protein) electrophoresis was an important method for the fractionation of protein extracts during the 1950s and early 1960s. It was carried out using columns packed with, for example, cellulose powder as a convection depressor, as in the “Porath column” (18). An innate limitation of preparative column electrophoresis is the joule heat developed during the course of the experiment. This means that the column diameter, if it is to allow sufficient cooling, should not exceed ~3 cm. Several hundred milligrams of protein can, however, be separated on such columns. Column zone electrophoresis has the advantage of allowing a precise description of the separation parameters involved and is, besides gel filtration, the mildest separation technique available for proteins. It can be recommended for special situations, but practical aspects and the excessive time required precludes its routine use. Methods for large and medium scale preparative electrophoresis have been developed, such as the flowing curtain electrophoresis of Hannig (19) and, more recently, the “Biostream” apparatus of Thomson (20).

Isoelectric focusing, the other main electrophoretic technique, separates proteins according to their isoelectric points (see Chapter 16). This technique gives very high resolution, but presents major difficulties as a preparative large or medium scale technique. Special equipment is required to allow cooling during the focusing. Proteins often precipitate at their isoelectric point, and this precipitate can contaminate the focusing fractionates proteins largely according to their isoelectric points and would therefore appear to be a more convenient alternative to preparative isoelectric focusing.

1.3.3 Chromatography

Separation by chromatography depends on the differential partition of proteins between a stationary phase (the chromatographic medium or the adsorbent) and a mobile phase (the buffer solution). Normally, the stationary phase is packed into a vertical column of plastic, glass, or stainless steel, and the buffer is pumped through this column. An alternative is to stir the protein solution with the adsorbent, batchwise, and then pour the slurry onto an appropriate filter and make the washings and desorptions on the filter.

Column chromatography has proved to be an extremely efficient technique for the separation of proteins in biological extracts. Since the development of the first cellulose ion exchangers by Peterson and Sober (23) and of the first practical gel filtration media by Porath and Flodin (24, 25) a wide variety of adsorbents have been introduced that exploit various properties of the protein for the fractionation. The more important of these properties, together with the chromatographic method for which they dominate the separation, are as follows:

1. Size and shape (gel filtration/size exclusion chromatography, SEC).
2. Net charge and distribution of charged groups (ion exchange chromatography, IEC).
3. Isoelectric point (chromatofocusing, CF).
4. Hydrophobicity (hydrophobic interaction chromatography, HIC; reversed phase chromatography, RPC).
5. Metal binding (immobilized metal ion affinity chromatography, IMAC).
6. Content of exposed thiol groups (covalent chromatography, CC).
7. Biospecific affinities for ligands, inhibitors, receptors, antibodies, and so on (affinity chromatography, AC).

The methods often have very different requirements with regard to chromatographic conditions, including ionic strength, pH, and various additives such as detergents, reducing agents, and metals. By appropriate adjustment of the buffer composition, the conditions for adsorption and desorption of the desired protein can be optimized. It should be stressed that the result of a particular chromatographic separation often depends on more than one parameter. In IEC, the charge interaction is the dominant parameter, but molecular weight and hydrophobic effects can also contribute to some degree, depending on the experimental conditions and type of solid phase used. In recent years the concept of multimodal, or mixed-mode adsorption chromatography, has received an increasing amount of attention, with several new products emerging on the market (see Chapter 4 for more detailed information).

Highly specific methods, such as those based on bioaffinity (e.g., antibody–antigen interaction) or those based on the use of in vivo fused tags such as (His)_6 or glutathione-S-transferase (GST), do in some cases give a highly pure
protein in a single step. Normally, however, several chromatographic methods have to be combined in order to achieve maximal purification of a protein from a crude biological extract. With the wide variety of chromatographic media available today, in combination with a modern computerized chromatography system, adequate purification can normally be achieved within a few days to a couple of weeks.

In recent years, columns containing a continuous, homogeneously porous solid phase have become available. See Chapters 2 and 9 for more information about these so-called monolithic column types. As in membrane adsorption techniques, the main advantage is the considerably reduced diffusion restriction, allowing high efficiency and also higher flow rates. The main disadvantage of both these techniques is the concomitant smaller surface area per unit adsorption medium volume, which will restrict the nominal column binding capacity.

All of the chromatographic methods mentioned above are treated in Part II of this book, which begins with a general description of the concepts used in protein chromatography.

### 1.3.4 Expanded Bed Adsorption

The problem of removing cells and cell debris from large volumes of whole cell cultures or cell homogenates has encouraged the development of technologies for the direct adsorption of target molecules from such feed stocks. In a fluidized bed, the adsorbent particles are subject to an upward flow of liquid that keeps them separated from each other. The resulting increased voidage allows the unhindered passage of cells and cell debris. In a typical fluidized bed there is a total mixing of particles and sample in the reactor, leading to incomplete adsorption of the target molecules unless the feed stock is recycled. Expanded bed adsorption is a special case of fluidized bed adsorption (26), and is primarily applied in a pilot- or production-scale environment (26–29).

### 1.3.5 Membrane Adsorption

The main argument for utilizing modified membranes as media for protein adsorption is to solve the problem of mass transport restriction in standard chromatography due to the slow diffusion of proteins in the pores of the large gel particles. In membranes, most pores allow convective flow, and the mass transport resistance is therefore minimized to film diffusion at the membrane matrix surface. The result is a more efficient adsorption–desorption cycle of target solutes, allowing considerably higher flow rates and thus considerably shorter separation times. The area has been reviewed by Thömmes and Kula (30). See Chapter 12 for more data regarding membrane separation.

### 1.4 FRACTIONATION STRATEGIES

#### 1.4.1 Introductory Comments

Before attempting to design a purification protocol for a particular protein, as much information as possible should be collected about the characteristics of that protein and preferably also about the properties of the most important known impurities. Useful data include approximate molecular weight and pI, degree of hydrophobicity, presence of carbohydrate (glycoprotein) or free –SH. Some of this information might be obtained already on a DNA level, if nucleotide sequence data are available, but is otherwise often collected easily by preliminary trials using crude extracts.

Criteria with regard to the stability of the protein to be purified should be established. Important parameters affecting structure are temperature, pH, organic solvents, oxygen (air), heavy metals, and mechanical shear. Special concern should be addressed to the risk of proteolytic degradation. Finally, it is the amount of protein to be purified per batch, and the required degree of purity, that to a high degree governs the techniques and methods used in the purification process.

According to a study of 100 published successful protein purification procedures (31), the average number of steps in a purification process is four. Very seldom can a protein be obtained in pure form from a single chromatographic procedure, even when this is based on a unique biospecificity. In addition to the purification steps there is often a need for concentrations and sometimes changes of buffers by dialysis or membrane ultrafiltration.

The preparation scheme can be described as consisting of three stages:

1. The preliminary or initial fractionation stage (often called the capturing stage).
2. The intermediate purification stage.
3. The final polishing stage.

The purpose of the initial stage is to obtain a stable, particle-free solution suitable for chromatography. This is achieved by clarification, coarse fractionation, and concentration of the protein extract. The purpose of the final stage is to remove aggregates and degradation products and to prepare the protein solution for the final formulation of the purified protein.

Sometimes one or two of these stages coincide. An initial ion exchange adsorption step can thus serve as a preliminary fractionation applied directly to the protein extract, or a gel filtration can give a product that is suitable as a final product. However, as the purposes of the three stages are different it is useful to discuss them separately.

The design of the preparation scheme will differ depending on the material at hand and the purpose of the purification.
If the starting material is very precious, one should favor high yield over speed and convenience. In cases where several different proteins are to be extracted from a single starting material, this of course also affects the planning of the work. Finally, the final step is designed so that the product will be suitable for its purpose, which can vary. These aspects will be discussed below.

### 1.4.2 Initial Fractionation

There are many methods for the clarification of protein solutions. Extracts of fungal or plant origin often contain phenolic substances or other pigments. These can be removed by adsorption to diatomite (diatomaceous earth, Celite), either batchwise or on a short column. In order to prevent oxidation and miscoloration, small amounts of ascorbic acid can be added to the crude plant extract.

Similarly, lipid material can be removed either by centrifugation, as the lipids will float, and one thus needs to extract the protein solution from below, or by a chromatographic procedure. Lipids adsorb to a number of materials. Aerosil, a fused silica, has been used for the adsorption of lipids, but agarose is sometimes a simple choice.

Contamination with nucleic acids can, in some cases, especially when preparing proteins from bacteria, constitute a problem due to their high viscosity. The classical way to solve this problem is to precipitate the nucleic acids. Streptomycin sulfate and polyethylenimine have been used as precipitants, as have protamine sulfate and manganese salts (32). Another way to solve the problem is to add nuclease, which cut the nucleic acids into smaller pieces, thereby reducing the viscosity. Another problem with nucleic acids or degradation products of nucleic acids is that, due to their low isoelectric points, they still are negatively charged at low pH. Anion exchangers strongly adsorb nucleic acids and are thus difficult to regenerate. The solution to this problem can in some cases be to perform two consecutive adsorption steps. The first is executed at a pH below the pI of the target protein, thus preventing it from binding to the ion exchanger. Often, a fairly small amount of the ion exchanger is required in this step, which is why it is economically motivated to discard the contaminated gel. In the second step using the same anion exchanger, the pH is increased to a value above the pI of the target protein, resulting in binding and subsequent elution using either a stepwise or continuous salt gradient.

#### 1.4.2.1 Clarification by Centrifugation and/or Microfiltration

The clarification of any cell homogenate is usually no problem on a laboratory scale, where refrigerated high-speed centrifuges operating at speeds from 20,000 rpm to 75,000 rpm, generating from ~40,000g to ~500,000g can be used. A useful review of centrifugation and centrifuges in preparative biochemistry is found in Reference 33. As a complement to centrifugation, in recent years, tangential or cross-flow microfiltration has received increased attention, especially for large-scale applications. For a review of the advantages of cross-flow microfiltration we suggest Reference 34. The area is also treated in more detail in Chapter 12.

#### 1.4.2.2 Ultrafiltration

Ultrafiltration has become a widely used technique in preparative biochemistry. Ultrafiltration membranes are available with different cut-off limits for separation of molecules from 1 kDa up to 300 kDa. The method is excellent for the separation of salts and other small molecules from a protein fraction with higher molecular weight and at the same time can effect a concentration of the proteins. The process is gentle, fast, and inexpensive. Ultrafiltration is treated in more detail in Chapter 12.

#### 1.4.2.3 Precipitation

Crude extracts are seldom suitable for direct application to chromatographic columns. Preparative differential centrifugation seldom results in a sufficiently clear solution. This is one reason why it is often necessary to use other means for clarification that simultaneously concentrate the solution and remove most of the bulk proteins. Such an initial fractionation step should also result in the removal of proteases and membrane fragments that sometimes bind the protein of interest in the absence of detergents. The classical means is to make a fractional precipitation. Bulk proteins in the solution are first precipitated together with residual particulate matter, and then the protein of interest can be precipitated from the resulting supernatant solution. Sometimes the protein of interest is allowed to remain in the mother liquor solution for direct application to chromatographic columns, for example, hydrophobic interaction adsorption of proteins in ammonium sulfate solutions and IEC of proteins in PEG mother liquors. The most commonly used precipitating agents are listed in Table 1.5, together with some of their properties. A typical precipitation curve is shown in Figure 1.2.

![Example of a precipitation curve, showing the amount of protein precipitated with a stepwise increase in ammonium sulfate concentration.](image-url)
Of the various methods available for protein precipitation, the classical ammonium sulfate has some disadvantages. The resulting protein solution often needs to be dialyzed to obtain an ionic strength that allows IEC. This problem is avoided when using PEG. Organic solvents, in particular ethanol and acetone, often produce extremely fine powder-like precipitates that are difficult to centrifuge and handle. They have also often been shown to cause partial denaturation of proteins, which can, for example, prevent subsequent crystallization. This is why organic solvents are not recommended as first-choice precipitating agents.

1.4.2.4 Liquid–Liquid Phase Extraction A radically different way of making an initial fractionation is by partitioning in an aqueous polymer liquid–liquid two-phase system (35). These systems often contain PEG as one phase constituent and another polymer, such as dextran or even salt, as the other. Under favorable conditions it is possible to obtain the protein of interest in the upper, normally the PEG phase. The contaminating bulk protein, as well as particles, will be collected in the lower phase and can be removed by centrifugation. Particles sometimes stay at the interphase and are thus also removed in the centrifugation step. Aqueous polymer two-phase systems have been shown to be effective tools for plasma membrane proteomics (36). By covalent attachment of affinity ligands to PEG molecules these can be used for affinity partitioning.

1.4.3 The Chromatographic Steps

1.4.3.1 Choice of Adsorbent Preliminary separation conditions for known proteins are easily extracted using data bases available over the Internet. For unknown (e.g., nonrecombinant) proteins, information regarding their chromatographic behavior can only be obtained by preliminary analytical-scale experiments, for example by gel filtration and by IEC using salt and pH gradients. Using these techniques, approximate values of molecular size and ionic properties such as isoelectric points are obtained, information that is fundamental to the further planning of the work. A more thorough survey of the behavior of the protein on various adsorbents can then be done using a panel of adsorbents. This can be carried out either in a panel of parallel columns or using tandem columns.

A classical parallel column approach was developed by Scopes (37) for a panel of dye adsorbents. In this case he used up to 20 small columns containing various dye adsorbents. The columns were equilibrated with a predetermined application or starting buffer. A small volume of the protein extract was applied to each column and the protein content (A280 absorbance) and activity in the effluent measured. A predetermined terminating buffer was then applied to each column, and the protein and enzyme activity in the effluent were then determined. A column where the bulk of the proteins, but not the activity, was adsorbed was chosen as a “minus column,” and an adsorbent where the reverse happened was chosen as a “plus column.” These two columns in combination effected a considerable purification of the desired substance in the actual preparation. In an earlier but similar approach, a panel of parallel columns was used by Shaltiel (38) for the evaluation of hydrophobic adsorbents. The technique can, however, be used for any set-up of adsorbents such as different ion exchangers, the same ion exchanger under different conditions, thiol-gels, metal-chelating gels, and so on. The elution of the columns can also be performed with more than two elution buffers. The purpose, however, is to get a quick idea of the behavior of a previously unknown protein and thus the set-up should not be enlarged beyond what can be handled easily in the laboratory.

If the adsorbents used have well defined and continuously increasing adsorption capacities for proteins, in general the panel can also be arranged as tandem columns. This approach was used by Porath and co-workers for the immobilized metal ion (IMAC) adsorbents (39). Here, three columns (e.g., Zn, Fe, and Cu) were connected in series and a sample was pumped through all of them. After washing with starting buffer, the three columns were disconnected and eluted separately, mostly using gradients. The approach requires that the first column adsorbs few of the proteins present, whereas the last adsorbs almost all of them. This technique is not as generally applicable as the use of parallel columns.

In Chapter 20, the use of high throughput methods in the development of industrial-scale protein purification processes will be discussed.

1.4.3.2 The Order of the Chromatographic Steps A priori, one would expect that the order in which the different chromatographic steps are applied in a protein purification protocol is of minor importance. The total purification factor should be constant and the product of the factors obtained in each individual step should be independent of the other steps of the protocol. In the ideal case, where each chromatographic technique is utilized optimally with regard to the resolution and recovery, that is, within the linear regions of the adsorption isotherms (see Chapter 2), with adequate sample volume to column volume ratios, and with no adverse viscosity effects, this is probably true. However the real-life situations are always far from ideal or at least such that adaptation to ideality becomes highly impractical. For example, a fractionation gel filtration step can be optimized to give very high resolution (Chapter 3), but only at the cost of time and sample volume. To choose fractionation gel filtration as the first step, when the sample volume might be much larger than the total volume of the column, means repetitive injections and excessive and impractical total process times, which would probably also be deleterious to the proteins in the sample solution. Likewise, to choose AC on immobilized monoclonal antibodies as the first step would
probably result in an extraordinarily high purification factor. However, the high cost of such adsorbents prohibits the use of large columns, which makes repeated injections of sample in smaller columns almost mandatory. This leads to long process times and the risk of product losses and/or modifications due to proteolytic attack. Proteolytic activity can also threaten the stability and life length of the actual immunosorbent. Furthermore, protein-based adsorbents are difficult to maintain to a sufficiently high degree of hygiene. There are limitations with regard to means for regeneration (washing) and sterilization (Chapter 9). This is why they should be saved for the later steps of the purification protocol.

The consequence of these considerations is that there are a number of practical rather than theoretical reasons why one should choose certain chromatographic techniques (31) for the early steps and others for the final steps of a protein purification process. The choice is primarily governed by the following parameters:

- the sample volume
- the protein concentration and viscosity of the sample
- the degree of purity of the protein product
- the presence of nucleic acids, pyrogens, and proteolytic enzymes in the sample
- the ease with which different types of adsorbents can be washed free from adsorbed contaminants and denatured protein.

The last parameter governs the life length of the adsorbent and, together with its purchasing price, the material cost of the particular purification step.

In light of what has been said above, the logical sequence of chromatographic steps would start with more “robust” techniques that combine a concentration effect with high chemical and physical resistance and low material cost. The obvious candidates are IEC and, to some extent, HIC. As the latter often requires the addition of salt for adequate protein binding, it is preferably applied after salt precipitation or after salt displacement from IEC, thereby excluding the need for a desalting step. Thereafter, the protein fractions can preferably be applied to a more “specific” and more expensive adsorbent. The protocol is often finished with a gel filtration step (Fig. 1.3).

It is advisable to design the sequence of chromatographic steps in such a way that buffer changes and concentration steps are avoided. The peaks eluted from an ion exchanger can, regardless of the ionic strength, be applied to a gel filtration column. This step also functions as a desalting procedure, which means that the buffer used for the gel filtration should be chosen so as to allow direct application of the eluted peaks to the next chromatographic step. The different chromatographies have, in practice, widely different capacities, even though it is possible to adapt several of the methods to a larger scale. However, in the initial stages of a purification scheme it is most convenient to start with the methods that allow the application of large volumes and which have the highest capacities. To this category belong IEC and hydrophobic interaction, but any adsorption chromatographic method can be used to concentrate larger volumes, especially in batchwise operations.

### 1.4.4 The Final Step

The purpose of the final step is to remove possible aggregates or degradation products and to condition the purified protein for its use or storage. The procedure will thus be different depending on the fate of the protein. Aggregates and degradation products are preferably removed by gel filtration. If the protein is to be lyophilized, this step is also suitable for transferring the protein to a volatile buffer (Table 1.1). This can sometimes be done by IEC, but more seldom by the other forms of chromatography. If the protein solution is intended to be frozen, stored as a solution, or used immediately the requirements for specific buffer salts might be less stringent.

Several of the adsorption chromatography steps might be designed in such a way that they result in peaks of reasonably high protein concentration. This is an advantage when gel filtration is chosen as a final step. Gel filtration will always result
in dilution of the sample and is therefore often followed by a concentration step.

If the protein is to be used for physical-chemical characterization, especially for molecular weight studies, gel filtration has the advantage of giving a protein solution of defined size and also in perfect equilibrium with a particular buffer. Biospecific methods, by definition, give a product that is homogeneous with respect to biological activity. This was taken advantage of for papain, where the enzyme eluted from a thiol column was twice as active as any previous preparation. Many of the early enzyme preparations apparently contained molecules in which the thiol necessary for activity was oxidized (see Chapter 8).

Proteins that, after purification and formulation, are intended for parenteral use in human beings must not contain endotoxins (lipopolysaccharides, LPS) or nucleic acids. The purification protocols must be designed so that these compounds are efficiently removed, and validation studies should be performed to prove this. To prepare sterile protein solutions, aseptic filtration is used.

1.5 MONITORING THE FRACTIONATION

Proper analysis is a prerequisite for successful protein purification. Most important is the establishment of a reliable assay of the biological activity. In addition, the protein content should be determined in order to be able to assess the efficiency of the different steps. It is beyond the scope of this chapter to go into details of the particular assay methods. This is covered by the special literature dealing with the activity in question—hormone, enzyme, receptor, and so on.

We recommend that each preparation be continually recorded in a purification table (Table 1.6). In combination with results from gel electrophoresis, for example, this will serve as a guide for judging the reproducibility and outcome of each preparation. In addition, each chromatography experiment should be accompanied by a suitable protocol such as the one exemplified in Figure 1.4. However, the need for measurements of biological activity and protein concentration—especially the latter—should not be allowed to delay the preparation, and in many cases it is sufficient to save aliquots for analysis at one’s convenience.

### 1.5.1 Assay of Biological Activity

In general, biochemical activities depend on the interaction between molecules. This can be measured in different ways. The classical method of enzyme catalysis is only one of these. In addition, the monitoring of the components can be done in several ways, such as spectrophotometry, measurements of radioactivity, and immunological methods. Examples of these include the following:

- enzyme activity by direct spectroscopy
- enzyme activity by secondary measurements on aliquots
- binding of ligand
- binding of antibody.

The immunological methods require that the protein studied has already been purified once to allow production of an antibody or an antiserum by immunization. The detection of the antigen–antibody precipitate can be done at almost any sensitivity down to the extreme sensitivity afforded by the use of sandwich techniques and radioactively or enzymatically labeled reagents (e.g., Chapters 17 and 18).

### 1.5.2 Determination of Protein Content

In general, a measure of protein content is obtained upon monitoring the effluent in chromatography by UV absorption. However, it is not always easy to relate these measurements to the protein content. In fact, the only certain measure of protein content is total amino acid analysis after hydrolysis. Strictly speaking, even this latter analysis suffers from some shortcomings, because tryptophan and cysteine normally have to be analyzed separately.

Large deviations from true protein values sometimes occur in the first steps in a purification scheme. The extract itself often contains substances that interfere with the protein analyses. An overestimation might result, especially if measurements of absorption at 280 nm are used, because the solutions are often turbid and absorbing substances of nonprotein origin are present. This in turn will make the calculated values of specific activity erroneous.

### TABLE 1.6 Example of a Purification Table

<table>
<thead>
<tr>
<th>Material</th>
<th>Volume (mL)</th>
<th>Protein (mg/mL)</th>
<th>Total Protein (mg)</th>
<th>Activity (U)</th>
<th>Total Activity (U)</th>
<th>Spec. Activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purif. Factor (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>500</td>
<td>14</td>
<td>7000</td>
<td>7</td>
<td>3500</td>
<td>0.5</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>First purif. step</td>
<td>50</td>
<td>10</td>
<td>500</td>
<td>60</td>
<td>3000</td>
<td>6</td>
<td>85</td>
<td>12</td>
</tr>
</tbody>
</table>

Activity (e.g., enzyme activity) is expressed as units, and specific activity as units per mg of protein (U/mg).
Three main procedures for protein determination are used routinely:

- spectrophotometry at 280 nm
- colorimetry by Lowry–Folin–Ciocalteau reagent
- dye binding with Coomassie Brilliant Blue G-250.

Each of these methods has its advantages and its disadvantages. UV-absorption measurements require knowledge of the extinction coefficient of the protein(s) to be measured. These vary widely. For example, in the low end there is serum albumin, with an optical density (OD) of 0.6 for a 1 mg/mL solution, and the extreme parvalbumin, with no
absorption at all in the 280 nm band. At the other extreme there is lysozyme, with an OD of 2.7 for a 1 mg/mL solution. These values arise due to a corresponding variation in the content of the aromatic amino acids tryptophan and to a lesser extent tyrosine. As a rule of thumb it is convenient to assume a mean extinction of 1.0 for a 1 mg/mL solution, and this is often sufficient for practical purposes. When the protein is purified, the extinction coefficient and the wavelength for maximum extinction should, however, be determined on a solution by spectral and amino acid analysis.

An alternative to measurements at 280 nm is the low wavelength measurements at 225 nm or below. This absorption is due to the peptide bond, which has a maximum at 192 nm but still a considerable absorption at 205 nm (50% of maximum or an OD of 31 for a 1 mg/mL protein solution) and at 220 nm an OD of 11. These measurements are, of course, even more sensitive to contamination and also require that buffers that are transparent in the low UV regions are used. The use of sensitive UV monitors at these wavelengths in chromatographic equipment thus allows an extreme sensitivity, but their use is not possible unless great care is taken to avoid contaminants and impurities in the buffer salts.

The Lowry methods (40) are less problematic but also less sensitive. Aliquots for analysis should have protein concentrations of 0.1 mg/mL or more. It is often a good alternative in the beginning of a purification procedure where direct UV measurement might be impossible due to turbid solutions. The same applies to the use of Coomassie Brilliant Blue. This method is 5–10 times more sensitive than the colorimetric one, but is more cumbersome to use (41, 42). The latter two methods are both destructive, whereas the UV method allows the sample to be recovered.

A complementary treatment of this topic can be found in Chapter 4, “Ion Exchange Chromatography” (Section 4.9.1).

### Analysis 1.5.3 Gel Electrophoresis

The gel electrophoresis techniques allow the investigator to get an idea of the complexity of the sample and, in particular, what the main contaminating species are. By using sieving electrophoresis, for example in the presence of SDS, and isoelectric focusing, a considerable amount of information about the sample can be obtained in a couple of runs. The amount of each component, its molecular weight, its isoelectric point and even its titration curve can be obtained (see Chapter 16). If an antiserum directed towards the complete mixture is available one can also see whether some of the components are immunologically related and thus also structurally related by means of crossed immunoelectrophoresis.

The gel electrophoresis techniques are introduced in Chapter 15.

### Analysis 1.6 THE FINAL PRODUCT

#### 1.6.1 Buffer Exchange

The high resolution chromatographic steps for protein fractionation usually result in a product that is not directly suitable for the intended use, storage, or distribution. The salt content may be too high, the pH of the protein solution may be unsuitable for long-term storage, the concentration of the protein may be too low, or the solution may contain desorption agents from an affinity chromatography step that must be removed before the protein can be used.

The classical way of changing the buffer composition of a protein solution is dialysis. The protein solution is here included in a dialysis bag consisting of cellophane or a similar semipermeable material. Salts and low molecular weight substances can diffuse through the membrane, whereas high molecular weight material remains within the dialysis bag. The bag is placed in a larger stirred vessel containing the desired buffer, which is changed several times.

A faster way of changing the buffer composition of protein solutions is by gel filtration on, for example, Sephadex G25 or BioGel™ P-6 equilibrated in the desired buffer. Proteins and other high molecular weight substances (>6000) elute at the void volume, whereas substances with lower molecular weights are retarded and thus separated from the proteins. The method is fast and, depending on the equipment and volume, the cycle time is often less than one hour. As in every type of column chromatography, the protein solution must not contain particles or colloidal material.

Ultrafiltration (dialfiltration) is a third way of changing the buffer composition of a protein solution. With this technique the protein solution is diluted with the desired buffer, concentrated to the original volume, diluted again, and so on. After a number of cycles the original buffer has in practice changed to the dilution buffer. The last concentration cycle may be driven longer so that the protein solution after the buffer change is concentrated.

#### 1.6.2 Concentration

Concentration is another operation often required after the final step in a protein purification procedure. Ultrafiltration is the most frequently applied technique for this purpose (see Chapter 12). Smaller volumes of protein solutions can alternatively be concentrated by inclusion in a dialysis bag, which is covered with a high molecular weight substance that cannot penetrate the dialysis bag but creates an osmotic pressure that drives the liquid out through the dialysis membrane. Polymers that are often used for this purpose are PEG and Ficoll™ (43).

All chromatographic techniques that adsorb protein can also be used for the concentration of protein solutions. Especially suitable is IEC because of its high capacity and
easy handling of the ion exchange medium. Other concentration methods that have also proved useful in large-scale applications are freeze concentration (44) and concentration using dry Sephadex (45).

1.6.3 Drying

Most biological processes occur in water solution, and one way to stop these is to freeze the protein sample. For minimum risk of inactivation or denaturation a storage temperature of –70°C or below is required. If the protein under study cannot stand repeated freezing and thawing, storage in aliquots is recommended. Another way to stop biological processes is to remove the water. The method used most for biologically active proteins is freeze-drying or lyophilization. In this method the protein solution is frozen below the eutectic point of the solution to ensure that all liquid is frozen. The frozen solution is then placed in a chamber that can be set under high vacuum. In the chamber or connected to the chamber is a condensing surface, a cold trap, with a temperature of less than –40°C. After the vacuum is applied, the protein sample is gently heated so that it does not melt to speed up the sublimation of water to the condenser. Normally, all proteins maintain their biological activity and are fully recovered upon adding water. A technique often used for commercially available biochemicals is to lyophilize aliquots of protein solution in ampoules.

1.7 LABORATORY EQUIPMENT

1.7.1 General Equipment

Laboratories for the preparation and separation of proteins may look very different, from the well-equipped special laboratory serving many research groups to the small laboratory with few people and limited resources. The large laboratory, in addition, often has dedicated service groups for special analyses, and so on.

For the successful preparation and separation of proteins, certain basic equipment is needed. Standard laboratory glassware will not be discussed. In the category of basic equipment will be two or three balances, one spectrophotometer, one centrifuge, one pH meter, as well as stirrers and micropipettes.

A good combination of balance equipment comprises two preparative balances—one a double-range digital balance 0–1200 g or 0–3000 g, with the facility to weigh with a resolution of 10 mg, and the other with the range 0–120 g or 0–300 g, with a resolution of 1 mg—and one analytical balance for the interval 0–150 g with a resolution of 0.1 mg. For the measurement of larger amounts of material, a simpler, and therefore cheaper, balance will suffice, for example, a balance for use in the food industry. It is also important that the balances are calibrated and that they are serviced regularly.

Spectrophotometers for protein work should cover the wavelength interval 190–800 nm. Absorption around 280 nm is used routinely for estimating protein concentration, whereas light in the visible region is often needed for measurement of different enzymatic activities. A double-beam UV–vis spectrophotometer equipped with an adjustable slit and a thermostated cuvette holder is a good choice. It is important that the wavelength setting is correctly calibrated and that the instrument shows low drift. Regular servicing of the instrument is recommended.

A refrigerated floor centrifuge is standard in a preparative protein laboratory. The largest rotor should take six flasks of 500 mL, whereas the smallest rotor should accommodate eight tubes of 50 mL. The maximum g-force for those rotors are normally about 15,000g and 50,000g at the tube bottom, respectively. An additional rotor for six flasks of 250 mL will increase the flexibility of the centrifuge. Some centrifuges accommodate zonal rotors with accessories for continuous operation, allowing larger volumes containing relatively low contents of fine particles to be centrifuged at high g-forces (up to 40,000g). The standard centrifuges normally need no or little service except changing of motor brushes. If the rotors are carefully maintained, including thorough cleaning after each run when liquid is found inside the rotors, they will function for many years. The rotors mentioned above are angle rotors and the flasks are normally filled completely. The special, completely tight lids that are available for most centrifuge flasks are strongly recommended. For small, easily centrifuged samples, a simple desk-top centrifuge without cooling is often a good complement to the larger high speed refrigerated centrifuge.

pH determinations are critical in the protein laboratory. Buffers should routinely be checked and the pH adjusted if necessary (alkaline buffers may absorb carbon dioxide from air if stored over extended periods of time). For certain enzyme assays equipment for monitoring the formation or consumption of protons is needed. The pH meter can then be equipped with an automatic burette that adds acid or base to hold the pH constant, and the consumption of acid or base is recorded. Combination electrodes are generally the most convenient. The linearity of the electrode changes with time and for accurate measurements the electrode has to be calibrated on both sides of the interval within which the pH will be determined.

At least two types of stirrers are integral parts of the basic laboratory equipment. First, magnetic stirrers with variable speed, at least one of which is equipped with a thermostat-controlled heating plate, are recommended. A variety of polytetrafluoroethylene (PTFE)- and glass-coated magnets are required. For extractions, propeller stirrers with interchangeable propellers and variable speed regulators are convenient. These should preferably be of the electronic type, which compensate for variation in load. Stirrers where the speed is controlled only with a variable resistor should be avoided.
Pipetting small volumes, up to 1 mL, is a standard laboratory procedure. Micropipettes of different types, most commonly with adjustable volume and with disposable tips, are used. Two or three pipettes of different sizes are normally sufficient. Check that the length of the pipette allows samples to be collected from the bottom of the longest type of test tube used in the laboratory.

For personnel safety the laboratory must have a ventilated hood or fume cupboard where procedures involving handling or preparation of hazardous substances can be safely performed. Even such a simple and common procedure as dissolving sodium hydroxide in water creates aerosols that are very irritating, and this procedure should be done in the hood.

1.7.2 Equipment for Homogenization

Even if the majority of starting material for protein purification is today obtained from fermentation of microorganisms and animal cell lines (recombinant or nonrecombinant), plant and animal tissues are sometimes chosen. For these, “Waring Blenders” or similar types of equipment and “Turrax”-type apparatuses are frequently used for the homogenization. The material is first cut into pieces with a knife or a pair of scissors to a size that suits the type of blender used. At the same time, the tissue or organ is trimmed and unwanted material like fat, ligaments, and vessels are discarded before grinding the material in the blender. The blender can be used both dry and wet. Plant material like seeds, for example, can be ground dry if the time of grinding can be kept short. Dry ice can be added to keep the temperature low. Wet grinding prevents the formation of harmful dust particles and the heat formed in the grinding procedure is dissipated by the liquid. In most homogenization equipment only a small part of the energy input is normally used for the disintegration procedure, the rest of the energy is lost as heat. A common household meat grinder is also useful in many instances, in combination with a Waring blender. Further homogenization is provided by the Elvehjem pestle-type homogenizers. There are different types (glass, Teflon®), sizes, and also motor-driven varieties of this homogenizer.

Microorganisms such as bacteria and yeast are much more difficult to disintegrate. The polysaccharide and proteoglycan cell wall withstands most homogenization procedures used for plant and animal cells. The well-cited overview by Wimpenny (46) for breakage of microorganisms is still valid for small-scale preparations (Fig. 1.5). For large-scale homogenization of microorganisms, which means kilogram quantities of cell paste, the most frequently used equipment

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**Figure 1.5** Principles and methods for cell breakage.
is the Dynomill (47) and the Manton–Gaulin (48) homogenizer. Both are designed for continuous operation and are available in various sizes for the breakage of from 100 g up to several kilograms of microbial cell paste per hour.

1.7.3 Equipment for Chromatography

1.7.3.1 Column Design In order to utilize the packed chromatographic gel particles optimally, the column should be designed so that it does not significantly contribute to band spreading or peak distortion. Most modern columns are of the closed type, with two fixed end pieces or with one fixed end piece and one adjustable adaptor. Sometimes it is convenient to use two adjustable adaptors, one at each end of the column. The end pieces or adaptors are equipped with porous frits made of, for example, sintered metal, glass, or plastic, the pore diameters and surface structures of which should be optimized to avoid clogging and abrasion of the chromatographic particles. The frits should be easily exchangeable. For larger-diameter columns the frits have to be combined with the flow distribution system. The prime requisites of such a system are that its volume should be negligible compared to the total column volume and that there should be no pressure drop in the system between the eluent inlet and the column wall. In many modern columns for low and medium pressure chromatography, the frits have been replaced by a combination of a fine mesh (10 μm) polyamide fabric and a coarse mesh (e.g., 0.93 × 0.61 mm) polypropylene support net. The flow distribution layer will then be only 1 mm in depth and the support net will reduce the dead volume by more than 30%. There is no indication that this uniform support net affects the sample application. This is the way many very large-diameter industrial columns are designed. For the largest columns (diameter, >400 mm) the fine mesh nylon fabric is in most cases replaced by a high quality stainless steel mesh. In most large-diameter industrial columns, the number of sample and eluent inlets and outlets have been increased to between four and six. The linear flow rate of the eluent in the column feed pipe is very high in large-diameter columns and it is recommended to prevent this jet from directly hitting the fine mesh fabric (when using a frit this problem is less pronounced), which could cause flow inhomogeneity and band distortion. The effect is reduced by the application of a small disc covering the support net under each pipe inlet.

The materials used in the parts of the chromatographic system that come into contact with the eluent should preferably be noncorrosive and compatible with all normal protein samples and eluent buffers/solvents used. However, they should also preferably be resistant to the conditions applied in the cleaning and maintenance of the packed gel particles. This includes high concentrations of sodium hydroxide and solvents such as alcohols. The cleaning-in-place (CIP) concept has become an important part of process design in biotechnology. In many instances, sterilization by autoclaving is preferred. Most laboratory column tubes are made of borosilicate glass or plastics with end pieces and adaptors made of polypropylene, nylon, or fluorocarbon. HPLC columns are normally made of stainless steel to withstand the mechanical stress.

The column is connected to the pump and to the monitors by small-bore tubing made of stainless steel or titanium in HPLC systems, or fluorocarbon, polypropylene, polyethylene, or nylon in medium or low pressure systems. The inner diameters of the tubings should be optimized for each application. They should be as small as possible without too much contribution to the pressure drop of the system. To avoid zone mixing, the tube length should be reduced as much as possible. Ideally, the column should be attached directly to the monitor cell on top of the fraction collector. In normal laboratory systems fluorocarbon tubing with an inner diameter of 1 mm is recommended.

1.7.3.2 Pumps and Fraction Collectors The traditional laboratory-scale pumps for low pressure systems (0.1 MPa) are of the peristaltic type, of which several brands are on the market. Care must be taken to keep the rollers and pumping tube clean. Otherwise, the tube life length is severely decreased. Peristaltic pumps are available with flow rates of a few milliliters per hour to cubic meters per hour. The pumping tube is available in several different materials, but for the protein work silicone tubing is recommended. Pumps with three or more channels can be used for gradient formation. The normal way to form a gradient is to use two connected vessels, with stirring in the output mixing vessel. In medium and high pressure chromatography, gradients are usually formed using two pumps with individually adjusted speed connected to a programmable controller.

For HPLC, most pumps are of the piston type and made of stainless steel or titanium, as are the sample injectors. In most cases they function according to the reciprocal displacement principle and are capable of pressures up to 35 MPa or more. Among medium pressure (5 MPa) pumps is the positive displacement type, in which a fluorocarbon-sealed piston made of titanium or stainless steel is allowed to move in a thick-walled borosilicate glass tube. When the piston reaches the end of the tube, a valve switches over to a second identical piston-equipped glass tube filled with eluent, which will continue feeding the column with eluent at the same pressure while the first is filled. Unless proper damping is provided, a pressure transient will occur, giving rise to spikes in the UV-monitor recordings at each piston change.

Traditional fraction collectors consist of a large plastic or metallic ring with holes bored at the periphery, moved by an electric motor controlled by a timer. This type of fraction collector requires a relatively large bench area. Alternatives are compact designs with the holes for the test tubes arranged
in a spiral or with the test tubes placed in racks. These collectors are controlled by microprocessors, and different tube sizes and fraction collection times can be programmed in advance. In large-scale chromatography, when the results are normally predictable from pilot experiments on a small scale, only a few fractions are needed and the fractions are obtained by using magnetic valves connected to the controlling equipment.

1.7.3.3 Monitoring Equipment The classical way to monitor a chromatographic experiment is to take fractions from the outlet of the column in a fraction collector and analyze each tube manually for the different substances one wishes to determine. This is very time-consuming work, and flow monitors connected directly to the column eluate have largely replaced this practice today. The most commonly used parameter in protein work is absorption at 280 nm, and a lot of detectors are available for this purpose. In new diode array monitors, a spectrum can even be obtained directly on the eluate. In IEC, the ionic strength is of interest and conductometers with flow cells are available. The pH of the eluate can also be monitored in, for example, by chromatofocusing using continuously working monitors. Even complicated enzyme assays can be made directly on-line by the use of autoanalyzers. Here a small part of the process stream is shunted through the autoanalyzer. This part of the sample is normally destroyed during the analysis.

1.7.3.4 Chromatography Systems During the last decade the use of stand-alone (nonsystem) components has been in decline, and very few laboratories are using these “separates” nowadays. So many new scientists expect a complete out-of-box solution to perform their protein purification. Within the systems market there is a broad product offering in terms of levels of automation and sophistication.

The introduction of the FPLC System (GE Healthcare Life Sciences, Uppsala) in 1982 meant a new way of thinking in the design of dedicated chromatography equipment for protein separation and purification. For the first time, a microprocessor-controlled and straightforward system approach was launched in which the parts and components were matching the performance of a new chromatography material. Parts in contact with sample and buffers are made of glass, plastic, or titanium to minimize corrosion and nonspecific adsorption of the sample components. In combination with prepacked columns containing the 10-μm-diameter MonoBead™ ion exchangers, which give high performance separations at moderate pressure drops, the FPLC System became extremely popular and has since been installed in several thousand biochemical laboratories.

Many successors to the FPLC System have been introduced by several manufacturers. In 1998, ÄKTAexplorer™ (GE Healthcare Life Sciences, Uppsala) was launched, which is a preassembled chromatography system configured for fast and easy development and optimization of a variety of purification methods using the “Adviser” included in its UNICORN™ software. This contains method development templates for media screening and method scouting, covering IEC, HIC, RPC, AC, and gel filtration chromatography, as well as purification protocols for recombinant proteins from *E. coli*, peptides, and oligonucleotides. Included is also a general purpose method for column cleaning. When a prepacked column is selected from those listed in the data base, the system automatically sets the running parameters to those best suited to that particular column. This allows direct and optimal use of the column without any previous special knowledge or experience. An automatic buffer preparation function covering a broad pH range eliminates the manual buffer preparation and titration needed for every pH change, particularly in IEC. ÄKTAexplorer can also manage system control and data access via a PC network, which gives a complete overview of operations. Results can be automatically saved on a server, and evaluation and generation of reports can be made locally or at remote PCs. The pumps have an exceptionally wide flow rate operation range (0.01–100 mL/min in isocratic mode and 0.5–100 mL/min in gradient mode) at pressures of up to 10 MPa, previously not covered by a single chromatography system. The whole system is mounted on a swivel platform and requires minimal bench space.

Following the successful introduction of the ÄKTAexplorer system, GE Healthcare Life Sciences AB introduced ÄKTAprimeplus, ÄKTAxpress and, recently, ÄKTAavant. ÄKTAprimeplus can be considered to be an “entry-level” complete instrument for simple, push-button purification, thus ideal for new protein scientists. ÄKTAxpress meets the needs of the scientist who must obtain high purity proteins but does not have the time or

![Figure 1.6 ÄKTA™ avant chromatography system. From GE Healthcare Life Sciences AB, Uppsala, Sweden. (See color insert.)](image-url)
skill to construct multistep purification schemes. Thus it offers the user a very simple interface to create sophisticated two- or three-step purifications employing affinity, ion exchange, and gel filtration techniques.

ÅKTAavant (Fig. 1.6) is a new generation of ÅKTA instruments. It is designed for the process developer who realizes that speed (productivity), security of operation, and scalability are critical factors. To this end, ÅKTA avant is equipped with an integrated “Design of Experiments” tool, which assists the user in designing statistically robust experiments to screen, optimize and test their “design and operating space.” Additionally, all buffers, using scalable buffer recipes, are titrated automatically and hence multiple pH environments can be automatically created for screening work.

A new software called UNICORN 6 enables the user to create chromatography methods with greater levels of complexity in quicker time than before. The temperature-controlled fraction collection is now built into the chassis of the instrument (giving greater control and minimizing the column tracking history to give greater operational safety. ÅKTAavant has been designed around, and optimized for, modern purification media intended to be used at a production scale. A new feature is also a flow velocity and pressure relationship function.

1.7.4 Equipment for Chromatographic and Electrophoretic Analyses

For characterization of the product at various stages of a protein preparation, in principle the same separation techniques that are used for preparation of the protein can be used. The techniques are, however, scaled down to the microgram-to-milligram scale, and sometimes a dedicated micro-preparative chromatographic equipment is preferred (49). Even more important are analyses by electrophoretic separation techniques. Equipment and methods for the different electrophoretic techniques are described in connection with the techniques themselves in Chapters 15–19.

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PART II

CHROMATOGRAPHY
INTRODUCTION TO CHROMATOGRAPHY

JAN-CHRISTER JANSON
Department of Physical and Analytical Chemistry, Uppsala University, Box 579, S-751 23 Uppsala, Sweden

JAN ÅKE JONSSON
Center for Analysis and Synthesis, Department of Chemistry, Lund University, Box 124, S-22100 Lund, Sweden

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2.1 INTRODUCTION: BASIC CONCEPTS AND VERSIONS OF CHROMATOGRAPHY

The term chromatography refers to a group of separation techniques characterized by a distribution of the molecules to be separated between two phases, one stationary and the other mobile. Molecules with a high tendency to stay in the stationary phase will move through the system at a lower velocity than those that favor the mobile phase.

The most common physical configuration is column chromatography, in which the stationary phase is packed or molded into a tube, a column, through which the mobile phase, the eluent, is pumped. The sample to be separated is introduced into one end of the column. The various sample components travel with different velocities through the column and are subsequently detected and collected at the other end. Other configurations, such as thin-layer chromatography and paper chromatography are also used, but are less commonly applied for protein separation.

In the context of this book, the mobile phase is always a liquid, most often an aqueous buffer. Consequently, these techniques are versions of liquid chromatography. For the separation of more volatile compounds, gas chromatography, in other words with a gaseous mobile phase, is an extremely powerful and widely applied technique.

For protein separation, several versions of liquid chromatography are used, differing mainly in the types of stationary phase (Table 2.1). One of these, gel filtration chromatography (also called size exclusion chromatography), is based on quite different principles than are other versions of liquid chromatography. Therefore, much of the theoretical description of that technique must be made separately. In this book, the basic principles of gel filtration chromatography will be described in Chapter 3, while other techniques will be dealt with briefly here. A thorough treatment of the theory and principles of chromatography is outside the scope of this book, but can be found in specialized texts (1–3).

2.2 THE STATIONARY PHASE

The stationary phase in a chromatographic experiment is composed of a porous matrix and imbibed immobile solvent. Typically, the solvent constitutes most of the stationary phase, often more than 90%, and such materials are generally referred to as gels. In protein chromatography the solvents are normally aqueous buffers, and the gel-forming materials are usually composed of hydrophilic polymers. Currently, two types of modern stationary phases are available. Traditionally, they are bead-shaped, with average particle diameters ranging from a few to ~100 μm. In recent years, so-called monolithic columns have been introduced onto the market, which are based on a continuous pore matrix structure, polymerized in situ, filling the whole column dimension. For early contributions to this area see References 4–7. A book entitled “Monolithic Materials: Preparation, Properties and Applications” was published in 2003 (8), and has become a standard reference of the status of this area. For a more recent account the readers are referred to a review by Jungbauer and Hahn (9).

In principle, one may distinguish between two types of gels: xerogels and aerogels. The xerogels are characterized by their ability to shrink and swell in the absence and presence of the solvent used, whereas the volume of an aerogel is independent of the solvent. Typical xerogels include cross-linked dextran gels (Sephadex™) and crosslinked polyacrylamide gels. Typical aerogels include porous glass, silica, and most gels based on macroreticular organic polymer gels, such as polystyrene gels and polymethacrylate gels.

2.2.1 Matrix Properties

In addition to being hydrophilic, an ideal general matrix for protein chromatography should not contain groups that spontaneously bind protein molecules. However, it should contain functional groups that allow the controlled synthesis of a wide variety of protein adsorbents. Furthermore, the matrix should be chemically and physically stable in order to withstand extreme conditions during derivatization and maintenance (regeneration, sterilization, etc.) and be rigid enough to allow high linear flow rates (5 cm/min or more) in columns packed with particles with diameters down to a few microns. Finally, the matrix substance should allow the production of gels with a broad range of controllable porosities.

A wide variety of materials have been used for the design of protein chromatography matrices. These can be classified as either inorganic materials, synthetic organic polymers, or polysaccharides. Among all three groups we find traditional standard chromatography media as well as modern high

<table>
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<td>Separation Principle</td>
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performance chromatography media. Examples include the following:

- **inorganic materials**
  - porous silica
  - controlled pore glass
  - hydroxyapatite

- **synthetic organic polymers**
  - polyacrylamide
  - polymethacrylate
  - polystyrene

- **polysaccharides**
  - cellulose
  - dextran
  - agarose

None of these materials fulfils all criteria for an ideal general matrix for protein chromatography: they are all compromises. A combination of hydrophilicity with chemical and physical inertness is best achieved by the use of alcohol hydroxyls or amido groups, so the most widely used standard chromatographic media for proteins are based on neutral polysaccharides and polyacrylamide.

Among the polysaccharides, cellulose (Figs. 2.1 and 2.2) is still used for the synthesis of protein ion exchangers, more than 50 years after its introduction by Peterson and Sober in 1956 (10), and is marketed under the trade names Whatman™ (Whatman BioSystems Ltd, Springfield Mill, Maidstone, Kent ME14 2LE, England, a subsidiary of GE Healthcare Life Sciences, Uppsala, Sweden) and Cellulofine® (manufactured by Chisso Corp. Ltd, Kumamoto, Japan). Crosslinked dextran (Figs. 2.1 and 2.3) was introduced in 1959 by Porath and Flodin (11) and is marketed under the trade name Sephadex (GE Healthcare Life Sciences, Uppsala, Sweden). This material is best known as a gel filtration medium (12), but is also widely used as a matrix for ion exchangers (13). An account of the history of the development of Sephadex is given in Reference 14. The neutral hydrophilicity of dextran gels makes them compatible with most proteins, and quantitative recovery is thus the rule. Their most important application area today is in desalting and buffer exchange of protein solutions. Protein fractionation by gel filtration is nowadays performed largely with composite gel matrices (see below).

Agarose (Figs. 2.1 and 2.3) is a low charge fraction of the seaweed polysaccharide agar. It was introduced as a medium for chromatography by Hjertén (15, 16) and marketed under trade names such as Sepharose™ and Superose™ (GE Healthcare Life Sciences), Ultrogel™ A (Pall Corporation, Port Washington, NY, USA) and BioGel™ A (BioRad Chemical Division, Richmond, CA, USA). Bead-shaped gels suitable for chromatography are formed by cooling 2–15% aqueous solutions of agarose dispersed in a nonpolar organic solvent in the presence of suitable emulsifiers (16). The agarose gel structure is an open three-dimensional network of fibers composed of spontaneously aggregated galactan helices (17) (Fig. 2.4). In a 4% agarose gel with an average diameter of 90 µm, the

![Figure 2.1 Partial structures of common gel-forming polymers.](image)
total surface area is approximately $5 \, \text{m}^2/\text{mL}$, with an average pore diameter of $\sim 30 \, \text{nm}$ (18). By chemical crosslinking (19, 20) of the spontaneously aggregated galactan polymers, the gel rigidity is improved considerably, allowing the manufacture and use of particles down to a diameter of $10 \, \mu\text{m}$ for a 12% agarose gel (21). The crosslinking does not change the gel pore structure, but the number of hydroxyl groups is reduced by $\sim 50\%$, which, however, does not significantly affect the binding capacity for proteins of ion exchangers and affinity chromatography adsorbents prepared from cross-linked agarose gels.

From a functional point of view it is convenient to distinguish between microporous and macroporous (or macroreticular) gel matrices. The microporous gels are prepared by point crosslinking of linear polymers such as dextran and polyacrylamide (Fig. 2.3). This type of gel lends itself ideally to molecular-sieving separations (size exclusion chromatography, gel filtration). At porosities suitable for protein chromatography these gels become impractically soft, and this is why they often participate in the design of composite gels (see below).

The macroporous gels are most often obtained from aggregated and physically crosslinked polymers. To this group belong agarose, macroreticular polyacrylamide, silica, and several synthetic organic polymers. These gels are best suited for the design of stationary phases intended for ion exchange chromatography, affinity chromatography, and other adsorption chromatographic techniques. By introducing microporous gel-forming polymers into the pores of macroreticular gels, composite gels are formed that combine the strength of and reduce the weakness of each separate gel moiety. Examples of such gels are Sephacryl™ (poly-$N,N’$-bisacrylamide-dextran, GE Healthcare Life Sciences).
Sciences) (Fig. 2.5), Superdex™ (agarose-dextran, GE Healthcare Life Sciences), Capto™ S, Capto Q, and Capto DEAE (agarose-dextran, GE Healthcare Life Sciences), MacroCap™ (polyacrylamide-dextran, GE Healthcare Life Sciences), Ultrogel™ AcA (agarose-polyacrylamide, Pall Corporation) and Ceramic HyperD™ (silica-polyacrylamide, Pall Corporation).

The advent of protein high performance liquid chromatography (HPLC) increased the demand for matrix rigidity. This gave rise to new derivatization procedures for porous silicato make it compatible with proteins, for example, through the introduction of diol silanols (22). Despite attempts to stabilize the silica surface by using, for example, zirconium oxide (23), the major weakness of this matrix is still its instability at alkaline pH. This has encouraged the development of rigid matrices based on porous synthetic organic polymers (MiniBead™, 3 μm; MonoBead™, 10 μm; Source™, 15 μm and 30 μm; polystyrene/divinyl benzene-based stationary phases; GE Healthcare Life Sciences) and agarose (Superose, agarose-based, 10-μm- and 13-μm-diameter stationary phases; GE Healthcare Life Sciences).

Hydroxyapatite represents a widely used inorganic matrix with high selectivity for a wide variety of proteins and also nucleic acids (24). Bead-shaped hydroxyapatite particles intended for HPLC with diameters of only a few microns are commercially available in prepacked columns from several sources.

2.2.1.1 Pore Size Distribution An important parameter to consider in the design of media for protein chromatography is the pore size distribution of the gel matrix network (25). This will affect the shape of the selectivity curve in size exclusion chromatography (see Chapter 3) as well as the relative diffusion coefficients of proteins when separated by various kinds of adsorption chromatography. One way to estimate the apparent relative pore size distribution of chromatographic gel media is by correlation to the elution behavior of well-defined fractions of a neutral and inert linear homopolymer such as dextran. The resulting calculated distribution coefficients \( K_d \) and \( K_{av} \) are then plotted against the hydrodynamic (viscosity) radius calculated for each dextran fraction using the equation \( R_H = 0.027M_0^{0.498} \), as suggested by Hagel (26). In Figure 2.6 are shown the apparent pore size distributions, obtained in this way, of a variety of common chromatographic media for proteins.

The behavior of Ceramic HyperD is remarkably deviant from the other gel media in this study. The pores are unexpectedly small in spite of its proven high capacity also for relatively high molecular weight proteins. This has been explained in terms of solid diffusion in this polyacrylamide-polystyrene coated silica composite (“gel-in-a-shell”). The phenomenon has been discussed in comparison to the behavior of the perfusion medium Poros™ 50 (27).
The apparent relative pore size distribution curve for 2% agarose (Sepharose 2B) is surprisingly similar to that of Poros MC. The data might be interpreted such that it should be possible to get perfusion effects also in this agarose gel. Figure 2.4 shows a scanning electron micrograph (SEM) of a 2% agarose gel. The SEM structure image indicates that an agarose gel might be characterized as being built up of randomly oriented linear fiber cylinders. Using equations derived from work by Happel and Brenner (28) for liquid flow through a network of randomly distributed, infinitely long, rigid and cylindrically shaped fibers, we have calculated that a pressure drop of approximately 10 bar/cm is required to force a perfusive liquid flow of 0.16 mL/cm²/min through a gel layer with a thickness of 1 cm (assuming a fiber diameter of 10 nm and a fiber volume of 2%), based on such a matrix structure. Figure 2.7 shows the equations used and the resulting graph obtained by plotting the flow rate as a function of the pressure drop through a 2-cm-diameter gel cylinder. Published data on the agarose gel structure (29) report an average matrix fiber diameter of 10 nm for a 2% (w/v) agarose gel. This suggests that it should be possible to force a reasonable flow of water through a 2% agarose gel of appropriate thickness without applying excessive pressure drops, provided that the gel matrix is rigid enough. If not, the agarose gel rigidity is known to be significantly improved by adequate crosslinking (20, 21).

2.2.2 Stationary Phase Technology

The shape, rigidity, and particle size distribution profile of the gel matrix are important parameters that govern the performance of the stationary phase. The original batch
polymerization procedures followed by grinding and sieve classification of the irregular particles have, with few exceptions (e.g., cheap silica variants), largely been replaced by bead polymerization technologies.

Crosslinked dextran gels, Sephadex, are thus produced by allowing aqueous solutions of dextran containing an excess of alkali to be dispersed in a nonpolar solvent containing a suitable emulsifier (30). The average droplet diameter and size distribution are controlled by the emulsifier concentration and the type and speed of the stirrer. Crosslinking occurs upon addition of a predetermined quantity of epichlorohydrin. The pore diameter and size distribution of the formed gel are governed by the concentration and the molecular weight distribution of the dextran and by the epichlorohydrin concentration.

Standard polyacrylamide gels (Figs. 2.1 and 2.3) for chromatography are formed by bead polymerization of aqueous droplets containing acrylamide (monomer) and methylenebisacrylamide (crosslinker) dispersed in an emulsifier-containing apolar solvent (31). The gel pore dimensions depend on the total concentration of monomer (% T) and the relative concentration of crosslinker (% C), respectively.

One major step forward in stationary phase technology was the bead polymerization technique developed by Ugelstad (32), which is based on controlled swelling of submicron particles in the presence of monomer, which gives rise to practically monodisperse beads (Fig. 2.8) of any desired diameter up to approximately 30 μm. Such particles are commercially available with diameters of 3, 10, 15, and 30 μm, and with controlled porosity (GE Healthcare Life Sciences).

\[
Q_1 = \frac{\Delta p}{L} \frac{\pi r^2}{8 \mu} (1 - c)^{1/4} \left(\frac{4}{c} - 1 - \frac{3}{c^2} \ln c\right)
\]

\[
Q_2 = \frac{\Delta p}{L} \frac{\pi r^2}{4 \mu} (1 - c)^{1/4} \left(\frac{c^2 - 1}{2c^2 + 1} - \ln c\right)
\]

\[
Q = \frac{Q_1 + 2Q_2}{3}
\]

**Figure 2.7** Equations for liquid flow through a network of randomly distributed, infinitely long, rigid and cylindrically shaped fibers. Adapted from Reference 28. The graph shows the corresponding theoretical flow rate as a function of pressure drop for a 2-cm-diameter and 1-cm-long cylinder built up of a 2% (v/v) network of 10-nm-diameter fibers.

**Figure 2.8** Scanning electron micrograph of Source™ (GE Healthcare Life Sciences) monodisperse PS-DVB beads. Scale bar, 10 μm.
As mentioned above, the development of new crosslinking procedures for agarose has enabled the use of this polysaccharide for synthesis of stationary phases that fulfill most of the requirements of a modern high performance matrix. Today, stationary phases are available based on beads of crosslinked agarose with average diameters of 10 μm (Superose, GE Healthcare Life Sciences), 30 μm (Sepharose High Performance™, GE Healthcare Life Sciences), 50 μm (Capto, GE Healthcare Life Sciences), 90 μm (Sepharose Fast Flow, GE Healthcare Life Sciences), and 200 μm (Sepharose Big Beads™, GE Healthcare Life Sciences), which cover the demands of protein chromatography from micrograms to several kilograms per cycle and with cycle times from a few minutes to less than two hours in adsorption chromatography applications such as ion exchange chromatography and affinity chromatography.

A procedure for the preparation of a new type of superporous agarose gel media has been reported (33). Agarose beads with diameters of 300–500 μm, containing perfusive pores with diameters of ~30 μm that allow convective mass transport, could be prepared using a double emulsification process.

Stationary phases intended for perfusion chromatography will be discussed in Sections 2.5.3 and 2.6.6.7 and monolithic bed designs will be discussed in Sections 2.5.4 and 2.6.6.8, respectively.

2.2.3 Introduction of Ligand Groups

The ideal base matrix for the synthesis of stationary phases for adsorption chromatography techniques is identical to the ideal gel filtration medium in all respects (as indicated in the beginning of Section 2.2.1), but it should not give rise to excessive mass transport constraints and should have an open, nonsieving and easily accessible pore structure, allowing unhindered diffusion of the protein molecules to their adsorption sites on the gel matrix polymer surface.

The surface should be covered with hydrophilic functional groups that do not themselves participate in protein binding, but should, on the other hand, be amenable to derivatization for the synthesis of ion exchangers and other adsorbents. However, all base matrices interact with at least some proteins under certain conditions. Crosslinking and derivatization procedures may also inadvertently introduce nonspecific adsorption sites, which may complicate interpretation of the purification data.

Through the introduction of new chemical structures on the surface of the matrix it is possible to design stationary phases that interact more or less specifically with a particular protein. Least specific are gels for ion exchange chromatography (IEC), which separates proteins primarily on the basis of their content and distribution of charged groups, and hydrophobic interaction chromatography (HIC) adsorbents, which interact with hydrophobic patches and crevices on the protein surface. Low specificity should, however, not be confused with low selectivity. Both IEC and HIC can be very selective indeed when time can be spent on the optimization of the operating conditions. The stationary phases with the highest specificities are found among the affinity chromatography adsorbents and particularly among immunoadsorbents based on immobilized monoclonal antibodies and adsorbents based on ligands obtained by screening biological combinatorial libraries (see Chapter 11). Medium specificities are obtained using so-called general ligands, that is, group-specific ligands such as co-enzyme analogs.

The synthesis of an ion exchanger usually takes place by a one-step chemical reaction in which a charged molecule containing a reactive group (often a halogende) is allowed to react with the alcohol hydroxyl-group-containing matrix (such as a polysaccharide) under strongly alkaline conditions. Most diethylaminoethyl (DEAE) and carboxymethyl (CM) ion exchangers are produced in this way. The synthesis of an affinity chromatography adsorbent, on the other hand, is normally a three-step procedure. In the first step the matrix is activated by the introduction of reactive groups. In the second step the ligand is covalently attached to the matrix by reaction with the activated group. In the third and last step the excess reactive groups are inactivated by blocking with a low molecular weight substance that displays low adsorptivity when coupled. Different coupling methods are discussed in some detail in Chapter 9. Some of the more general approaches are mentioned below for hydroxyl-group-containing matrices. For synthetic organic polymer-based matrices and for silica gels, special techniques are required.

As with ion exchangers, the activation reactions normally take place at alkaline pH. The most frequently used reagents for hydroxyl-group-containing matrices are N-hydroxysuccinimide, CNBr (introducing reactive cyanoesters), bis-epoxides and divinylsulfones. These can then be reacted with nucleophilic groups such as thiols, amines, and alcohols. Blocking is often carried out with an excess of ethanolamine. Several alternatives to these coupling methods are available, also for binding via carboxylates. Amino groups are the most commonly used functional groups for coupling of low-molecular-weight ligands as well as proteins. Carbhydrates (sugars, oligosaccharides) can be attached to epoxides and vinylsulfones via their hydroxyls.

2.2.4 Ligand–Protein Interactions

The binding of a dissolved protein to an immobilized ligand arises because of one or several of the following interactions:

- ion–ion or ion–dipole bonds
- hydrogen bonds
- dispersion or van der Waals forces
- aromatic or π–π interactions
- hydrophobic effect or hydrophobic interaction.
Sometimes, covalent bonds are formed, as in covalent chromatography (Chapter 8), or metal ligand bonding, as in immobilized metal ion affinity chromatography (Chapter 7). The hydrophobic interaction differs from the others in that it has a large entropic component.

The system solution-protein-stationary phase at each moment minimizes its free energy, and this gives rise to the binding of the protein to the ligand. This interaction is normally the result of the formation of many secondary bonds, and several of the abovementioned factors might contribute. Each one of these might be weak, often around 1 kcal/mol, but several of them, in combination, add up to a considerable binding constant. A $K_D$ of $10^{-5}$, which gives rise to retardation, corresponds to a binding energy of 7 kcal/mol in standard conditions according to

$$
\Delta G = RT \ln K_D
$$

and thereby 4–8 weak interactions (see, e.g., Ref. 34). A general treatment of the subject is given in Reference 35.

The surface of globular proteins typically has $\sim 45\%$ hydrophobic residues and $55\%$ charged or hydrophilic uncharged residues accessible to surrounding water (Fig. 2.9). In the latter groups, the positive charges of lysine, arginine, and histidine side chains, as well as the $\alpha$-amino group, change according to pH (Table 4.2). The negative charge of glutamic acid and aspartic acid side chains and the $\alpha$-carboxyl group are also pH-dependent. Of these, the histidine side chain and the $\alpha$-amino groups titrate within the pH region that is commonly used in chromatography. The cysteine thiol side chain is not typically available in extracellular proteins, but might be in intracellular proteins. It does not play a role as an anion, although it has a $pK$ of $\sim 8.5$. Other hydrophilic groups such as asparagine, glutamine, serine, and threonine, as well as peptide bonds, are mostly involved in hydrogen-bonding interactions.

Although these interactions determine the strength of the binding of proteins, the specificity of binding is to a large extent explained by the fit between the ligand and the protein. This is particularly true in the case of affinity chromatography. But even if other types of chromatography might to a larger extent depend on a single one of the interactions discussed, it is certainly not a sufficient explanation for protein selectivity. Ion exchange thus depends on ionic forces and HIC on hydrophobic interactions. However, net charge or net hydrophobicity cannot alone explain the differences in retardation of different proteins. Here, the distribution of the interacting groups on the protein surface and therefore the fit between the protein and the ligand is of major

![Figure 2.9](image)
importance, as well as secondary interactions such as hydrogen bonding.

The different interactions are each influenced by the solvent in some particular way. Increased ionic strength thus decreases ionic interactions, whereas hydrophobic interactions are favored. The different ways to elute adsorbed proteins are discussed in detail for each type of chromatography. It should, however, be kept in mind that because a single type of interaction is seldom responsible for the binding, nonconventional approaches are sometimes worth trying.

2.3 CHROMATOGRAPHIC THEORY

2.3.1 Chromatographic Quantities

A large number of quantitative concepts are used to describe a chromatographic experiment. Some of the most significant of these will be discussed here.

2.3.1.1 Retention Parameters

A chromatogram is a plot of concentration at the column exit versus time or eluent volume. The volume that has passed the column from the introduction of sample until the emergence of a certain component as a peak in the chromatogram is the retention volume, \( V_R \) (see Fig. 2.10).

Instead of retention volume, retention time, \( t_R \), is often used, which may be easier to measure. Obviously, \( V_R = F_C t_R \), where \( F_C \) is the volumetric flow rate. The numerical value of the retention volume depends largely on the column used. To obtain a normalized retention quantity, the retention factor, \( k \), is generally used. It is equal to the number of void volumes, \( V_0 \), needed to elute the compound of interest minus one. Thus,

\[
    k = \frac{V_R}{V_0} - 1 \quad \text{or} \quad V_R = V_0(1 + k). \quad (2.1)
\]

In Figure 2.10 an alternative x-scale is graduated in units of \( k \). Often, the retention factor is referred to as the capacity factor and written as \( k' \). This is, however, in variance with official recommendations for the terminology of chromatography. In this chapter, the terminology recommendations by IUPAC (36) is followed where relevant.

Another normalized retention measure is the retardation factor, \( R_F \), which is the ratio between the velocity of the actual component and that of the mobile phase. Consequently,

\[
    R_F = \frac{V_0}{V_R} = \frac{1}{(1 + k)}. \quad (2.2)
\]

\( R_F \) is used particularly in noncolumn versions of chromatography (e.g., thin layer chromatography). When plotted in a chromatogram as in Figure 2.10, a scale in units of \( R_F \) is nonlinear.

The void volume or hold-up volume, \( V_0 \), is usually seen as the volume of mobile phase in the column. With liquid chromatographic columns packed with small particles or gels, this simple correspondence is unfortunately not exact, because small molecules penetrate the pores to a greater extent than large molecules, and therefore are distributed in a larger volume of liquid. Even in the absence of physical or chemical interactions with the stationary phase itself, this effect leads to the separation of molecules of different sizes: molecules of a particular size will emerge at the column outlet after passage of a volume that is equal to the column volume that is available to that size. This is the principle of gel filtration chromatography, where the porosity characteristics of the materials are chosen to maximize this effect. In other types of chromatography, the effect should be minimized by using pores that are wide enough to accommodate all molecules of interest. This is especially important for chromatography of proteins. The concept of hold-up volume was recently discussed in an IUPAC recommendation paper (37).

2.3.1.2 Peak Shape and Width: The Theoretical Plate Concept

A separation of two compounds is possible only when their velocities (\( R_F \) values), and therefore their \( k \) and \( V_R \) values, differ by some amount. As is obvious from Figure 2.11, additional parameters related to the width of the peaks are needed to specify the resolution of two compounds.

To describe the width of chromatographic peaks, the concept of (theoretical) plate number, \( N \), is commonly used (38). This term is related to fractional distillation and is somewhat misleading, because there are no plates, either theoretical or otherwise, in a chromatographic column. The proper definition is based on statistical theory,

\[
    N = \frac{k^2}{\sigma^2}. \quad (2.3)
\]

Figure 2.10 Relation between various retention parameters.
where $\mu^2$ and $\sigma^2$ are the mean and variance of the chromatographic peak, respectively. In practice, one of the following formulae is used to calculate $N$ from a chromatogram:

$$N = 5.55 \left( \frac{t_R}{w_{1/2}} \right)^2 \quad (2.4a)$$

$$N = 16 \left( \frac{t_R}{w_b} \right)^2 \quad (2.4b)$$

$$N = 6.28 \left( \frac{t_R h_p}{A_p} \right)^2. \quad (2.4c)$$

Here, $w_h$ and $w_b$ are the width of the peak at half the peak height, $h_p$, and at the base, respectively (see Figure 2.12), and $A_p$ is the area of the peak.

To apply these formulae, it is not important whether $t_R$ or $V_R$ is used. It is, however, crucial that the widths are measured in the same units at $t_R$. Typically, they are measured in centimeters, directly from the recorder paper using a ruler. Equation 2.4c is intended to be used with an electronic integrator or a computer system, which usually supplies values for $t_R$, $h_p$, and $A_p$, but often no peak widths.

Equations 2.4 are derived from the Gaussian (normal) probability curve, which is a fair description of a chromatographic peak in simple cases. It should be emphasized that they are only valid in isocratic chromatography and when the peak is reasonably symmetrical. For tailing peaks, which are often obtained, it is not meaningful to calculate the number of theoretical plates.

The theoretical plate concept is also used widely to characterize the performance of a chromatographic column. To a first approximation, all (symmetrical) peaks in a chromatogram show roughly the same plate number. Consequently, this number can be considered as a property of the column used. A large plate number means narrow peaks and thus a "good" column.

As the plate number is approximately proportional to the column length $L$, column quality can also be expressed in terms of theoretical plates per meter column or height of a theoretical plate (HETP), $H$, which is defined as

$$H = \frac{L}{N}. \quad (2.5)$$

Thus, for a good column, the value of $H$ is small.

2.3.1.3 The van Deemter Equation

According to the theory originally developed by van Deemter and co-workers (38) for gas chromatography, the plate height $H$ is a sum of three independent contributions, which depend on the flow rate of the mobile phase according to the equation

$$H = A + \frac{B}{u} + C \cdot u. \quad (2.6)$$

Here, $u$ is the linear flow rate, and $A$, $B$, and $C$ are constants. A plot of the van Deemter equation will show a minimum at a certain flow rate (Fig. 2.13). This is the optimal flow rate at which a maximal plate number is obtained. In practice, especially for liquid chromatography of macromolecules, the optimal flow rate is often impractically low, and columns are operated at considerably higher flow rates. The third term in the van Deemter equation then becomes especially important.

A detailed interpretation of the terms is complex but rewarding. The classical work in this field is Giddings’ "Dynamics of Chromatography" (1). For another more recent and fairly complete treatment the reader is referred to Reference 3.

Briefly, the first term, $A$, the eddy term, describes peak broadening resulting from the complicated geometry in a packed column. In other words, some molecules will find a relatively straight path through the column, whereas others may follow a longer, more tortuous path. The second term,
B/u, results from diffusion of sample molecules along the column. The third term, C·u, originates from various kinetic parameters, such as slow transfer of molecules into and out of pores or within the stationary phase and noninstantaneous equilibrium.

Both the A term and the C term depend on the diameter \(d_p\) of the column packing particles. A is directly proportional to \(d_p\), but the relation for C is more complex. The contribution to the C term of slow mass transfer in pores, and so on, is proportional to \(d_p^2\), whereas the nonequilibrium contribution is independent of \(d_p\) (1). Using smaller particles thus leads to a lower plate height and a larger plate number. Note that the effect might be largest for the C term, thereby moving the minimum in the van Deemter plot towards higher flow rates and decreasing the plate height, especially for high flow rates, thereby permitting efficient separations in shorter times. The price to be paid is the necessity of using high pressure pumps to force the mobile phase at high flow rates through columns with high resistance. This is the reason why the P in HPLC can mean “performance,” “pressure,” and “price.”

In order to facilitate comparisons among columns of different types the concepts of reduced plate height, \(h\), and reduced flow rate, \(v\), are often used. They are defined as

\[
h = \frac{H}{d_p} \quad \text{and} \quad v = \frac{u \cdot d_p}{D_M}, \tag{2.7}
\]

where \(D_M\) is the diffusion coefficient to the solute in question in the mobile phase. The adoption of reduced parameters removes the influence of \(d_p\) and \(D_M\) from the A and B terms in the van Deemter equation, but the C term will still depend on these parameters.

All the above treatment assumes that the liquid flow is laminar. There is evidence that at very high liquid flow rates, turbulence will occur. This leads to greatly improved mass transfer, so the C term will go through a maximum and eventually decrease, resulting in very small plate numbers at high flow rates, which obviously is a potentially desirable situation. This mode of operation is called turbulent flow chromatography, and it is mainly applied for very fast separation of small compounds (39).

2.3.1.4 Resolution In a chromatogram of a complex sample, a great number of peaks are to be separated, but it is only necessary to consider one pair of peaks at a time. The peak resolution, \(R_S\), of this pair is defined by

\[
R_S = \frac{\Delta t}{2\left(\omega_{b1} - \omega_{b2}\right)} \tag{2.8}
\]

that is, the distance between the peak maxima divided by the mean peak width (Fig. 2.14). Just as for Equations 2.4, retention volumes can be used instead of times. For \(R_S < 1\), the peaks are incompletely separated, and for \(R_S = 1\), they just touch each other at the base, but for \(R_S > 1\), there will be a stretch of baseline between the peaks. Optimal separation conditions are reached when \(R_S \geq 1\) for all pairs of interest. If \(R_S\) is considerably greater than 1 for all pairs, the resolution is unnecessarily good and the separation can be made faster.

Upon combination with Equation 2.4b the resolution can be written

\[
R_S = \frac{\alpha - 1}{\alpha + 1} \cdot \frac{k}{1 + k} \cdot \frac{\sqrt{N}}{2}, \tag{2.9}
\]

where \(k\) with a bar over is the mean of the retention factors for the two peaks, \((k_1 + k_2)/2\), and \(\alpha\) is the relative retention or separation factor, \(k_1/k_2\).

The resolution depends, according to Equation 2.9, on three more or less independent factors: the selectivity, the retention, and the plate number. To optimize the resolution, each of these factors should be considered.
The selectivity, \((\alpha - 1)/(\alpha + 1)\), is usually the most important. For example, an increase of \(\alpha\) from 1.01 to 1.02 (i.e., only 1% change) will double the resolution. On the other hand, if \(\alpha = 1\), no separation is possible. The retention, \(k/(1+k)\) increases with \(k\), but when \(k\) is larger than 5 the increase is marginal and will mainly result in slower separation. If \(k < 1\), the resolution is unnecessarily low. Finally, an increase in the plate number \(N\), for example by using a longer column, will increase resolution. However, a fourfold increase in column length is necessary to double the resolution.

Facing an inadequate resolution, the most effective remedy would probably be to change the chromatographic conditions, aiming at a higher relative retention. Changes in pH or in the composition of the eluent are the usual approaches tried to increase \(\alpha\). A mere increase of column length might be impractical, and would considerably increase separation time (but not necessarily the total time spent on the entire task, especially if only a limited number of runs are to be made!).

If the problem is the opposite and more pleasant one, to speed up an unnecessarily effective separation, the use of a shorter column will be the best solution, offering a considerable time saving on each run. A higher relative retention is also beneficial, as it permits the use of still shorter and faster columns.

2.3.2 Retention in Adsorption Chromatography

Most versions of chromatography used for protein separation (except gel filtration chromatography) may be more or less adequately treated together under the term adsorption chromatography. This implies that the sample molecules are adsorbed onto the surface (or in a thin surface layer) of the stationary phase. The precise nature of the adsorption forces varies among the techniques and can, for the moment, be disregarded.

2.3.2.1 The Adsorption Isotherm

The central concept in adsorption is the adsorption isotherm, which is a plot of the sample concentration on the adsorbing surface of the stationary phase versus the sample concentration in the mobile phase. These concentrations are written \(C_S\) and \(C_M\), respectively. Concentration \(C_S\) may be expressed either in units of moles per surface area or moles per gram of adsorbent. \(C_M\) is expressed in moles per litre.

A general shape of such a plot is shown in Figure 2.15. The curved shape of the isotherm originates from competition among sample molecules for adsorption sites. The most simple description of this competition is given by Langmuir (40). Assume that the adsorbent has a fixed number of equal ligands or adsorption sites, \(S\), to which the sample protein molecules, \(P\), bind one-to-one in a reversible way. The following equilibrium then applies:

\[
P + S \rightleftharpoons PS; \quad K = \frac{[PS]}{[P][S]}, \quad (2.10)
\]

where \(K\) is an association constant. Clearly \([PS] = C_S[P] = C_M\) and \([S] + [PS] = Q\), the adsorbent or binding capacity, that is, the number of sites per unit surface area (or weight). This has nothing to do with the capacity factor \(k\) as defined in Equation 2.1. We can easily solve the equilibrium equation for \(C_S\), which leads to Langmuir’s adsorption isotherm equation:

\[
C_S = \frac{Q \cdot K \cdot C_M}{1 + K \cdot C_M}. \quad (2.11)
\]

For most adsorption equilibria we must consider the competition of two different counterligands, one of which is the sample molecule, \(P\), and one is a component, \(E\), of the eluent:

\[
P + ES \rightleftharpoons E + PS; \quad \frac{[PS][E]}{[P][ES]} = K_{P/E}. \quad (2.12)
\]

Here, \([PS] = C_S\), \([P] = C_M\), and \([PS] + [ES] = Q\), and \(K_{P/E}\) is a selectivity constant, the quotient of the relevant association constants.

From this, we again obtain Equation 2.11 with \(K = K_{P/E}/[E]\). For a fixed eluent concentration the Langmuir equation is thus also valid for competition equilibria. The model is also immediately applicable to an ion-exchange equilibrium, where \(P\) signifies the protein ions, \(E\) is an ion in the eluent, and \(Q\) is the ion exchanger capacity.

In practical protein purification applications, there may be (and usually are) interfering molecules of several kinds in the sample. Also in this case, the Langmuir model still applies, but with a conditional constant that incorporates the influences of the competing molecules.
Because the natures and concentrations of interfering sample components are generally unknown or at least incompletely known, the value of measured $K_{P/E}$ values for prediction of retention volumes is limited.

The assumptions upon which the derivations above are based may not be entirely applicable in all cases: the adsorption sites may be unequal, there may be multisite binding (certainly relevant for macromolecules), and there may be interactions (e.g., repulsion) between adsorbed molecules. In these cases the Langmuir model is not quantitatively correct, but in practice it often has a reasonable semiquantitative validity.

2.3.2.2 Chromatographic Retention In one of the first papers on chromatographic theory (41), the relation between the retention volume and the adsorption isotherm was derived:

$$V_R = V_0 + A_S \frac{dC_S}{dC_M}.$$  \hspace{1cm} (2.13)

Here, $A_S$ is the total area of the adsorbent in the column or, alternately, the weight of the adsorbent, depending on the definition of $C_S$ (see above). Inserting Equation 2.11, we obtain, after differentiation,

$$V_R = V_0 + \frac{A_S \cdot Q \cdot K}{(1 + K C_M)^2},$$  \hspace{1cm} (2.14)

which is the proper expression of the retention volume, assuming a Langmuir adsorption isotherm.

If the concentration $C_M$ of the sample is small enough, the denominator in the second term of Equation 2.14 is practically unity, leading to

$$V_R = V_0 + A_S \cdot Q \cdot K = V_0 + n_T \cdot K,$$  \hspace{1cm} (2.15)

where $n_T$ is the total number of adsorption sites in the column. From Equation 2.1, we find for the retention factor that

$$k = \frac{n_T}{V_0} \cdot K.$$  \hspace{1cm} (2.16)

The retention volume here is independent of sample concentration and is solely determined by column parameters and by the equilibrium constant $K$. As mentioned in Section 2.3.1.1, $V_0$ may vary slightly with molecular size, which influences the retention.

This case is termed *linear chromatography*. In analytical applications of chromatography this is the preferred case. It is characterized by a simple theoretical description of peak retention and dispersion (broadening). The concepts related to theoretical plates as described above are valid. The term *linear* refers to the fact that it is equivalent to the assumption of a linear adsorption isotherm. In reality, the isotherm must be curved (see Fig. 2.15), but at sufficiently low concentrations the curvature becomes negligible.

In many cases, especially when chromatography is used for preparative purposes (which is important in the context of this book), the assumption of linearity is not valid due to the relatively high concentrations of sample. If this happens, the retention volume varies with sample concentration $C_M$, as described by Equations 2.13 and 2.14. This leads to asymmetric tailing peaks, as parts of a peak with low concentrations are retarded more than the parts with high concentrations. In Figure 2.16, tailing peaks of different sizes are shown. The width of a tailing peak depends partly on the dispersion factors as described below, but these are interrelated with the peak broadening due to the tailing itself in an intractable way. Consequently, theoretical plates and related quantities are not applicable to tailing peaks and should not be calculated. It is impossible to treat this general problem of nonlinear chromatography in a mathematical way.

Observe that although the component of interest may not be present at high concentration, other components may, and often are. All components can then compete for the same adsorption (ion exchange) sites and, consequently, influence each other’s retention.

The conditions prevailing when a mixture of proteins is chromatographed are very complex, and attempts to calculate retention volumes from batch experiments or chromatography of pure compounds can be very inaccurate.

An erroneous interpretation of retention in nonlinear chromatography, usually implicitly expressed as a nonconstant retention factor, is found in several texts. The retention factor can be written as (Eq. 2.16)

$$k = \frac{A_S}{V_0} \cdot \frac{C_S}{C_M}.$$  \hspace{1cm} (2.17)

This is correct in the case of linear chromatography, and is often stated as an alternate definition of $k$. However, this is
not generally true, and applying Equation 2.14 to nonlinear chromatography is dangerous. It would imply that

\[ V_R = V_0 + A_S \cdot \frac{C_S}{C_M} \]  (2.18)

instead of the correct Equation 2.13. The difference between Equations 2.13 and 2.18 is conceptually difficult and leads seemingly to a paradox. The matter was clarified by Helfferich (43).

2.4 CHROMATOGRAPHIC PROCEDURES

2.4.1 Sample Introduction

The usual way to perform a chromatographic experiment involves plug injection, for example, the introduction of a small volume of sample at the beginning of the column. The width of this plug obviously influences the width of the resulting peaks. In theoretical discussions, the width of the plug is usually assumed to be negligible. However, in preparative chromatography wider plugs may be tolerated, thereby permitting the introduction of a larger volume of sample. See the example in Figure 2.17.

As the introduction of larger amounts of sample may cause overloading and nonlinear conditions (see Section 2.3.2.2) the effect is generally more complex than an increase in peak widths.

A special case is frontal chromatography, in which the pure eluent is exchanged for a solution of sample, which is pumped into the column. The sample will appear, after some time, as a more or less steep concentration step in the detector. The midpoint of the step corresponds to the retention time and the height of the fully developed step to the initial sample concentration. With several components, several steps will build up and only the fastest component will be (partly) separated from the other components. If the sample is again exchanged for pure eluent, negative steps will occur and the result is equivalent to a large plug. Figure 2.17c is an example of such a frontal chromatogram. The technique is used mostly for determination of physicochemical parameters by chromatography, where it has some advantages, and for sampling and preconcentration of dilute samples. It may also unintentionally be encountered in preparative chromatography after introduction of excessively wide plugs. In the latter case, it is important to realize that a chromatogram such as that in Figure 2.17c contains two components, not three.

2.4.2 Chromatographic Development

2.4.2.1 Isocratic Elution The simplest mode of chromatographic development is the isocratic elution mode, in which all conditions are held constant throughout the experiment. Each component of the sample will thus travel through the column according to Equations 2.13–2.15.

The retention times of the various components and therefore the degree of separation is determined by the corresponding values of \( K \). As seen above (Section 2.3.2.1), this constant may be interpreted in several ways, depending on the physical process on which the separation is based. Generally, a necessary condition for a successful isocratic elution is that all sample components elute in a reasonable time and that compounds of interest are not eluted too early (which would destroy resolution, c.f. Eq. 2.9). This can only be accomplished for a narrow range of sample types, and the application of isocratic elution to a wide range of sample types leads to disturbing trade-offs regarding separation power and time. This is sometimes referred to as the general elution problem. To solve this problem, it is necessary to make use of gradient elution, where the composition of the eluent is changed during development. The change may be continuous (usually linear) or stepwise, and the object is to decrease \( K \) successively for each component with time.

2.4.2.2 Gradient Elution This increase in “elution power” may be accomplished in various ways for different chromatographic techniques. In reversed phase liquid chromatography, the polarity of the eluent is decreased, thereby decreasing the partition coefficients. In ion exchange chromatography, the concentration of an eluent ion is increased, leading to a decrease in the apparent \( K \) (see Eq. 2.12). In several other techniques the successive addition of competing compounds will affect the apparent \( K \) in the desired direction.

In the beginning of the chromatographic run, a low elution power is chosen. Then, the components that are most loosely

![Figure 2.17](image-url)
bound to the adsorption (etc.) sites will elute under favorable conditions. Other more strongly held compounds will be successively eluted as the elution power is increased. The result is usually an increased resolution over a wide range of sample compounds.

The retention times in a gradient elution run cannot be calculated directly from Equation 2.15 (and similar equations), because the parameter $K$ varies with time. If the variation of $K$ with time is known, the retention time can, in principle, be calculated by integration, a procedure that is rarely applied.

The plate number concept as defined previously is not relevant in the case of gradient elution, and the application of Equation 2.4 to a gradient chromatogram leads to gross overestimates of $N$. Beware of excessive column performance claims produced in this way!

2.4.2.3 Displacement Chromatography An extreme case of gradient elution is displacement chromatography, which is based on competitive binding between the sample components themselves and an additional compound, the terminal displacer or developer, which is more strongly adsorbed than any of the proteins in the sample. During the course of the experiment the continuously added terminal displacer will push the sample components in front of itself, forcing them to displace each other, thereby forming a so-called displacement train in which the different molecules will arrange themselves in the order of their interaction strength with the adsorption sites of the column. In order to improve the resolution between the sample components, spacers (i.e., molecules with intermediate adsorption strengths) are usually added. Examples of such compounds are the carrier ampholytes used in isoelectric focusing or carboxymethyl dextrans. A detailed description of displacement chromatography is given in Reference 44. A review of displacement chromatography of proteins is given in Reference 45 and of peptides in Reference 46. A brief discussion can also be found in Chapter 4 of this book (see Section 4.8.2.5).

2.4.3 Determination of the Column Capacity and Association Constants

The chromatographic retention depends, as seen above (e.g., in Eq. 2.14), on essentially two parameters other than column dimensions: namely, the column binding capacity $Q$ and the (apparent) association constant $K$.

The capacity $Q$ for a particular protein is a complex function of several parameters: matrix composition and matrix pore structure, particle diameter and particle size distribution profile, protein molecular weight and solubility, forward and backward rate constants for the binding reaction, bulk-, film-, and gel pore diffusion constants of the protein, and finally, the event of possible competitive binding and displacement effects of other proteins present in the sample solution. It is convenient and useful to distinguish between the nominal binding capacity of a particular stationary phase such as an ion exchanger and its dynamic or functional binding capacity. The nominal binding capacity of an ion exchanger can, for example, be determined by an acid–base titration.

This value, however, does not reveal how much protein will bind under normal operating conditions in a running column. This can only be measured by chromatographic methods and can for a single column vary considerably for different proteins. For a single protein the dynamic binding capacity varies significantly with the flow rate used during adsorption and washing.

The determination of the dynamic, or functional, binding capacity is usually performed by frontal chromatography (see Section 2.4.1) under isocratic conditions. Figure 2.18 shows typical fronts in nonlinear adsorption chromatography.

A positive step-injection, that is, the exchange of pure eluent for a solution of sample with concentration $C_0$, will produce a sharp front at the outlet as shown in Figure 2.18b, whereas a negative step-injection (removal of the sample feed) produces a tailing, diffuse concentration step (Fig. 2.18a). It is easiest to treat the second case, as the tailing slope follows Equation 2.14.

The area $A$ in Figure 2.18 is found by integration:

$$A = \int_0^{C_0} V R \cdot dC_M.$$ (2.19)

If we arrange the experiment to make $K$ very large, with Equation 2.14 we obtain

$$A = V_0 C_0 + A_5 Q.$$ (2.20)

Area $A$ in Figure 2.18b corresponds to the amount of sample that has been held on the column, whereas the analogous area

![Figure 2.18](https://example.com/figure218.png)

**Figure 2.18** Nonlinear chromatographic fronts.
in Figure 2.18a corresponds to the same amount that is released when sample feed is removed. Consequently, these areas are equal. The capacity \( Q \) can easily be calculated from a sharp frontal chromatogram, as the area \( A \) in such a case will be nearly rectangular,

\[
Q = \frac{C_0}{A_S} (V_R,\text{front} - V_0),
\]

(2.21)

provided that \( K \) is large. \( V_R,\text{front} \) is the retention volume measured from the start of sample introduction to the sharp front. In order to obtain a larger \( K \), we should choose a suitable sample component (we intend to measure a property of the column, so there is a choice of sample) and an eluent with low eluting power, free from competing compounds. To obtain valid values of \( Q \) it is advisable to repeat the measurement under different conditions. In some cases, the binding equilibrium might be slow. This is often the case in affinity chromatography. The result is that the shape of the front is not sharp but rounded. The measurements described are still valid, provided that area \( A \) is calculated by integration of the recorder signal.

Determination of the association (partition) coefficients, \( K \), can in some cases be performed by nonchromatographic methods. Hutchens and Yip determined binding constant and capacities of several proteins to IMAC gels by means of Scatchard plots, that is, equilibrium binding (47). The values obtained agreed nicely with those derived from frontal chromatography. The chromatographic determination of \( K \) is simple: the retention volume of a peak obtained after the injection of a narrow sample plug of low concentration directly gives (Eq. 2.15) \( A_S \cdot Q \cdot K \). If \( A_S \cdot Q \) is determined as described above, \( K \) is easily calculated.

## 2.5 CHROMATOGRAPHIC TECHNIQUES

### 2.5.1 General Comments

Modern column chromatography utilizes sophisticated equipment to obtain high resolution separations. However, for some applications this might not be necessary. The simplest way to carry out an adsorption experiment is batchwise, that is, by mixing the adsorbent with the protein sample and choosing proper conditions for adsorption and subsequent desorption. This has the advantage of being applicable with large volumes of protein solution, which do not necessarily need to be clear, and is sometimes of use at an early stage in a purification procedure or simply to test whether adsorption occurs under the conditions selected.

Column chromatography can be performed as low pressure (or standard), medium pressure, or high pressure liquid chromatography (HPLC). Medium pressure techniques also include fast protein liquid chromatography, for example the chromatography system FPLC™ (GE Healthcare Life Sciences). For desalting experiments, adsorption tests, and so on, simple column chromatography equipment is often sufficient. High resolution results can also be obtained with standard, low pressure equipment provided the selectivity of the column packing material is sufficiently high. High resolution fast techniques require small diameter beads and equipment able to withstand the often high pressures necessary to force the buffer or solvent through the column.

In all three techniques, the size, and thus the capacity, of the chromatographic column can vary considerably. Medium scale (from about 1 mg to 1 g of protein) is usually the easiest to handle. Microgram scale requires sensitive analytical techniques, high purity of buffers and solvents (although less volume is consumed), and special care often has to be taken to avoid adsorption to the walls of vessels. Equipment for large scale chromatography runs into other difficulties, in particular the high cost of larger amounts of modern HPLC media. However, most industrial process chromatography separations of proteins today are based on 90-μm-diameter standard media such as Sepharose Fast Flow. Useful information on large scale chromatography can be found in References 48–53.

The most crucial point in column chromatography is to achieve a good column packing. This is therefore treated in some detail in Section 2.5.2 of this chapter. FPLC and HPLC columns are normally delivered prepacked from the manufacturers. However, most other gels are delivered in bulk and must be packed in the user’s laboratory.

### 2.5.2 Packing of Columns

In any chromatographic experiment the result obtained can never be better than the quality of the column packing allowed. This is why it always pays off in the long run to learn how to pack the most commonly used stationary phases. One should be particularly cautious when packing columns for gel filtration and other isocratic techniques. Detailed packing instructions are usually provided by the manufacturers of a particular gel material. Here only some general principles will be discussed.

The packing techniques used differ depending on the type of stationary phase. The most important discriminating parameter is the rigidity of the gel matrix. It is thus convenient to distinguish between soft, semirigid, and rigid gel matrices. Particle shape, diameter, and size distribution are also important parameters to consider in column packing. The first step is to mount the column with its extension tube on a steady laboratory stand and to ensure that the column tube is perfectly vertical. The stationary phase slurry is degassed to remove all trapped air.

**Rigid gel materials** such as silica with particle diameters in the range 5–15 μm are preferably slurry packed in dry acetone or chloroform. Slurry concentrations around 10% and packing pressures up to ~300 kg/cm² usually give satisfactory results.
Semirigid gels such as Sephacryl HR, Sepharose FF, Superose, and Capto are preferably packed in two steps. In the first step, a homogeneous slurry (10–50%) containing all the stationary phase intended for the column is poured into the column fitted with an extension tube. The extension tube is connected to a pump, which is adjusted to a medium high flow rate. For a 45-µm Sephacryl HR this means ~30 cm/h for columns between 40 and 100 cm in length. When the bed has settled, the second step in the column packing involves a doubling of the flow rate to, in this case, ~60 cm/h. The packing of the column should not be considered complete until 4–5 column volumes of packing buffer have been pumped through. The crucial feature of this procedure is that it prevents the formation of a plug of hard packed gel at the bottom frit of the column, which will inevitably occur upon packing in one step at constant pressure with an initial high flow rate. This plug will block the flow and give rise to badly packed columns.

Every column should be tested for packing quality. A zone of acetone (0.5% V₁) at 30 cm/h is suitable for this purpose. The reduced plate height should fall in the range 2–3, at the lower end for experienced column packers and at the upper end for beginners.

The packing buffer composition for semirigid gels does not seem to be critical. Similar results are obtained with distilled water and with various buffer salt solutions. Most convenient is to use the slurry obtained by shaking the original bottle in which the gel is delivered, and dilute with distilled water to the desired slurry concentration. The column bottom frit or filter mesh should be wetted and all air removed. Ethanol (20%) is recommended for this purpose. A few centimeters of this solution can be left in the column before the addition of the gel slurry.

A critical point in the column packing is the application of the adaptor on top of the packed bed. As a general recommendation one should allow the adaptor to compress the upper part of the bed by ~5 mm. Some workers prefer to pack the columns upside down towards the adaptor. In this way they get the best possible starting conditions for the sample zone, which is especially desirable in gel filtration. The bottom end piece is then allowed to compress the packed bed a few millimeters as described above.

For the short columns (5–15 cm in length) normally used in various types of adsorption chromatography (ion exchange chromatography, affinity chromatography, etc.) packing quality is less critical. Often, one-step procedures based on either constant flow or constant pressure give equally satisfactory results. Larger diameter, semirigid stationary phases such as derivatives of Sepharose FF (90 µm) give too little flow resistance to be packed efficiently with normal laboratory pumps. This is why many workers pack this matrix using compressed air or nitrogen as a pressure source and regulate the flow with a needle valve at the column outlet. Sepharose FF is preferably packed in two steps in the way described above. The bed is thus allowed to settle at a linear flow rate of ~3 cm/min and the final packing takes place at ~5 cm/min. One should be aware of the danger of using pressurized vessels and never allow the pressure to exceed the ratings of the equipment used. For normal laboratory columns a pressure drop of 2 kg/cm² is sufficient to give efficient packing of Sepharose FF. As a general precaution, the column and accessories should always be placed behind a protective screen or cover during column packing. Some workers claim that the packing of Sepharose FF is facilitated by the presence of 0.05–0.1% Tween20.

Soft gel matrices such as cellulose, Sephadex with higher G-numbers, noncrosslinked agarose gels such as Sepharose, and conventional polyacrylamide gels with low degrees of crosslinking are, in principle, packed in the same way as the semirigid gel materials with the exception that the flow rates used are considerably lower and are never allowed to approach the maximum flow rate obtainable in a particular column. Neither, of course, should the operating flow rate exceed the packing flow rate of the bed.

2.5.3 Perfusion Chromatography

In perfusion chromatography (54), column packing particles with large pores, passing through the entire particle are used. Similar materials have also been termed “gigaporous” (55). A part of the mobile phase, ~5%, may flow through these pores. This significantly decreases the resistance to mass transfer caused by diffusion in pores, because the molecules to be separated are brought to the inner of the particle by convection, not only by diffusion. The result is a smaller C term in Equation 2.6, and the possibility for rapid separations without the need for especially small particles and the accompanying need for high pressure. These advantages are especially noted for chromatography of macromolecules due to the low diffusion coefficients of large molecules.

Under optimized conditions, perfusion chromatography can also be performed in agarose gel media. Thus, a procedure for the preparation of superporous agarose gel media has been reported (33). Agarose beads (300–500 µm diameter) containing perfusive pores with diameters of ~30 µm, allowing convective mass transport, could be prepared using a double emulsification process.

2.5.4 Membrane Chromatography and Chromatography using Monolithic Columns

In parallel to perfusion chromatography, high performance membrane chromatography and chromatography using continuous polymer beds (monolithic columns) have been developed. In both these cases, micropore diffusion restriction is largely eliminated because all of the mobile phase flows through the macroporous polymer matrix network. However, as relatively large pores are required to allow
efficient flow-through in these systems, the total column binding capacity is reduced and this is why these systems are less useful from a large scale preparative point of view. For a comprehensive treatment of chromatography using monolithic columns the reader is referred to publications by Jungbauer and co-workers (References 56–61, of which Reference 61 is a recent review).

2.5.5 Field-Flow Fractionation (FFF) for Protein Separation

Field-flow fractionation (FFF) is a separation technique that was invented and developed by J. Calvin Giddings in 1966 (62). The separation is performed in open channels by the interaction between a parabolic flow profile and a perpendicular field. This “field” is most often a perpendicular liquid flow, but gravitational or thermal fields can also be applied. Instrumentation for FFF is commercially available (63).

FFF is not strictly a version of chromatography, as separations are performed in one phase only. It is an important complement to chromatographic techniques, as it is suited for separation of large to superlarge molecules and particles, from proteins, ribosomes and other organelles, to entire cells. There are also many technical applications, for example in the field of polymer science. Several current reviews give details about the technique and its applications in biological sciences (64–66).

2.6 ON THE HISTORY OF PROTEIN CHROMATOGRAPHY

The purpose of this review is to give an overview of the events that led to the development of the most important column packing materials, techniques, and methods for protein chromatography. For more detailed information the reader is referred to the original literature.

2.6.1 Contributions up to around 1950

Pioneers such as Willstätter, Northrop, Sumner, Kunitz, Kraut, Grundmann, Zechmeister, Turba, and others were restricted to low resolution techniques and methods such as salt precipitation and batch adsorption in their efforts to develop protein purification procedures. For batch adsorption they used solid phases such as alumina hydrates (Al₂O₃·nH₂O, e.g., bauxite), Al-silicates (bentonites), Mg-silicates (soapstone, etc.), silicas (SiO₂·nH₂O), Ca- and Zn-carbonates, Ca-phosphate, iron oxides (hematite, Fe₂O₃), and Al,Mg-silicate (florisil). Early attempts regarding chromatography of proteins were based on the use of these adsorbents, often mixed with various filter aids. A thorough review of these early contributions was published by Fritz Turba in 1954 (67).

In a review with 771 references, entitled “Analytical Chemistry of the Proteins” published in Advances in Protein Chemistry, Volume 2, 1945, the chromatography pioneers, and Nobel Laurates of 1952, A.J.P. Martin and R.L.M. Synge wrote

“...So far the chromatographic methods have not been sufficiently widely used to permit of any final assessment of their value, although the beginning is most promising. Of the adsorption chromatographic methods, Wieland’s acid-alumina method for the dicarboxylic acids is the only one to have been used on proteins ...”

Obviously, protein chromatography was in a very early stage of development in the middle of the 1940s.

In another review of 1951 by Zechmeister and Rohdewald (68) entitled “Some Aspects of Enzyme Chromatography, III. Chromatography as a Step in the Purification of Enzymes” the authors stated

“... So far as we know chromatography alone will usually not lead to preparations of highest purity or crystalline enzymes from a crude extract. However, in combination with other procedures such as precipitation, salting out etc. it represents a valuable tool and offers, under favourable circumstances, a short cut on an otherwise lengthy road ...”

In yet another review from 1953 by Schwimmer and Pardee (69) entitled “Principles and Procedures in the Isolation of Enzymes,” the authors wrote

“... Some workers feel that chromatography will not have general applicability to the precise separation of proteins because of the extremely large molecular weights of the proteins and their subsequent slow diffusion ...”

Also in 1953, Zittle (70), in a review entitled “Adsorption Studies of Enzymes and Other Proteins” drew the following conclusions:

“... reveals several factors that impose limitations on the chromatography of many proteins. These factors are the frequent encountered irreversibility of adsorption, the slow rate of adsorption and the nonlinearity of adsorption curves ...”

Zittle also made the following visionary conclusion:

“... It appears that a search for specific adsorbents might lead to results that would further broaden the application of this method. A study of adsorbents simulating the specific substrates of particular enzymes is seriously needed to determine the nature of the phenomena and whether this type of adsorbent can be made to order ...”

Fifteen years later, the vision by Zittle would turn out to be a reality by the introduction of affinity chromatography by...
Cuatrecasas, Wilchek, and Anfinsen in their classical paper of 1968 (71).

In the early 1940s, Tiselius introduced the fundamental chromatographic principles of frontal analysis, elution analysis, and displacement development (72). Tiselius preferred the term analysis over the term chromatography, which at the time he regarded a misnomer. However, he later changed his mind. These studies were followed up by the introduction of the modifications “carrier displacement” (73) and “gradient elution” (74).

Tiselius’ first attempts to apply these principles to proteins used frontal analysis for salting-out of ovalbumin, serum albumin, and immunoglobulin to silica in the presence of varying concentrations of ammonium sulfate (75, 76).

### 2.6.2 Ion Exchange Chromatography and Hydroxyapatite Chromatography

Thanks largely to the “Manhattan Project,” porous organic polymer ion exchangers based on monomers such as styrene, methacrylic acid, and divinyl benzene became commercially available during the 1940s. The weak cation exchanger Amberlite™ IRC-50, a co-polymer of methacrylic acid and divinyl benzene, was used in some of the first published protein separations, the purification of low molecular weight basic proteins such as cytochrome c (77) and ribonuclease A (78). Neutral proteins such as hemoglobins were also successfully separated using the same ion exchanger (79).

However, the major early breakthrough in protein chromatography was the introduction of the DEAE- and CM-cellulose ion exchangers by Sober and Peterson (80, 81) and calcium phosphate (hydroxyapatite, Ca$_{10}$(PO$_4$)$_6$(OH)$_2$) by Tiselius and co-workers (82, 83). Somewhat later, strong anion exchangers (quaternary ammonium) were introduced by Porath (84).

In the early 1960s, ion exchangers based on crosslinked dextran gels (Sephadex) were introduced by Pharmacia (85) and quickly found several applications, not least in the plasma protein separation area. An industrial batch method using DEAE–Sephadex A-50 as a polishing step in IgG production was therefore developed. For a review see the paper by Björling (86). The major drawback of the Sephadex ion exchangers, their significant shrinking and swelling at high and low ionic strengths, respectively, was overcome when ion exchangers based on crosslinked agarose, such as DEAE–Sepharose CL-6B and SP-Sepharose CL-6B, were put on the market by Pharmacia Fine Chemicals AB (Uppsala, Sweden) in the middle of the 1970s. Ten years later, these were followed up by the second-generation agarose ion exchangers based on the Sepharose Fast Flow series, produced using an improved crosslinking manufacturing process. The physical characteristics of these 90-μm (average) beaded agarose particles enabled, for the first time, industrial-scale chromatography of proteins. Thus, DEAE–Sepharose FF was, immediately after its launch, packed in a 300 L column and used as the first step in the purification process for recombinant human growth hormone at the biopharmaceutical company Kabi (Stockholm, Sweden).

In 2001, further improvement of the agarose crosslinking production process, giving rise to considerably increased gel rigidity, led to the third-generation ion exchangers. These were market introduced as Capto Q, Capto DEAE, Capto S, Capto adhere, and Capto MMC. The first three mentioned are equipped with a dextran-based surface extender enabling increased binding capacity. The last two are equipped with multimodal ligands containing, in addition to charged groups, hydrophobic and hydrogen bond forming ligands. Capto MMC is also called “salt tolerant,” in that it makes possible protein binding at much higher salt concentrations than conventional ion exchangers. It is therefore possible to apply cell culture supernatants of, for example, *Pichia pastoris* directly to the ion exchange column without the need for dilution (87).

In 1978, a new concept in ion exchange chromatography, termed chromatofocusing, was introduced by Sluyterman and co-workers (88, 89). The publications were soon followed up by the introduction to the market of adequately designed so-called polybuffer ion exchangers (PBE™ 118 and PBE 96) and matching polybuffers for elution (Polybuffer™ 74 and Polybuffer 96). Chromatofocusing is the chromatographic equivalent of isoelectric focusing, in that it separates proteins according to their isoelectric points. The chromatograms are composed of very narrow peaks enabling the resolution of proteins that differ in pI by less than 0.05 pH units (90).

### 2.6.3 Gel Media Based on CrossLinked Dextran, Polyacrylamide, and Agarose

At the Faraday Society’s discussion meeting on “Chromatographic Analysis” in Reading, UK, in 1949, the topic of molecular sieve effects came under scrutiny and, among other speakers, Tiselius (91) and Synge (92) expressed the hope that such effects could be exploited for separation. The first published accounts on the use of molecular sieving as a separation method were reported in 1954 by Deuel and Neucorn (93) using crosslinked galacto-mannan (locust bean gum), in 1955 by Lindqvist and Storgård (94), and in 1956 by Lathe and Rutherford (95), both groups making use of potato starch grains. The commercial breakthrough of the technique came in 1959 following introduction into the market of crosslinked dextran, Sephadex by Pharmacia, following the initial discovery by Porath and Flodin (11).

The story behind the development deserves a special mention. In 1941, Tiselius and co-workers at the Department of Physical Chemistry and Biochemistry, Uppsala University, Sweden, embarked on a cooperation project with the Swedish Sugar Corporation aiming to characterize the
macromolecules in sugar beet juice. How this study led to the discovery of dextran by Tiselius’ student Björn Ingelman and subsequently to the development of the gel filtration media Sephadex by Jerker Porath at the Institute of Biochemistry, Uppsala University, and Per Flodin at Pharmacia in Uppsala, Sweden, in 1959, is described in detail in Reference 14.

Other important gel media were developed in the laboratory of Tiselius in the early 1960s, and quickly became subject to commercialization. Stellan Hjertén introduced crosslinked polyacrylamide (96) and agarose (15, 16). After Pharmacia had declined an offer by Hjertén, the production process for polyacrylamide gels was adopted by BioRad (Richmond, CA, USA), giving rise to the BioGel P series of gel filtration media. The agarose gel production method was adopted by both BioRad and by Pharmacia, who introduced the BioGel A (1966) and Sepharose (1967) series of chromatography products, respectively. Soon after, a crosslinking procedure for agarose gels was patented and published by Jerker Porath and co-workers (19) and the Sepharose CL (1975) and later Sepharose FF (1984) series of products were introduced on the market by Pharmacia Fine Chemicals AB (now GE Healthcare Life Sciences).

### 2.6.4 Development of Affinity Chromatography

In the middle of the 1960s, in an attempt to prepare media for the immobilization of peptides and proteins (enzymes), a research associate of Jerker Porath, Rolf Axén, substituted Sephadex with amino groups that were subsequently reacted (activated) with cyanogen bromide (CNBr). It turned out that a much higher degree of activation was obtained than could be explained by the amount of amino groups present in the gel. In a control experiment it was shown that the same degree of activation was obtained with unsubstituted Sephadex (97). In their seminal paper of 1968, Pedro Cuatrecasas, Meir Wilchek, and Christian B. Anfinsen used the CNBr-activation technique for the covalent coupling of amino group-containing ligands to the agarose media Sepharose 4B (71). In this paper, for the first time, the concept of affinity chromatography was introduced, thus laying the foundation for a breakthrough technique for protein separation and purification. The development of affinity chromatography was further facilitated by the introduction to the market of CNBr-activated Sepharose 4B by Pharmacia Fine Chemicals AB in 1971. Examples of other pre-activated affinity chromatography products that were introduced in the early 1970s include epoxy-activated Sepharose 6B, activated CH-Sepharose 4B, tresylactivated Sepharose 4B and thio- propyl Sepharose 6B.

During the 1970s, a number of affinity products based on group-specific ligands were introduced on the market. The first was ConA-Sepharose, for the purification of glucose- and mannose-containing glycoproteins (98, 99). This was later followed by AMP-Sepharose 4B and ADP-Sepharose 4B for NAD- and NADP-dependent dehydrogenases, respectively. These so-called general ligands were suggested by Klaus Mosbach and co-workers at Lund University, Sweden (100). Another highly popular adsorbent was Blue Sepharose, which was shown to possess a most useful variety of selectivities depending on the source of the raw material. For a thorough treatment of dye ligand affinity chromatography the reader is referred to the excellent monograph by Scopes (101). However, the most commercially successful of all turned out to be Protein A-Sepharose CL-4B for the specific purification of IgG in one step directly from blood plasma, serum, or hybridoma cell culture supernatants. Soon after its introduction to the market by Pharmacia in 1975, it became the best-selling product of the company.

Another breakthrough in the field of protein separation and purification was the introduction in 1975 by Porath and co-workers of immobilized metal ion affinity chromatography (IMAC) (102). The technique added another dimension to protein purification by the fact that it is primarily the content of exposed histidine residues in the protein that govern the separation (103). The technique was further developed by Hochuli and co-workers by introducing the concept of His-tagging in combination with new chelating groups for highly selective separation and purification of recombinant proteins expressed in *Escherichia coli* (104, 105).

### 2.6.5 Development of Hydrophobic Interaction Chromatography

Soon after the spacer concept was introduced as an essential element in the design of affinity chromatography adsorbents (71, 106), reports on the anomalous behavior of spacer-containing ligands started to appear in the literature (107–109). The binding observed could be ascribed to the hydrophobic spacer rather than the ligand. Several authors independently realized the potential of this new separation principle (110–115), and hydrophobic interaction chromatography was soon recognized as a powerful tool in protein separation. The paper by Porath and co-workers is the first demonstration of the salt-promoting effect on protein adsorption to a hydrophobic support substituted by hydrophobic ligands. Pure hydrophobic interaction can only be achieved using adsorbents lacking nonhydrophobic binding sites and is characterized by an increase in binding capacity by increasing ionic strength as well as temperature. For a more detailed treatise of hydrophobic interaction chromatography please refer to Chapter 6.

### 2.6.6 High Performance Liquid Chromatography (HPLC) of Proteins

Early reviews of HPLC of proteins were written by Regnier and Gooding in 1980 (116), primarily covering the early developments in size exclusion chromatography and ion
exchange chromatography, and by Hearn in 1982, a treatise also covering reversed phase separations (117).

The first protein HPLC media were based on porous glass and porous silica. Thus, in the middle of the 1960s, Haller (118–120) introduced a method to prepare controlled pore glass with a very narrow pore diameter distribution. High production costs and low reproducibility reduced the general acceptance of these materials. The majority of the early applications of HPLC in the protein area were based on macroporous silica, with pore diameters normally up to 300 Å. The most sophisticated of these were spherical and prepared according to two different principles. Unger (121) developed one type of spherical controlled porosity silica (marketed by Merck AG, Darmstadt, Germany, under the brand name LiChrospher TM) by emulsion polymerization of polyethoxy-silane. The other type was developed by Kirkland (122, 123), who agglutinated submicron silica particles to microspheres of uniform diameter. The pores of these were formed by the spaces created between the fused microparticles. These bead-shaped media, under the brand name Zorbax TM, were developed at the R&D laboratories of E.I. DuPont de Nemours & Co. (Wilmington, DE, USA), and are currently produced and marketed by Agilent Technologies Inc. (Wilmington, DE, USA).

2.6.6.1 Size Exclusion Chromatography (SEC) The first SEC-HPLC media for protein chromatography were based on surface modified controlled pore silica. Here, pioneering contributions were primarily made by groups headed by Unger, Hashimoto, and Regnier. Unger developed 1,2-dihydroxy-3-propoxypropyl modified silica (124–126), which became commercially available as LiChrosorb Diol TM (Merck, Darmstadt, Germany). Regnier and co-workers introduced the glycercylpropyl coating procedure of porous silica (127–131). The corresponding supports were given the brand name SynChropak TM GPC and were at the time produced and marketed by SynChrom Inc. (Lafayette, IN, USA).

In 1978, Hashimoto, Kato, and their co-workers at the Central Research Laboratory, Toyo Soda Manufacturing Co., Ltd., Tonda, Shinnanjo City, Yamaguchi Prefecture, Japan (present name Tosoh) introduced the TSK Gel Type PW prepacked columns for SEC-HPLC (132–134). There was no mention at the time of the chemical composition of these media other than “GPC columns packed with microspheres of hydrophilic polymer gels.” In 1980, the silica-based SEC media TSK Gel Type SW were introduced to the market (135, 136).

2.6.6.2 Ion Exchange Chromatography The first IEC-HPLC media for protein chromatography were based on surface modified controlled pore glass and silica, respectively. Regnier and co-workers prepared such supports by substituting controlled pore glass (137) or porous silica (138–140) with a layer of covalently bonded glycercylpropylsilyl groups followed by reaction with triglycidylglycerol and substitution with charged groups. Because the principle structural unit in the coatings was glycerc, these primary coatings were called glycophases. In a subsequent paper is described the synthesis of IEC-HPLC supports based on controlled pore silica covered with a thin layer of polyethyleneimine (PEI) being reacted with a variety of crosslinking agents (141).

2.6.6.3 Reversed-Phase Chromatography There was some early hesitation among the scientific community regarding the possibility of using totally hydrophobic media for protein separation. Hancock, in an early review of the subject, made the following statement (142):

“Although the separation of amino acids and small peptides by reversed-phase HPLC is becoming an accepted procedure, the application of this technique to the purification of proteins still requires careful evaluation. The biological activities of many proteins are sensitive to denaturation by extremes in pH, by contact with organic solvents or high salt concentration, by adsorption to glass or hydrophobic moieties, or at an air–water interface.”

However, the early literature did give a clue to the future with a description of the successful chromatography of insulin (143) and growth hormone (144). Subsequently, Eli Lilly published a description of a highly successful manufacturing process based on RP-HPLC (145).

At the end of the 1970s, few reports had been published on the use of reversed phase chromatography for the separation and purification of proteins. A claim of priority in this area has been put forward by Rubinstein (146) based on his work with the purification of leucocyte interferon (147, 148), hormone precursors (149) and human fibroblast interferon (150). However, at about the same time several other groups, more or less independently of each other, made important contributions to the development of RP-HPLC of proteins: Hancock et al. (1978) (151), O’Hare and Nice (1979) (152), Nice et al. (1979) (153), Lewis et al. (1980) (154), Pearson et al. (1982) (155), Benedek et al. (1984) (156), Geng et al. (1984) (157).

2.6.6.4 High Performance Liquid Affinity Chromatography (HPLAC) Towards the end of the 1970s, attempts were made to introduce high performance liquid affinity chromatography for protein separation. To this end, Mosbach and co-workers in 1978 suggested the term HPLAC, and substituted 10-μm porous silica particles with a variety of affinity ligands (158, 159).

2.6.6.5 Monodisperse PS-DVB Chromatography Media The late Norwegian polymer chemist, professor John Ugelstad (1921–1997) developed the theory and
invented the technique of activated swelling for making monodisperse porous polymer particles (160–164). The neat polymer particles are manufactured by Dyno Particles A/S, Lilleström, Norway, and chemically modified to a variety of chromatographic gel media at GE Healthcare Life Sciences AB. Prepacked columns of these are known under the trade names MiniBeads™ (packed with Mini™ Q, Mini S, etc.), MonoBeads™ (packed with Mono™ Q, Mono S, Mono P (for chromatofocusing), etc.) and Resource™ (packed with Source Q, Source S, etc.).

2.6.6.6 Agarose Gel Media for HPLC of Proteins The first accounts of using agarose gels for HPLC of proteins were published by Hjertén and co-workers in 1981 (165, 166). However, independently and in parallel, the development of new crosslinking procedures for the manufacturing of 10-µm beaded agarose was taking place in the R&D laboratories of Pharmacia Fine Chemicals AB, leading to the introduction of the Superose series of media in 1982 and a publication in 1985 (167). These products are now manufactured and marketed by GE Healthcare Life Sciences.

2.6.6.7 Perfusion Chromatography When the average pore size of PS-DVB column packings exceed around 6000 Å, part of the flow through the column will pass through the particles. It has been estimated that ~5% of the mobile phase flows through the porous particles in a perfusion column. Even so, this will enhance the intraparticle mass transfer, leading to faster equilibria establishment in adsorption chromatography of proteins. This phenomenon was discovered and studied by Afeyan and co-workers in the late 1980s and became known as perfusion chromatography (168–171). The company PerSeptive Biosystems was founded, and a series of Poros™ flow-through pore media was launched in 1990. These products are now manufactured and marketed by Applied Biosystems (Carlsbad, CA, USA).

2.6.6.8 Chromatography using Monolithic Columns The first example of using monoliths, that is, continuous polymer beds, in protein chromatography was reported by Hjertén and co-workers in 1989 (4) using an in situ molded 0.6-cm-diameter gel plug of a co-polymer of acrylic acid and N,N'-methylenebisacrylamide. The plug was strongly compressed to a bed height of 3 cm in order to increase its resolution power. The column was used for demonstrating ion exchange chromatography of a variety of model proteins at a flow rate of 0.5 mL/min, generating a column back pressure of 1.4 MPa. The resolution obtained with the continuous gel bed was shown to be roughly independent of the flow rate. Analogous results had previously been obtained using compressed beds of nonporous agarose (172, 173).

In 1991, Tennikova and co-workers (174) reported the synthesis of what can be regarded as a pre-stage of monoliths, namely 1-mm-thick macroporous polymeric high performance chromatography membranes. The membranes were synthesized by free radical co-polymerization of glycidyl methacrylate and ethylene dimethacrylate in the presence of porogenic solvents using a procedure similar to that used for the synthesis of beads. The epoxide groups were further derivatized to different functional groups for protein separation.

In 1992, Svec and Fréchet (5) pioneered the introduction of the first in situ polymerized continuous-rod monoliths for protein chromatography based on co-polymerization of glycidyl methacrylate and ethylene dimethacrylate. The bed height was 3.0 cm and the i.d. 0.8 cm. The bed was diol-functionalized, followed by amino-functionalization, in order to introduce 1-(N,N-diethylamino)-2-hydroxypropyl groups for ion exchange chromatographic experiments using model protein mixtures. In a subsequent paper the same group reported on the synthesis and use of a macroporous styrene-divinyl benzene polymeric rod for reversed-phase chromatography (175).

For a recent review of the area of monolithic columns for preparative chromatography of proteins the reader is referred to the article by Jungbauer and Hahn (9).

2.6.7 Continuous Chromatography

Many attempts have been made over the past 50 years to develop continuous chromatography columns. Most of the designs are based on an annular bed or on an array of annularly arranged parallel columns continuously fed with samples in a cyclic manner. For more details of the development and application of continuous chromatography designs, methods, and applications the reader is referred to the following publications: Svensson (1949) (176), Svensson et al. (1955) (177), Fox et al. (1969) (178), Scott et al. (1976) (179), Canon et al. (1978) (180), Barker et al. (1977) (181), Uretschlager et al. (2001) (182), Hahn and Jungbauer (2001) (183), and Buchacher et al. (2001) (184).

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3

GEL FILTRATION: SIZE EXCLUSION CHROMATOGRAPHY

LARS HAGEL
GE Healthcare Bio-Sciences AB, SE-751 84, Uppsala, Sweden

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3.1 INTRODUCTION

Molecular sizing properties of natural materials were noted already in the 1940s, but the first attempts to separate biomolecules by size were described in 1955 by Lindqvist and Storgårds and Lathe and Ruthven (1–3). The separation material used was swollen maize starch, which, due to its limited mechanical strength, could only be run at very low flow rates. In the late 1950s, Porath and Flodin noticed that crosslinked dextran, when used as a stabilizing medium in
column electrophoresis experiments, demonstrated size-separating properties (4). The importance of this discovery may perhaps best be recognized by the following citation from Unger and colleagues: “The evolution of gel filtration as a method for sizing biopolymers according to their hydrodynamic volume and molecular weight started in 1959, when Porath and Flodin developed crosslinked dextrans as size exclusion chromatography (SEC) packings and advocated the novel technology” (5). The discovery led in 1959 to the introduction of Sephadex™ (from Separation Pharmacia Dextran), which was composed of dextran crosslinked with epichlorohydrin to enhance mechanical stability (6). With the introduction of a commercial product designed for molecular size separations and having different purposely made separation ranges (due to the degree of crosslinking), the new technique, named gel filtration as suggested by Arne Tiselius, was soon applied to various tasks such as desalting of protein solutions, purification of protein mixtures, and the determination of molecular mass distributions of aqueous polymers such as clinical dextran. Several types of beaded matrices, based on agarose, and polyacrylamide, to mention just a few materials, were subsequently developed and marketed for molecular separations by size (7, 8). In 1964, the technique was extended to nonaqueous solutions, initially for the purpose of determining molecular mass distributions of organic polymers through the introduction of a polystyrene-based matrix by Moore (9). He called the technique gel permeation chromatography.

The separation of solutes by their molecular size has, in addition to gel filtration and gel permeation, been given a variety of designations, and gel chromatography, exclusion chromatography, molecular sieving chromatography, sterically exclusion chromatography, and size exclusion chromatography are synonymously used in older literature (10). Kaj Pedersen, a colleague of Tiselius, had already discussed in 1962 the phenomenon behind gel filtration and similar size-based separation techniques, and had proposed the name exclusion chromatography (11). Today, there seems to be a general consensus to use the term size exclusion chromatography as a general designation of the separation principle, because it is a mechanically correct descriptive term of the process, in accordance with the proposal by Pedersen.

However, gel filtration is still widely used as a designation of SEC in aqueous solvents, and particularly for biomolecule applications.

Many models have been proposed to explain the separation mechanisms in SEC, and the validities of these have been thoroughly discussed (11–13). The separation process is schematically illustrated in Figure 3.1. The separation may simply be regarded as occurring as a result of the different amount of time different solutes stay within the liquid phase that is entrapped by the porous matrix. This time is of course related to the fraction of the pores that is accessible to the solute. The interpretation of this fraction in terms of pore dimensions and pore structure, together with various expressions for solute size, results in slightly different equations for relating the distribution coefficient to the size of the solute. Interestingly, all these equations propose a linear relationship between the logarithms of the two parameters (13). A more general approach was outlined by Casassa, who used a stochastic model to relate the nonavailable fraction of the pores and the dimension of the solute (14). It can be concluded that regions of inaccessibility of the pore volume result in a loss in entropy of the molecules (15). This loss in entropy is due to the smaller number of possible conformations of the molecules within the pores as compared to an equal volume segment outside the pore. In this case, a linear relationship between the logarithm of the distribution coefficient and the logarithm of solute size is also expected. The relationship between the distribution coefficient, \( K_D \), and the loss in entropy is for ideal SEC given by

\[
K_D = \exp(-\Delta G^o/RT) = \exp(-\Delta H^o/RT + \Delta S^o/R)
\]

because no enthalpy effects take place (i.e., no surface interaction generating heat of transfer as for adsorptive chromatographic processes). It can be noted that the distribution coefficient in ideal SEC will vary from \( K_D = 1 \) for \( \Delta S^o = 0 \), that is, no change in number of conformations between free solution and pore space (e.g., for small solutes) to \( K_D = 0 \) for \( \Delta S^o \ll 0 \), that is, great loss in number of conformations (e.g., for large solutes).

Although SEC is an uncomplicated and straightforward technique, there are some points worth consideration before starting experimental work. The actual sample may require a particular pH, solvent, ionic strength, additives, or pretreatment to yield a true solution. The next step is to select a SEC support that will cope with the chosen solvent and pH, and that has a suitable separation range. Possible adsorption properties of the support must also be considered and are in general to be eliminated if separations based purely on size are required. The nature of the sample, that is, target protein and impurities and demands on resolution, separation time, and sample load may require sophisticated optimization strategies. The result is affected by the choice of SEC support, column dimensions, and the packing efficiency of the column, as well as running conditions. Obviously, different separation situations, such as desalting, preparative fractionations, or analytical separations require different strategies for optimization. Economic factors and the possibilities of scaling up the SEC step will be important in industrial SEC.

A thorough description of classical laboratory techniques for gel filtration has been given by Fischer (10). An extensive
Figure 3.1 Fundamentals of SEC. (a) View of a simple experimental set-up with buffer and sample flasks and a column packed with SEC support being eluted with the aid of gravity flow. (b) Illustration showing the packed bed and one selected bead during sample injection (top), separation (middle), and elution (bottom) of a three-component mixture. Proteins injected into the bed are separated according to decreasing size due to a reduction in available pore volume for larger molecules. (c) SEC chromatogram, where $V_0 = \text{void volume between the support particles}$, $V_t = \text{total liquid volume of the bed}$, and $V_c = \text{total geometric volume of the column}$. $V_i = \text{pore volume, calculated from } V_t - V_0$, and $V_s = \text{matrix volume of the support calculated from } V_c - V_t$. $V_R = \text{retention volume of the protein}$, column plate number $N = 5.54 \cdot (V_R/w_h)^2$, where $w_h$ is the peak width at half peak height, and $A_s = b/a$ is the asymmetry factor at 10% peak height.
description of SEC has been presented by Yau and co-workers (16), and a review of modern SEC has been given by Hagel and Janson (17). Reviews of recent papers dealing with SEC have been regularly published by Barth and co-workers (18, 19). An extensive compilation of offerings from different suppliers of columns and supports for SEC and their applications is available, edited by Wu (20).

Solute may be separated according to size by techniques other than SEC. Filtration has traditionally been used for size separation of solutes, and modern filters with sharp cut-off provide alternatives for buffer exchange and desalting (9). In hydrodynamic chromatography, the solutes are separated in the interstitial volume between porous or nonporous beads (11, 21). A related technique, termed slalom chromatography, is applied for the separation of elongated solutes such as DNA, which is trapped in the interstices between the particles in a packed column, and the solutes are eluted in order of increasing size (22). Field flow fractionation is very suitable for the separation of larger solutes and is therefore a complementary technique to SEC (23). The use of these techniques for size separation of biomacromolecules in comparison to SEC is briefly reviewed in Section 3.7. SEC resins may also be used for applications other than initial size separation, for example, for refolding of inclusion bodies or breaking up of macromolecular aggregates formed by weak association forces (24).

The aim of this chapter is to describe some fundamental practical and applied theoretical aspects of experimental work with SEC regarding biomacromolecules, with special reference to the separation of proteins. However, owing to the interest in the separation and characterization of larger molecules such as plasmid DNA, as well as smaller molecules such as peptides and carbohydrates, the applicability of SEC for these solutes will be discussed when appropriate. A good review of high performance SEC of peptides was recently presented by Irvine (25).

### 3.2 BASIC CONSIDERATIONS

SEC has proven to be a valuable tool for a variety of applications, such as desalting of a reaction mixture after tagging (group separation), industrial purification of a monomer form of a recombinant protein from multimers (fractionation), or accurate determination of molecular size (analysis). The general goal of SEC is to separate components that differ in size, and a general view of the basic factors that will affect the separation is depicted in Figure 3.2. Separation is influenced by the selectivity (i.e., peak-to-peak distance), zone broadening (i.e., column efficiency), and any extra-column effects (e.g., sample volume or mixing chambers). These are the factors to take into account in order to optimize a SEC separation, and different experimental parameters will be more or less important for different applications of SEC, as we shall see below. An inherent factor that will influence the separation and hence the interpretation of the result in analytical SEC is the shape and density of the molecule, and therefore a review of the influence of properties governing the apparent SEC size of biomolecules is also given.

#### 3.2.1 Separation Efficiency Given by Selectivity and Zone Broadening

A separation strategy should, in general, be focused on obtaining a high selectivity (i.e., peak-to-peak distance) rather than a high efficiency (i.e., narrow peaks), unless peak dilution is a critical factor. The selectivity of a SEC material is, in contrast to other types of supports (e.g., adsorbents for ion-exchange or reversed-phase chromatography), not adjustable by changing the composition of the mobile phase (as long as this change does not influence the solute shape or pore structure). The selectivity of the SEC support is thus an inherent property of the material, and the separation volume of the column is limited by the total pore volume of the packed particles.

The selectivity curve of the separation material is obtained by plotting the elution volume, or some function thereof, versus an expression of the solute size. Very often, the distribution coefficient, $K_D$, is related to the logarithm of the molecular mass, $M_r$, of the solute. $K_D$ is a column-independent variable, and is calculated from the elution, or retention, volume, $V_R$, the extra-particle void volume, $V_0$, and the pore volume of the bed, $V_t$, according to

$$K_D = \frac{V_R - V_0}{V_i} = \frac{V_R}{V_i} - \frac{V_0}{V_i}.$$

![Figure 3.2](image)
where $V_s$ is the total liquid volume of the bed (see Fig. 3.1). $K_D$ is an expression of the relative pore volume sensed by the solute (i.e., 1 for very small molecules and 0 for very large molecules). The plot of $K_D$ versus log $M_t$ will yield a sigmoid selectivity curve, which in the middle range may be approximated by

$$K_D \approx a - b \log M_t. \quad (3.2)$$

The determination of these parameters is discussed in Section 3.7.3. The slope of the selectivity curve, that is, the value of $b$, depends on the width of the pore size distribution of the material, and $a/b$ is related to the mean pore size (see Section 3.7.3). A narrow pore size distribution will result in high selectivity, that is, a large value of $b$, but a small separation range for the material, because $0 < K_D < 1$ for ideal SEC. The sigmoid nature of the selectivity curve reduces the practical working range of the support to $\sim 0.1 < K_D < 0.9$, and a SEC material with highest selectivity will span a separation range of three decades in protein molecular mass.

### 3.2.1.2 Zone Broadening

The selectivity of SEC is determined by the pore size distribution of the support and is therefore not influenced by the running conditions (as long as solute hydrodynamic dimensions or swelling/shrinking of the resin are not affected). However, the column efficiency is affected by the running conditions, and much effort has been expended to find an adequate description of the various phenomena that control zone broadening in order to optimize the experimental conditions.

The chromatographic zone width of a solute can be related to the plate height by (27)

$$H = \frac{L}{N} = \frac{L}{\left(\frac{V_R}{\sigma}\right)} = \frac{L}{\left(\frac{4 \cdot V_R}{w_b}\right)} \quad (3.3)$$

where $H$ is the height equivalent to a theoretical plate (sometimes denoted HETP) for the solute, $N$ is the number of plates per column length $L$, and $w_b$ is the base width (i.e., $4\sigma$) of the elution profile. The plate height in Equation 3.3 is the sum of contributions to peak broadening from different parts of the chromatographic system. The extracolumn dispersion can, with the aid of a proper experimental set-up (see Section 3.6.5), be neglected compared to column effects.

The plate height equation for a chromatography column was described long ago by van Deemter (28) and later adapted to size exclusion by Giddings and Mallik (29). Several other plate height equations have been derived starting from slightly different assumptions (e.g., the empirical Knox equation has gained wide popularity), but a review of the various equations shows that the van Deemter equation is presently the most accurate one for describing zone broadening in SEC (30). This equation is generally written as

$$H = A + B/u + C \cdot u. \quad (3.4)$$

The first term arises from multiple path dispersion of the solute, the second term describes the effect of axial diffusion of the solute, and the third term is due to nonequilibrium conditions in the separation process at the interstitial linear velocity $u$. The terms are given by (27, 31)

$$A = \sum_i (1/2\lambda_id_p + D_m/\omega_0a_i^2u)^{-1},$$

$$B = 2[\gamma_mD_m + \gamma_mD_m(V_R/V_0 - 1)],$$

$$C = V_0/V_R(1 - V_0/V_Ra_i^2d_p^2/(30\gamma_mD_m))$$

where $\lambda_i$ and $\omega_0$ are geometrical factors of order unity, $d_p$ is the average particle diameter of the support, $D_m$ is the diffusion coefficient of the solute in the mobile phase, $\gamma_m$ and $\gamma_p$ are obstruction factors to diffusion in the extraparticle space and the pores, respectively, and $V_0/V_R$ is the ratio of zone velocity to mobile phase velocity. Fortunately, several simplifications can be made to the expressions. Thus, due to the slow diffusion of proteins, the second term of $A$ can be neglected compared to the first term. The value of $\gamma_m$ was found to be close to 0.6 (27). The plate height equation for macromolecules can thus be approximated by

$$H = 2 \cdot a_i \cdot d_p + \frac{2}{u} \cdot \left[0.6 \cdot D_m + \gamma_p \cdot D_m \cdot \left(V_R/30\gamma_mD_m \right) \right] + u$$

$$\cdot \frac{V_0}{V_R} \cdot \left(1 - V_0/V_R\right) \cdot d_p^2$$

$$\cdot \frac{30 \cdot \gamma_p \cdot D_m}{30 \cdot \gamma_p \cdot D_m}. \quad (3.5)$$

The different shapes of the van Deemter plot for small solutes and large macromolecules at the flow rates commonly used in SEC of proteins are illustrated in Figure 3.3. The contribution from the $B$ term is seen to be very small for macromolecules, and this term is negligible compared to the $C$ term at high reduced velocities (e.g., $d_p\mu/\gamma_mD_m > 5$). This condition is fulfilled for most SEC materials under the experimental conditions commonly used, and the plate height equation for macromolecules may in this case be reduced to

$$H = 2 \cdot \lambda \cdot d_p + u \cdot \frac{V_0}{V_R} \cdot \left(1 - V_0/V_R\right) \cdot d_p^2 \cdot \frac{30 \cdot \gamma_p \cdot D_m}{30 \cdot \gamma_p \cdot D_m}. \quad (3.6)$$
in solution, \( D \). The effective pore diffusivity, \( D_{\text{eff}} \), describing the flux of proteins through pores having uniform cylindrical shape, is given by (35)

\[
D_{\text{eff}} = \frac{D \cdot K_D \cdot \epsilon_p}{\tau} \cdot \left[ 1 - 2.104 \cdot \left( \frac{R}{r} \right) + 2.09 \cdot \left( \frac{R}{r} \right)^3 - 0.95 \cdot \left( \frac{R}{r} \right)^5 \right].
\]

(3.7)

where \( R/r \) is the ratio of solute to pore radius (this is equal to \( 1 - \sqrt{K_D} \) for a cylindrical pore model), \( \epsilon_p \) is the particle porosity, and \( \tau \) is the tortuosity factor used to compensate for variations in effective pore length. The value of \( \tau \) may be obtained from batch experiments, as suggested by Liapis and Arve (36). However, when no data are available, \( \tau \) is arbitrarily set to \( 1/\epsilon_p \) (B.H. Arve, Pharmac 41 AB, Uppsala, personal communication, 1987). The restricted diffusion coefficient is given by \( D_s = D_{\text{eff}}/K_D \epsilon_p \). This relationship was, with slight modifications, used by Ackers and Steere to calculate apparent pore radii of membranes (37). Another version of the expression for \( D_s \) where the tortuosity factor was ignored was proposed for predictions of zone broadening in SEC experiments (38). Unfortunately, none of the equations yields data in sufficient agreement with experimentally found obstruction factors (39–41). Diffusivities of macromolecules in pores was found to be 5–20% of the free diffusion. The difficulties in obtaining a general expression for hindered diffusion may be elucidated in a work where a simple relationship was derived from the gel model of Ogston, that is, \( D_s/D = \exp[-(\ln K_{av})^{1/2}] \) was fitted to experimentally determined diffusion coefficients of proteins in Sepharose™ (42). The authors obtained a good fit to the experimental data only in the high \( K_{av} \) range (i.e., \( K_{av} > 0.8 \)) and, in fact, the simple relationship \( 3 \times K_{av}/4 \) seemed to provide a better general fit to their data in the investigated range \( 0.3 < K_{av} < 0.9 \). The model applied gave geometric estimates of fiber thickness and radius of cavities of half the expected values as compared to other investigations. Also, the authors noted that the fit to the theoretical curve was not as good as that obtained earlier for other types of supports, which indicates inherent differences between the SEC materials. Thus, the lack of applicable models for the pore structure of size exclusion supports prevents the establishment of accurate relationships for hindered pore diffusion from a theoretical basis. In a recent report, Lenhoff and colleagues used fairly advanced Brownian dynamics simulation and network modeling to assess pore diffusion (43). They found that the models could predict restricted diffusion of small solutes in Sepharose materials, but under-predicted the diffusivity of larger solutes (44). More research is needed to find appropriate expressions to describe the

This equation may be used to predict the zone broadening of a protein, of known molecular weight, under various experimental conditions. An identical conclusion regarding zone broadening of polymers in SEC was made by Dawkins (33). Figure 3.3 also illustrates that the minimum value of the plate height is independent of the solute diffusivity. It can be shown that the flow rate giving this minimum is proportional to \( D_{av}/d_p \) (see Section 3.6.3).

The value of \( \lambda \) can be estimated from the minimum reduced plate height of any solute. We have found \( \lambda \) to be close to 1 for experimental results on many size exclusion columns (e.g., Fig. 3.3), although it may be inferred that \( \lambda = 0.5 \) will be in accordance with a minimum of \( H = 2d_p \). Yamamoto and colleagues have also reported that a value of \( \lambda = 1 \) is in accordance with experimentally found axial dispersion in columns packed with Sephadex as well as ion-exchange sorbents (34).

The value of \( \gamma_c \) expresses the hindered diffusion of solutes within the porous network, \( D_s \), as compared to free diffusion

Figure 3.3 Zone broadening in protein SEC, as measured by the plate height, \( H \), for small and large solutes as a function of eluent velocity. A, B, and C are the terms in the van Deemter equation as calculated from Equation 3.5 with \( d_p = 33 \mu m \) for (a) cytosine and (b) myoglobin. Dots represent experimental data for Superose™ 6 prep grade (32).
effective diffusivities of solutes in porous networks, particularly for large solutes for which restrictions on free diffusion may be anticipated to be regulated by local matrix properties, such as the availability and connectivity of larger pores. It is questionable if a general formula for hindered diffusion applicable to all solutes and type of matrices can be found. Therefore, the relationships reported by various groups must still be regarded as empirical and only applicable to the type of matrix and solute tested. One work describing hindered diffusion of proteins suggested that the simple expression \( \gamma_s \approx K_D/4 \) for \( 0.2 < K_D < 0.8 \) may be used. It is worth noting that even the pessimistic estimate of \( \gamma_s \approx K_D/4 \) probably yields estimates of the hindered diffusion of high molecular weight solutes that are too high, as the experimental broadening is often larger than anticipated from the van Deemter equation (e.g., for thyroglobulin chromatographed on Superose™ 6 prep grade) (45).

The diffusivity (cm\(^2\)/sec) of globular proteins may be derived from a formula given by Tanford as (46)

\[
D_{25,H_2O} \approx 2.6 \cdot 10^{-5} \cdot M_r^{-1/3}, \quad (3.8)
\]

which is in good agreement (better than 6%) with experimentally found data (40, 41). The influence of temperature \( T \) and viscosity \( \eta \) may be estimated from (17)

\[
D = 8.89 \cdot (T/\eta) \cdot M_r^{-1/3}, \quad (3.9)
\]

By using the relationships given above, the zone broadening of any solute of known molecular weight may be estimated. The approach is illustrated in Figure 3.3, which shows that the experimentally found data are in reasonably good agreement with the predicted plate height.

### 3.2.1.3 Extra-Column Effects

The influence of sample volume on zone broadening can be estimated by comparing the width of the injected sample plug of volume \( V_{\text{inj}} \) (47) and the final width of the peak as calculated by the plate height equation:

\[
\sigma_{\text{total}}^2 = \sigma_{\text{column}}^2 + \sigma_{\text{injection}}^2 \approx V_b^2 \cdot \frac{H}{L} + \frac{V_{\text{inj}}^2}{K_{\text{inj}}}, \quad (3.10)
\]

The theoretical value of the injector constant \( K_{\text{inj}} \), that is, 12 for a square-wave pulse, has been obtained with carefully designed injection devices (48), but values of 1–5 have been reported for ordinary valves (47, 49). We found a value of 5 to be in accordance with experimental results (50).

The relative contribution to the total variance, \( \sigma_{\text{rel}}^{(2)} = \sigma_{\text{injection}}^2 / \sigma_{\text{total}}^2 \), will then theoretically result from a sample volume of

\[
V_{\text{sample}} = \sqrt{\frac{\sigma_{\text{ref}}^2}{1 - \sigma_{\text{ref}}^2} \cdot A_C \cdot \sqrt{L} \cdot (1 + K_D \cdot \frac{V_i}{V_0})} \cdot \frac{V_0}{V_C} \cdot \sqrt{h \cdot d_p} \cdot \sqrt{K_{\text{inj}}}. \quad (3.11)
\]

This formula confirms the general expectations that high values of the cross-sectional area \( A_C \), the column length, the pore volume, and \( K_D \) will support a high sample volume. However, it is not always realized that a material of small particle size will only allow applications of small sample volumes if optimal performance is to be retained. From Equation 3.11 it is seen that the injection volume needs to be adjusted to a level proportional to the square root of the particle size if \( \sigma_{\text{rel}} \) is to be kept constant. Thus, although sample volumes of 1–2% of the bed volume do not impair the performance of traditional protein SEC supports with a particle size of 100 \( \mu \)m (10), this is certainly not true for 10-\( \mu \)m material, where 0.3% is a more realistic figure if the advantages of the 10-\( \mu \)m support are to be retained (50). Mixing chambers and dead volumes in connectors, adaptors, or other parts of the fluid-handling system after the sample application point may add to the total system zone broadening. However, such zone broadening is to be minimized, as described in Section 3.6.5.

By using Equations 3.3, 3.10, and 3.21, an approximate theoretical simulation of the chromatogram that is useful for qualitative studies may be obtained (this is also useful for preparative situations) as presented by Hagel and Janson (17).

### 3.2.2 Optimizing the Separation for Group Separation, Fractionation, or Analysis

The ultimate goal for any separation is the acceptable resolution of a set of sample components. Resolution is measured as the peak-to-peak distance divided by the peak width:

\[
R_s = 2(V_{R2} - V_{R1})/(w_{b2} + w_{b1}). \quad (3.12)
\]

A resolution factor of 1.5 is required for a total separation (in practice) of Gaussian-shaped peaks of equal concentration. With \( w_b = 4\sigma \), it can be seen that \( R_s = 1.5 \) means that the (Gaussian) peaks are separated by \( 6\sigma \) and from a table of statistics we can see that this yields a 99.9% separation of the peaks (and that \( R_s = 1.0 \) would in this case mean a 97.7% separation; however, as noted below, a resolution factor of 1.0 is, in theory, sufficient for total separation of square-wave-shaped peaks).
The peak-to-peak distance is related to the selectivity and the pore volume of the SEC material, that is, Equation 3.1 can be rearranged to

\[ V_R = V_0 + K_D \cdot V_i \]  
(3.13)

and

\[ V_{R2} - V_{R1} = (K_{D2} - K_{D1}) \cdot V_i. \]  
(3.14)

These relationships set the basis for the general optimization strategy of SEC. A SEC material having a maximum difference between \( K_{D2} \) and \( K_{D1} \), that is, maximum selectivity for the components of interest, is sought. Selecting a SEC resin of high pore volume \( V_i \) further enhances the resolution. Finally, for experimental conditions yielding a low zone broadening, \( n_h \) is chosen. In addition, total pore volume will be proportional to the bed dimensions, and particle size will influence zone broadening. The strategy for optimization is of course related to the separation situation. Thus, group separation where the difference in molecular size is large (e.g., a factor of 10) or fractionation where the size difference may be a factor of 2–5, and analytical applications where an array of molecular masses may need to be separated and characterized will require different optimization strategies to yield the required performance.

The sample volume will contribute considerably to the width of the sample peak unless the volume is small compared to the volumetric dispersion caused by the column itself. Whereas this fact means keeping the concentration and volume as low as detector sensitivity permits in analytical SEC the balance in fractionations are made with respect to volume, viscosity, and flow rate to yield the desired throughput and in group separation the maximum permissible volume is applied since sample dispersion, caused by excessive flow rate, is normally not an issue.

One inherent disadvantage of SEC is that the sample is not adsorbed and hence not concentrated as in ion exchange chromatography (IEC), affinity chromatography (AC), or hydrophobic interaction chromatography (HIC). On the other hand, this also means that no re-equilibration step is generally required between sample applications, which increases the throughput (see Section 3.7).

### 3.2.2.1 Group Separation

Buffer exchange and desalting are two examples of group separation. This mode of SEC is normally the most favorable, allowing large freedom as to the choice of experimental parameters. The pore size of the SEC material is ideally chosen to totally exclude the larger solute while allowing total permeation of the smaller solute, that is, \( K_{D2} - K_{D1} = 1 \). The total pore volume is therefore used for the separation, and large volumes of sample can be handled. Furthermore, by applying large samples the relative influence of sample dispersion is low, which results in low sample dilution, as illustrated in Section 3.7.1 (Fig. 3.12). The dilution effects often seen with SEC are therefore less pronounced in group separation. Figure 3.12 also shows that the peak shape is retaining the square-wave shape of the injection zone (if using a proper injection technology) and therefore a resolution factor of 1.0 is sufficient to yield a complete resolution between the solutes, under ideal conditions. The productivity, that is, the amount of purified material per unit time and bed volume of group separation can be very high, up to 150 g/L support and hour, as shown in Figure 3.4. Hence, the optimization strategy will be to select a material with suitable exclusion limit and a maximum pore volume. Column dimensions are selected to cope with the volume to be purified and the flow rate can be as high as the SEC material allows. Group separation of small volumes will require a good sample application device and minimization of extra-column zone broadening.

### 3.2.2.2 Fractionation

Separation of a monomer form of a protein from dimers and oligomers is a typical example of fractionation. The difference in molar mass is rather small and the separation will require further optimizations compared to group separation. Often, the SEC support is chosen so that the solutes permeate the porous structure to different extents. The difference in elution volumes may, in the linear part of the selectivity curve, be expressed in terms of molecular mass from Equations 3.1 and 3.2 as

\[ \Delta V_R = -V_i \cdot b \cdot \Delta \log M_r. \]  
(3.15)

The resolution of two adjacent components with molecular masses \( M_{11} \) and \( M_{12} \) is, with the aid of Equations 3.3 and

*Figure 3.4 Productivity of group separation. Calculation of purified amount of a protein (e.g., BSA) excluded from the SEC support from a salt impurity at a protein concentration of 25 g/L. The decrease in productivity with column length illustrates the theoretical reason for using wide and short columns for desalting, as long as system dispersion effects are small (which will not be the case for columns that are too short). Copyright General Electric Company – all rights reserved. Reproduced from Reference 51 with kind permission.*
The resolution is thus affected by the differences in the sample molecular masses \( \log M_1/M_2 \), the porosity-dependent quotient \( b/(V_0/V_1 + K_D) \), the column length \( L \), and the plate height \( H \). Equation 3.16 involves the simplification \((V_0/V_1 + K_{D1})H_1^{1/2} + (V_0/V_1 + K_{D2})H_2^{1/2} \approx 2(V_0/V_1 + K_D)H^{1/2} \), where \( K_D = \frac{1}{2}(K_{D1} + K_{D2}) \) and \( H = \frac{1}{2}(H_1 + H_2) \). The equation can, by setting \((V_R - V_i)/V_i = k\), be shown to be analogous with the resolution equation used in reversed-phase chromatography, for example (see Chapter 5).

From Equation 3.16 it can be seen that increased resolution is favored by increasing the slope of the selectivity curve, the column length, and the bed permeability (i.e., \( V_i/V_0 \)), by decreasing the particle size, and, in most cases, the flow rate. Operating at low values of \( K_D \) will theoretically also increase the resolution (the gain in selectivity is larger than the loss in efficiency). The effects of these parameters are illustrated in Figure 3.5, which shows that a large value for the slope of the selectivity curve is an important factor for achieving resolution [Fig. 3.5a(i, iv)] and that a low value may be compensated for by a large value of the permeability [Fig. 3.5a(ii, iii)]. The positive effect of operating at a low value of \( K_D \) (i.e., at \( K_D \approx 0.2 \)) is illustrated in Figure 3.5b. However, the effect is small compared to the positive effect of a large pore volume.

Because the slope of the selectivity curve, \( b \), is directly related to the shape of the solute (expressed as \( d \log R/d \log M_i \); see Section 3.2.3), the resolution of rod-shaped molecules is higher than the resolution of globular solutes of similar molecular masses. In a study of the theoretical limits for the resolving capacity of SEC it was concluded that separation of dimer and monomer forms of a globular protein should readily be obtained on a 30-cm column packed with 10-\( \mu \)m beads, yielding 10,000 plates (52). However, separation of proteins differing in molecular mass by less than 20% would be difficult also with columns of extreme plate counts (i.e., plate counts of 100,000). On the other hand, separation of rod-shaped molecules such as DNA of intermediate size that differ by 10% in molecular mass might be possible with highly porous SEC supports. These figures seem to set the limits for the resolution in fractionation of biomolecules using size exclusion.

It is important to realize that the above conclusions were made by assuming that the sample volume would not influence zone broadening. In preparative fractionation the sample volume is kept as high as possible to allow high throughput without severely affecting the purity of the collected fractions.

The optimal sample volume for preparative SEC of macromolecules, \( V_{\text{inj, opt}} \), may be inferred from Equations 3.6 and 3.10 as (53).

\[
V_{\text{inj, opt}} \approx [V_{\text{feed}} \cdot K_{\text{inj}} \cdot V_i \cdot V_0 \cdot d_p^2/(15D_m)]^{1/3},
\]

Figure 3.5 Factors affecting the resolution of SEC. (a) Resolution as a function of particle size, \( d_p \), of the support at various slopes of the selectivity curve, \( b \), and bed permeabilities, \( V_i/V_0 \), of the support.

| Curve: (i) (ii) (iii) (iv) |
|-----------------|---|---|---|---|
| \( b \) | 0.4 | 0.2 | 0.4 | 0.2 |
| \( V_i/V_0 \) | 1.0 | 2.5 | 0.5 | 1.0 |

Calculated from Equation 3.16 with \( K_{D1} = 0.5 \), \( L = 30 \) cm, and \( H = 2d_p \). (b) Resolution as a function of distribution coefficient \( K_D \) at various permeabilities of the support. \( V_i/V_0 = (i) 2.0 \), (ii) 1.5, (iii) 1.0, (iv) 0.5. Calculated from Equations 3.5 and 3.16 with \( d_p = 10 \) \( \mu \)m, \( D_m = 7 \times 10^{-7} \) cm²/sec, \( L = 30 \) cm, \( u = 1 \) cm/min, \( M_1/M_2 = 10 \), and \( b = 0.3 \).
where \( V_{\text{feed}} \) is the volume of sample that is to be processed per hour. The nominal solvent velocity for this condition is given by \( u_{\text{nom}} = L(V_{\text{feed}}/V_{\text{inj, opt}}) \). Thus, in order to utilize the inherently lower zone broadening of a SEC material of smaller particle size, a lower sample volume should be used, the feed should be divided into more fractions, and each run carried out at high velocity to yield the highest productivity. The opposite is true for SEC materials of larger particle sizes.

A simple way to increase the productivity of a separation that gives a high resolution factor (i.e., \( R_s > 1.5 \)) is to utilize the inherent separation power for an increase in sample volume. From Equations 3.10 and 3.12 it is easy to show that the maximum sample volume that can be applied to give a resolution of \( R_s \) between the components of interest, from an initial resolution of \( R_{s1} \) obtained at very low sample volumes (i.e., when the zone broadening is only governed by the column zone broadening \( \sigma_z \)), is given by

\[
V_{\text{inj}} = \frac{w_b}{4} \sqrt{5 \cdot \left( \frac{R_{s2}}{R_{s1}} \right)^2 - 1}. \tag{3.18}
\]

In most cases, \( R_{s2} \) is set to 1.5 to give complete resolution between the sample of interest and the impurity; therefore, from calculating the resolution and peak width under analytical conditions the maximum sample volume under these conditions can easily be calculated. If the other running conditions are to be optimized, for example, fluid velocity, then the strategy outlined above must be used.

### 3.2.2.3 Analytical SEC

The resolution increases significantly and the separation time decreases (c.f. Section 3.6.3) when using very small particle sizes (below 5 \( \mu \)m), something that is advantageous in analytical SEC. However, the trade-off is a substantial increase in column backpressure, which requires special requirements regarding the instrumentation used if the objective is very fast separations. Under such conditions the risk of temperature effects due to frictional heat or shear degradation of elongated solutes, caused by the high fluid velocity in the narrow void channels, must be considered. Furthermore, an increase in the pressure resistance of a material is mostly achieved by increasing the matrix content, and this is likely to have an adverse effect on adsorptive properties and resolution (due to low pore volume). The inherent limitation of particle size for SEC is set by the large pore size necessary for separating macromolecules and the requirement for a large ratio of void channel radius (which is related to particle radius) to pore radius to avoid size exclusion effects in the extra-particle space. Guiochon and Martin calculated the optimal particle size for SEC of macromolecules as 1–2 \( \mu \)m at a modest separation time of 1 h (54). However, as shown by Verzele and colleagues, it is very difficult to pack such small particles efficiently, which seems to suggest an optimal particle size of \( \sim 5 \mu \)m with present packing technology (55). Also, it may be noted that columns of small particle size SEC supports are predominantly supplied prepacked, that these columns are shorter than standard laboratory columns (because the material is expensive), and that the increased matrix volume results in a low separating volume. The total effect is that the peak capacity of columns for SEC expressed as

\[
r_{\text{SEC}} = 1 + \left( \frac{V_i}{V_j} \right) \cdot \frac{\sqrt{N}}{4 \cdot R_s} \tag{3.19}
\]

does not exceed 13 for microparticulate SEC supports (e.g., 4-\( \mu \)m Zorbax™ GF-250) in short columns or for laboratory materials (e.g., 33-\( \mu \)m Superose 6 prep grade) in long columns. Furthermore, traditional supports such as Sepharose CL 6B yield competitive peak capacities due to the high pore volume and the longer columns used (45). The peak capacity of an optimized SEC system is illustrated in Figure 3.6. The advantage of using supports of small particle size is the reduction in separation time (see Section 3.6.3).

### 3.2.3 Size of Biomolecules

The retention of solutes is, in ideal size exclusion, governed solely by the differences between the solute dimensions and the pore dimensions. The relationship between size and molecular weight of solutes is strongly dependent upon solute shape, which may be illustrated by the relationship between the radius of gyration, \( R_g \), of a solute and its relative molecular mass \( M_r \):

\[
R_g \propto M_r^a \tag{3.20}
\]

where \( a \) = 1 for rods, \( a \approx 1/2 \) for flexible coils, and \( a \approx 1/3 \) for spheres (56). Thus, a compact protein will occupy less volume than a flexible coil and much less than a rigid rod upon rotation in a fluid, provided the masses are identical. The influence of the solute shape on the retention in size exclusion is illustrated in Figure 3.7. It is readily seen that calibration versus molecular mass is only meaningful for solutes of similar shapes. The influence of shape on the slope of the selectivity curve may be inferred from Equation 3.2 by \( b = dK_D/d \log M_r = dK_D/d \log R 
\times d \log R/d \log M_r \), where the first term is the true selectivity of the support, that is, the change of distribution coefficient with a change in solute size, and the second term gives the change in solute size with a change in molecular mass (i.e., exponent \( a \) in Eq. 3.20).
The shapes of solvated proteins vary considerably; there are spherical proteins, slightly asymmetrical globular proteins, rod-shaped fibrous proteins, and denatured flexible coil structures. The frictional coefficient, \( f \), of a solvated protein, obtained from sedimentation or diffusion experiments, may be compared to the theoretical frictional coefficient, \( f_0 \), of a nonsolvated sphere of equal volume. It is tempting to interpret deviations of the frictional ratio, \( f/f_0 \), from unity as deviations of the protein shape from a sphere. This is facilitated by the theoretical relationship between the frictional ratio of an ellipsoid of rotation and the geometry of the particle (e.g., \( f/f_0 = 1.25 \) corresponds to a prolate ellipsoid with an axial ratio of 1:5) (46). However, the frictional ratio is a function of solute solvation as well as asymmetry (and surface roughness), so interpretation of the shape of, for example, globular proteins as ellipsoidal hydrodynamic particles may not be realistic. It has been concluded that globular proteins (e.g., with \( f/f_0 < 1.25 \)) are neither highly solvated nor highly asymmetric (46). The situation may be

---

**Figure 3.6** Peak capacity of analytical SEC as illustrated by analysis of a polymeric dextran sample having a weight average molar mass of 1000. Column: two columns 50 \( \times \) 1 cm (i.d.) packed with Superdex\textsuperscript{TM} peptide. Total separation time: 6 h for this extreme separation. Copyright General Electric Company – all rights reserved. Reproduced with kind permission.

**Figure 3.7** Selectivity curves for solutes of different shape: globular proteins (filled squares), representing compact spheroids; dextran fractions (filled circles) representing flexible polymers; DNA restriction fragments (open squares) representing rod-shaped molecules. Reprinted from Reference 57 by permission of American Chemical Society.
illustrated by reference to apoferritin. This molecule has a perfect spherical shape but yields a frictional ratio of 1.29 due to the large degree of solvation of this hollow sphere-shaped molecule (58).

Information on shape may be obtained by comparison of data from hydrodynamic measurements (e.g., intrinsic viscosity and sedimentation or diffusion coefficients) with the radius of gyration from light scattering. A size exclusion column calibrated with such reference substances may be utilized to provide structural information about solutes of known molecular weight (c.f. Fig. 3.7). Conversely, the column may be used for the estimation of molecular weights of compounds with shapes similar to the shapes of the reference substances.

Tanford and co-workers have proposed the calibration of a Sephadex column by plotting $K_D$ versus protein Stokes radius for the accurate (i.e., ±5%) estimation of the molecular mass of proteins in a detergent solution (59). The Stokes radius, $R_{St}$ is defined as the radius of a sphere that would have the same frictional coefficient as the protein (46). The molecular mass of a compact globular protein may be inferred from the Stokes radius through

$$\log M = \log \left(\frac{R_{St}^3}{v}\right) + 0.140 \pm 0.041,$$  \hspace{1cm} (3.21)

where the partial specific volume, $v$, if unknown, may be tentatively assigned a value of 0.74 (58).

The size of solutes may also be estimated from viscosity data, yielding an expression of the hydrodynamic volume:

$$V_h = \left[\eta\right] \cdot \frac{M}{v \cdot N_A},$$  \hspace{1cm} (3.22)

where $[\eta]$ is the intrinsic viscosity (i.e., volume of molecules per unit mass), $N_A$ is Avogadro’s number, and $v$ is the Simhas factor. The value of this factor depends on the shape of the molecule and is 2.5 for spheres and >2.5 for ellipsoids (an axial ratio of 1:5 corresponds to $v \approx 5$). For flexible polymers and spherical solutes, a viscosity radius ($R_\eta$ or $R_h$) may be calculated from the hydrodynamic volume by $R_h = (3/4V_h/\pi)^{1/3}$. This estimate is often called the hydrodynamic radius. However, $R_{St}$ is also obtained from hydrodynamic measurements, so the term hydrodynamic viscosity radius is preferred for $R_h$ or $R_\eta$. The following relationship between the molecular weight of compact globular proteins and hydrodynamic volume may be calculated from Tanford (46):

$$V_h \approx 1.544M_r.$$  \hspace{1cm} (3.23)

The geometric radius calculated from this formula, assuming a spherical shape of the protein, will yield an underestimate of the size (because the formula was derived for globular proteins). Assuming a spherical shape of proteins (by assuming a value of 2.5 for the Simhas factor in the calculation of hydrodynamic volume) yields $R_h \approx (0.82 \pm 0.02) \cdot M_r^{1/3}$. Squire calculated a similar relationship for solvated proteins assuming a spherical shape (i.e., $R_h \approx 0.794 \cdot M_r^{1/3}$) (60). Solving Equation 3.21 for $R_{St}$ yields $R_{St} \approx (0.808 \pm 0.025) \cdot M_r^{1/3}$. These relationships, derived from different assumptions, indicate that a reasonably accurate estimate of the radius of compact globular proteins is given by

$$R \approx 0.81M_r^{1/3}.$$  \hspace{1cm} (3.24)

As pointed out in the work of Tanford and co-workers, it was not known, earlier, whether partition in the SEC support was responsive to Stokes radius or viscosity radius. However, observed differences between the two estimates were less than 10% for globular proteins, ~15% for random coils, and up to 100% for fibrous proteins (59). This is in qualitative agreement with Figure 3.7; that is, the differences can be attributed to the different shapes of the molecules. In another study they found that denatured proteins of quite different conformations eluted according to identical calibration curves when the solute size was inferred from viscosity data (61). Benoît and colleagues proposed the hydrodynamic volume as the retention decisive parameter for the solute in SEC (62). This approach has been shown to be applicable to a wide variety of solute shapes and solute–solvent systems (60–68). Calibration of a column using hydrodynamic volume (or viscosity radius) has therefore been termed universal calibration. This type of calibration curve is illustrated in Figure 3.8, which shows that globular proteins and rod-shaped virus particles elute according to the same curve. A similar result would not have been obtained if retention had been plotted versus $R_{St}$ (59). Other work has indicated that universal calibration is only applicable to compact solutes (i.e., globular proteins) and flexible polymers, and that rigid rods (i.e., DNA of intermediate length) will deviate from a universal calibration curve (70). As the length of DNA increases, the flexibility increases, which is the explanation for the behavior observed in Figure 3.8 (70).

Thus, the general applicability of size estimates increases in the following order: molecular mass (or molecular weight) $\ll$ Stokes radius $<$ hydrodynamic volume (or viscosity radius). The radius of gyration is proportional to the viscosity radius of spherical solutes and flexible polymers, but not to that of rigid macromolecules. The use of a well-defined polymer, such as dextran or poly(ethylene glycol) (PEG), to calibrate the column according to the hydrodynamic volume seems to be the most appropriate procedure for the characterization of an unknown solute with the aid of size exclusion. However, calibration using a polymer sample requires that
the size related to the peak retention volume is known or else the column may be calibrated using an iterative procedure (see Section 3.7.3).

The solute size is often related to the distribution coefficient in a log-linear plot. Various relationships between size and $K_D$ that have been found useful are given in Table 3.1. Many of the relationships are claimed to yield linear calibration curves, which probably reflects that variations in

![Graph: Universal calibration for evaluation of sample viscosity radius. Columns: TSK 6000 PW (upper curve), TSK 5000 PW (middle curve), and Superose 6 (lower curve). Sample: tobacco mosaic virus (TMV) dimers (open triangles); TMV, spectrin, tropomyosin, and ovomucoid (filled circles); DNA and α-actinin (crosses); proteins (open circles). Curves reproduced from Reference 69 with permission of the author and the publisher.](image)

**Figure 3.8** Universal calibration for evaluation of sample viscosity radius. Columns: TSK 6000 PW (upper curve), TSK 5000 PW (middle curve), and Superose 6 (lower curve). Sample: tobacco mosaic virus (TMV) dimers (open triangles); TMV, spectrin, tropomyosin, and ovomucoid (filled circles); DNA and α-actinin (crosses); proteins (open circles). Curves reproduced from Reference 69 with permission of the author and the publisher.

**TABLE 3.1 Relationships used for Calibration of Analytical SEC Columns**

<table>
<thead>
<tr>
<th>Relationship</th>
<th>Shape</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_D^{1/3} = a - b \cdot M_r^{1/2}$</td>
<td>Linear</td>
<td>Porath (71)</td>
</tr>
<tr>
<td>$K_D = \exp(-aR_{St} + bR_{St}^2)$</td>
<td>Nonlinear</td>
<td>Laurent and Killander (72)</td>
</tr>
<tr>
<td>$K_D = a + b \cdot \log M_r$</td>
<td>Partly linear</td>
<td>Granath and Kvist (73)</td>
</tr>
<tr>
<td>(\text{erfc}(1/K_D) = a + b \cdot R_{St})</td>
<td>Linear</td>
<td>Ackers (74)</td>
</tr>
<tr>
<td>$K_D = a + b \cdot \log ([\eta]M_r)$</td>
<td>Partly linear</td>
<td>Benoit et al. (62)</td>
</tr>
<tr>
<td>$\log(1 - K_D) = a + b \cdot \log M_r$</td>
<td>Linear</td>
<td>Basedow et al. (15)</td>
</tr>
<tr>
<td>$1/V_r = a + b \cdot R_{St}$</td>
<td>Linear</td>
<td>Davis (75)</td>
</tr>
</tbody>
</table>

*Note: $a$ and $b$ are operational constants, erfc is the error function, $R_{St}$ is the Stokes radius of the solute, and $[\eta]$ is the limiting viscosity number of the solute.*

solute properties are sufficiently large to disguise the sigmoid nature of the curve. The largest linear portion of the calibration curve is obtained from a single-pore-size support, but significant deviations from the linear portion are noticed for $K_D < 0.1$ and $K_D > 0.9$ (76). When selecting the mode of calibration it is important to examine critically the validity of the data for the calibration probes. Parameters such as shape and density of the molecule and the experimental conditions for obtaining data on Stokes radius have been used to explain disparities between different sets of experimental results (62, 63, 67).

The conformation of proteins may be normalized to random coils to avoid the ambiguities sometimes involved with less well-defined solute shapes. Denaturing media promote disruption of noncovalent bonds, and the native protein reverts to the state of a random polymer coil. As urea is a poor denaturing agent for many proteins, the use of 6–8 M guanidine hydrochloride (GuHCl) is preferred (61, 78). The disulfide bonds are broken by reductive cleavage with mercaptoethanol or dithiothreitol, and subsequent re-oxidation is prevented by carboxymethylation (78). Proteins may also be denatured with the aid of low concentrations of detergents, such as sodium dodecyl sulfate (SDS) (61, 78, 79). It should be realized that this treatment will yield a protein–detergent complex of a size that is considerably larger than that of the native protein, and that charged groups are introduced when using ionic detergents (80). The hydrodynamic properties of the protein–SDS complex indicate that the complex is a rod-like particle (81). The shape of the complex will grow more spherical as the molecular weight of the protein decreases, and the contribution of SDS to the size of the complex may dominate. This sets a lower limit of ~15,000 to the molecular weight of proteins that may be estimated by SEC in SDS (61). Procedures for fast analytical SEC in GuHCl and SDS (0.1%) have been presented and shown to offer attractive alternatives to SDS gel electrophoresis (82, 83).

Membrane proteins are solubilized in aqueous solutions with the aid of detergents. A SEC method for determining molecular mass and the size of protein–detergent complexes using alkyl-maltose and CYMAL has recently been presented (84).

Proteins may be modified with other solutes to simplify purification (e.g., by tagging) or enhance the clinical efficacy of biopharmaceuticals (e.g., covalently attaching PEG). This will of course influence the SEC dimensions of the modified protein. A general method for calculating the viscosity radius of PEGylated proteins applicable to SEC experiments has been devised by Fee and van Alstine (44).

Because proteins are charged macromolecules, the effective size of proteins will vary with pH and solvent ionic strength. Quite dramatic changes (e.g., fivefold) in the SEC radius of bovine serum albumin (BSA) was reported due to the influence of ionic strength on the Debye length of the protein (85).
3.3 SELECTION OF THE SEC SUPPORT

The first constraint on the choice of SEC support lies in the solvent and pH required to provide a good solvent for the sample. Most SEC materials are compatible with the common solvents and pH values used (pH 2–12) to dissolve proteins, with the exception of silica-based materials. The importance of selecting a SEC support with optimal properties increases with the complexity of the separation problem. For difficult separation problems the resolution of the material will be critical, and in these instances properties such as bead size, selectivity (given by the pore size distribution), and separation volume (available pore volume) become important. Another factor to take into account is the effect of the sorption properties of the matrix under running conditions. Although virtually no support can be expected to be completely free from sorption properties, the nature and degree of these properties varies with the nature of the matrix (86–88). Sometimes, these properties have been used to achieve increased separation of the sample components (88–90). However, it is important to realize that these mixed-mode separations may lack reproducibility, and also that they should not be classified as SEC!

3.3.1 Characteristics of some Available SEC Supports

Since the advent of the crosslinked dextran, Sephadex, in 1959, supports based on agarose, polyacrylamide, and combinations of these materials have been commercially exploited. These traditional supports are characterized by large bead sizes (typically 100–250 μm), high pore volume (>95%), and high elasticity and deformability. Porous glass may, although rigid, be included in this category due to the large bead size. The chemistry and properties of these SEC supports have been thoroughly described (10, 20, 88) and compared (86). Data for some of these materials are compiled in Table 3.2.

The materials presently used for high resolution SEC of proteins are based on silica, hydrophilized vinyl polymers, or highly crosslinked agarose with bead sizes typically between 5 and 50 μm. The smaller particle size yields more efficient columns, resulting in narrower peaks, and may be used to achieve higher resolution and/or faster separations. However, these supports are more expensive than traditional materials and are often only available as prepacked columns. The chromatographic properties of columns prepacked with silica-based supports have been reviewed by Pfannkoch and co-workers (87). The support properties and application of the theoretical aspects of chromatography will be discussed with special reference to the supports designed for high resolution SEC. Characteristics of some available SEC supports are given in Tables 3.2 and 3.3.

3.3.2 Chemical and Adsorptive Properties

The pH resistance of standard SEC supports approximately covers the range pH 2–10. High resolution SEC materials have a somewhat higher pH stability (pH 1–14), with the exception of silica-based materials (see Tables 3.2 and 3.3). To reduce the dissolution of silica at high pH, a pre-column packed with silica may be placed before the injector. To improve the stability of silica, treatment with zirconium has been suggested. However, deterioration of even this material was noted with prolonged use at pH 7 (69).

The surface of traditional supports prepared from natural polymers (e.g., agarose or dextran supports) predominantly contains hydroxyl groups, and provides a good noninteracting surface for hydrophilic proteins. Unfortunately, the hydrophilicity is reduced somewhat by the introduction of crosslinking reagents (e.g., epichlorohydrin). Matrices composed of styrene-divinylbenzene copolymer must be chemically treated to increase the hydrophilicity of the surface before the material is suitable for aqueous SEC. The surface of silica and porous glass must also be derivatized or coated to prevent excessive adsorption of proteins.

The presence of charged groups may be due to small amounts of natural occurring acidic groups in the raw materials (e.g., sulfate groups in agarose or carboxylic acid groups in dextran). Ionic sites may be introduced by acid or alkaline hydrolyses of the matrix by prolonged exposure to an extreme pH.

The structure of the matrix may yield specific interactions such as aromatic adsorption of, for example, tryptophan-rich peptides, on Sephadex G-25, G-15, and G-10 (94). The effect is assumed to be caused by interaction between aromatic amino acids and the ether bridges introduced by the crosslinker (95). Biospecific interactions where the matrix resembles an enzyme substrate or an affinity site may also be noted, for example, interaction between lectins and glucosidic sites of supports.

Adsorption properties of standard SEC supports have been utilized advantageously to improve the separation of substances, and the basic principles of the phenomena have been fully described (95–103). Nonspecific interactions between samples and high resolution supports are mostly due to ionic interaction with residual silanol groups or carboxylic acid groups, and hydrophobic interactions with the coating or the crosslinking sites. Pfannkoch and colleagues tested various interactions by measuring the distribution coefficients of citrate, arginine, and phenylethanol at ionic strengths from 0.026 to 2.40 in a phosphate buffer at pH 7 (87). Deviations of the distribution coefficient from unity indicate anionic exclusion, cationic adsorption, and hydrophobic effects. Large differences between different coated silica materials have been noted.

The mechanism underlying the interaction between protein and SEC supports was discussed from the theory of...
potential barrier chromatography, where the total effect is a sum of contributions from the electric double-layer interaction, van der Waals forces, and repulsive short-range interactions (102, 103). It was concluded that true size separations were in some cases only achieved under conditions where the different forces, for example, hydrophobic and ionic, were balancing each other (103).

Changes in the mobile phase pH or ionic strength may induce conformational changes of proteins, leading to the exposure of hydrophobic groups, which may interact with

| TABLE 3.2 Some Traditional Supports for SEC of Proteins and Peptides |
|-----------------------------|------------------------|-----------------|-----------------|------------------|
| Media                        | Type             | Supplier | Particle Size of Hydrated Beads (μm) | Fractionation Range for Globular Solutes (kDa) | pH Stability |
| Sephadex™ Dextran            | 1                | 20–100, 100–300² | 1.5–30          | G–50            |
| Sepharose™ Agarose           | 1                | 45–165    | 10–4000         | 6B              |
| Sepharose CL Agarose         | 1                | 45–165    | 10–4000         | 4B              |
| Ultrogel™ Agarose            | 2                | 60–200    | 70–40,000       | 2B              |
| Bio–Gel™ Agarose             | 3                | 60–140    | 25–2400         | A6              |
| Bio–Gel Polyacrylamide       | 3                | 40–80, 80–150, 150–300 | 1–500          | A–0.5m          |
| Sephacryl™ Dextran/bisacrylamide | 1               | 40–80, 80–150, 150–300 | 1–500          | S–200 HR        |
| Ultrogel Agarose/acrylamide  | 2                | 60–140    | 1–15            | AcA 202          |

²Calculated from the stated dry bead diameter from:

\[ d_{p,wet} = d_{p,dry}(1 + Wr \cdot \rho)^{1/3} \]

where \( Wr \) is water regain of the bead, and \( \rho \) is the density of the matrix.

Note: Data are given as stated by the suppliers: 1, GE Healthcare (Uppsala, Sweden); 2, Réactifs IBF (Villeneuve la Garenne, France); 3, Bio-Rad Laboratories (Hercules, CA, USA).
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<tr>
<th>Media</th>
<th>Type</th>
<th>Supplier</th>
<th>Particle size, µm</th>
<th>Fractionation Range of Globular Solutes, kDa</th>
<th>Molecular Mass Selectivity ( \frac{\Delta K_D}{\Delta \log M_r} )</th>
<th>Operation range, kDa</th>
<th>void Fraction ( V_0 / V_c )</th>
<th>pH Stability</th>
<th>Buffer Composition</th>
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<td>Agarose/dextran</td>
<td>1, 13, 34</td>
<td>0.1–7</td>
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<td>1–14</td>
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<td>0.84</td>
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**Bulk Media**

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<td>Superdex 75 prep grade</td>
<td>1</td>
<td>34</td>
<td>3–70</td>
<td>2.1</td>
<td>1–14</td>
<td></td>
</tr>
<tr>
<td>Superdex 200 prep grade</td>
<td>1</td>
<td>34</td>
<td>100–600</td>
<td>2.2</td>
<td>1–14</td>
<td></td>
</tr>
<tr>
<td>Fractogel™ TSK HW-55S Vinyl polymer</td>
<td>2</td>
<td>25–40</td>
<td>1–1000</td>
<td>0.18</td>
<td>0.96</td>
<td>2.17</td>
</tr>
<tr>
<td>Superose 12 prep grade Agarose</td>
<td>1</td>
<td>20–40</td>
<td>1–2000</td>
<td>1–600</td>
<td>0.32</td>
<td>0.92</td>
</tr>
<tr>
<td>Superose 6 prep grade Agarose</td>
<td>1</td>
<td>20–40</td>
<td>5–40,000</td>
<td>5–10,000</td>
<td>0.24</td>
<td>0.97</td>
</tr>
</tbody>
</table>

*a* Data as stated by the suppliers: 1, GE Healthcare (Uppsala, Sweden); 2, Tosoh Biosoience (Tokyo, Japan); 3, Micra Scientific (Northbrook, IN, USA); 4, Showa Denko (Tokyo, Japan); 5, Bio-Rad (Hercules, CA, USA); 6, Analytical Inc. (Chadds Ford, PA, USA).

*b* For globular proteins, calculated from $0.1K_D^{0.9}$.

*c* Data from Reference 87.

*d* Data calculated from Reference 91.

*e* Toyopearl™ HW type.

Source: Data also obtained from Reference 20.
the matrix. Also, lipophilic interactions are often encountered for small macromolecules such as peptides, where no tertiary structure aids in shielding these groups from a hydrophilic environment.

A general recommendation for all types of materials is to use a buffer with intermediate ionic strength (e.g., 0.15 M) to suppress ionic effects, but still not promote hydrophobic interactions. This will also effectively mask the Donnan effect, that is, the increase of electrolyte concentration in the pores caused by the presence of macro-ions in the void volume (104). Buffers commonly used for the different materials are given in Table 3.3, and more detailed information on buffer preparation and additives is found in Section 3.5.

### 3.3.3 Selectivity Curve and Separation Range

The selectivity of a SEC material is, in contrast to other types of supports (e.g., adsorbents for ion-exchange or reversed-phase chromatography), not adjustable by changing the composition of the mobile phase (as long as this change does not influence the solute size or shape or the pore structure). The selectivity of the material is thus an inherent property, and the separation volume of the column is limited by the total pore volume of the packed particles.

The selectivity curve of the separation material is obtained by plotting the elution volume, or some function thereof, versus an expression of the solute size, as described in Section 3.2.3. The plot of the distribution coefficient, \( K_D \), versus log \( M_r \), will yield a sigmoid selectivity curve, which in the middle range may be approximated by

\[
K_D \approx a - b \log M_r.
\]  

(3.2)

A narrow pore size distribution will result in high selectivity, that is, a large value of \( b \), but a small separation range for the support because \( 0 < K_D < 1 \) for ideal SEC. The selectivity curve may be expressed as a function of solute size and this can be used for calculating an apparent SEC pore size distribution of the support as discussed in Section 3.7.3. The sigmoid nature of the selectivity curve reduces the practical working range of the support to \( 0.1 < K_D < 0.9 \). It is important to realize that the exclusion limit quoted by many manufacturers is often an extrapolated value from the selectivity curve and will therefore only give an indication of the size of molecules that will be excluded from the matrix, and thus found in the void volume, and not the ultimate separation range. Data on selectivity and separation range for some materials are found in Tables 3.2 and 3.3. The separation range may be extended by the combination of materials with different pore sizes. If the bead sizes (and densities) of the materials are similar they may be mixed before packing, but it is more usual to pack the two materials separately in the same or different columns. The supports are often placed in order of descending pore size. However, it has been shown that the column arrangement may be optional and is preferentially random (105). It is very important to use materials with overlapping selectivity curves or else artifacts may be created (106, 107).

### 3.3.4 Pore Volume of SEC Supports

The total pore volume of a material packed in a column \( V_t \) can be calculated by subtracting the void volume \( V_0 \) from the total liquid volume \( V_t \) of the bed. The matrix or support volume \( V_s \) is obtained by subtracting the total liquid volume from the geometric volume of the column \( V_c \); that is, \( V_s = V_c - V_t \) (c.f. Fig. 3.1). The particle porosity or the relative pore volume of the material is expressed by

\[
V_{rel} = V_t / (V_c + V_t) = (V_t - V_0) / (V_c - V_0).
\]  

(3.25)

The particle porosity may vary from 95 to 50% for materials of different matrix volumes (see Table 3.3). A high pore volume will result in a high separation volume of the column because solutes are eluted between \( V_0 \) and \( V_0 + V_t \) in normal SEC. Pore volume and pore size (distribution) belong to the two most important characteristics of a SEC material.

### 3.4 PACKING THE COLUMN

For many purposes it is possible, or even recommended, to use a prepacked column. This is especially true for SEC materials with very small particle sizes (<15 μm), which may be difficult to pack with standard laboratory equipment to achieve optimal packing performance. In some cases, the availability of ready-packed columns, for example, small disposable columns for desalting, column arrays for high throughput SEC, or ordinary laboratory columns for fractionation may offer a cost-efficient solution. In yet other cases the desired combination of SEC material and column dimension may not be available or the skilled user may prefer to control all steps of the purification process, including packing and re-packing the SEC column.

#### 3.4.1 Column Materials and Accessories

Although most column materials are suitable for the aqueous buffer solutions commonly used in protein chromatography, there are often limitations in the use of organic solvents or extremes of pH with different materials. The resistance increases in the order acrylic plastic < polypropylene < glass. However, it is important to realize that the plungers, seals and other components may have a lower solvent resistance than the column tube. When using steel columns, special precautions should be taken to reduce problems with corrosion; for example, extreme pH and the use of halide ions.
should be avoided. Recommendations for use should be provided by the manufacturer. Columns suitable for large scale SEC where the adaptor is adjusted by hydraulic assistance have been introduced. A selection of columns suitable for SEC of proteins is shown in Table 3.4.

The column of choice should preferably be transparent, pressure- and solvent-resistant, and equipped with an adjustable top adaptor. The dimensions may be chosen according to the application at hand, but most laboratory columns have an inner diameter of 4–16 mm and a length of 25–70 cm. The choice of inner diameter may be based on the desired sample load or considerations of the extra dispersion effects from the wall region, which extends 30 particle diameters away from the wall (108). It is thus safe to use an 8-mm-i.d. column for 100-μm beads, but not for a 1000-μm material. The length of the column is primarily chosen according to the resolution that is required (c.f. Eq. 3.16). Before packing or repacking the column, all parts should be thoroughly cleaned. The bed support filters should be new and have a mesh size that prevents the passage of small particles of the SEC material. Touching the surface of nets and filters should be avoided, because a fingerprint may cause uneven sample application and a poor separation. It is also wise to inspect the bed support parts for burrs and so on, to ensure the best possible sample application after the column is packed. The use of screens instead of frits was found advantageous to avoid clogging and extra-sample dispersion (109).

### 3.4.2 Packing Procedure

The SEC material should be pretreated according to the manufacturers’ instructions. However, it is often advantageous to repeatedly (i.e., 2–3 times) decant the diluted slurry to remove fines. The slurry must be made, and kept, homogeneous before pouring the slurry into the packing reservoir. Packing should be started immediately to avoid settlement and the potential creation of inhomogeneities in the packing reservoir or column.

An efficiently packed column will have a low, nonseparating void volume, resulting in a homogeneous bed, which prevents channelling, disturbed flow paths, and possible rearrangement of the bed. The ways to achieve an efficient packing seem in general to be derived empirically. Thus, small rigid beads are often packed at high pressures (>4 MPa) in solvents that are chosen to prevent interparticle interactions. Various techniques have been used to pack soft and semirigid materials (32, 110, 111). This situation probably reflects the many parameters influencing the packing density and bed stability, parameters that will be unique for each type of support (55). A guideline on how to pack these materials may be given by the pressure–flow curve that is obtained from running a flow gradient through the column (32). The pressure drop over the bed is proportional to the fluid velocity (see Section 3.4.4). At sufficiently high velocities, the beads of semirigid materials are compressed (and particles of rigid materials may be partly crushed) by the large cumulative drag force acting on the particles. In the compression region (most likely to be found at the column outlet) the flow resistance of the packed bed increases dramatically as a result of a decrease in void fraction due to local compression of nonrigid beads. It seems to be most appropriate to pack semirigid materials at a constant flow rate close to, but before, the compression region. Packing at a constant flow rate will generate a column with a uniform packing density due to the constant friction force acting on the particles (112). It has been suggested that columns should be packed in the compression region to produce a bed with a low nonseparating void volume. However, the effect on the average void fraction is

### TABLE 3.4 Empty Columns Suitable for Self-Packing of Protein SEC Columns

<table>
<thead>
<tr>
<th>Column Type</th>
<th>Supplier</th>
<th>Column Dimensions (i.d. × L, mm)</th>
<th>Pressure Specification</th>
<th>Tube Material</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Laboratory Columns</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C Column System</td>
<td>A</td>
<td>10 × 100 to 26 × 1000</td>
<td>Up to 0.1 MPa</td>
<td>Borosilicate glass</td>
</tr>
<tr>
<td>Econo-column</td>
<td>B</td>
<td>5 × 50 to 50 × 700</td>
<td>Up to 0.1 MPa</td>
<td>Borosilicate glass</td>
</tr>
<tr>
<td>XK-columns</td>
<td>A</td>
<td>16 × 200 to 50 × 1000</td>
<td>Up to 0.5 MPa</td>
<td>Borosilicate glass</td>
</tr>
<tr>
<td>Tricorn™ columns</td>
<td>A</td>
<td>5 × 200 to 16 × 500</td>
<td>3–5 MPa</td>
<td>Borosilicate glass</td>
</tr>
<tr>
<td>G-columns</td>
<td>C</td>
<td>1 × 250 to 44 × 1000</td>
<td>3–7 MPa</td>
<td></td>
</tr>
<tr>
<td><strong>Large Scale Columns</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INdEX™</td>
<td>A</td>
<td>100 × 500 to 200 × 950</td>
<td>0.3 MPa</td>
<td>Borosilicate glass</td>
</tr>
<tr>
<td>Vantage</td>
<td>C</td>
<td>60 × 500 to 250 × 1000</td>
<td>0.7 MPa</td>
<td>TPX (polymer)</td>
</tr>
<tr>
<td>Superformance</td>
<td>D</td>
<td>50 × 500 to 450 × 1000</td>
<td>0.2–1.4 MPa</td>
<td>Glass</td>
</tr>
<tr>
<td>BPG-series</td>
<td>A</td>
<td>100 × 500 to 300 × 450</td>
<td>0.4–0.8 MPa</td>
<td>Borosilicate glass</td>
</tr>
</tbody>
</table>

*Note*: Suppliers: A, GE Healthcare (Uppsala, Sweden); B, Bio-Rad Laboratories (Hercules, CA, USA); C, W. R. Grace & Co., Amicon Division (Danvers, MA, USA); D, Merck (Darmstadt, Germany).
small, and the increase in backpressure and irregular flow paths caused by partial blockage of the bed by collapsed particles may be disadvantageous. Furthermore, an impaired stability of the compressed bed, resulting in a reduced column lifetime, may also be expected.

It is important to stabilize the bed after the top adaptor has been inserted by running the column at a somewhat higher flow rate (32, 112). This second step probably predominantly influences the upper, more loosely packed, part of the bed, and may be performed using constant flow rate or constant pressure because the bed is already formed.

To obtain a perfect sample application it is essential to obtain a homogeneous and well-packed zone at the inlet of the column. This is easily achieved by packing the column towards the inlet adaptor (using two adaptors) (53). Running a column in the reverse direction to the packing direction is generally not recommended for rigid materials such as silica, because this may cause rearrangement of particles in the bed. This effect is normally not seen with elastic semirigid materials.

### 3.4.3 Evaluation of Column Packing

Many of the supports for high resolution SEC are only available as prepacked columns, and there is an increasing demand for columns prepacked with more traditional materials. As shown by Potschka (69), the efficiencies of similar prepacked materials vary substantially in the hands of a user, and the evaluation of prepacked columns may be as necessary as it is for user-packed columns to ensure optimal performance.

The column packing may be evaluated by running a low molecular weight noninteracting solute such as acetone, cytidine, sodium nitrate, glucose, and D₂O at a high fluid velocity (c.f. Fig. 3.3) and calculating the reduced plate height from

\[
h = \frac{L}{5.54 \cdot \left( \frac{V_R}{w_h} \right)^2 \cdot d_p},
\]

where \(w_h\) is the peak width at half peak height. For an efficiently packed column, the reduced plate height should be close to 2 (113). Reduced plate heights of 1.5 have been reported for very efficiently packed columns (39, 40). It is important to keep the sample volume low when checking the column efficiency, especially for columns of high plate count, as illustrated by Figure 3.9. If the peak shape is not symmetrical the plate count must be calculated from the second moment of the distribution of solute molecules, that is, the elution curve. The quality of the peak shape may be expressed by the peak asymmetry factor, \(A_s\), (Fig. 3.1), which should be close to unity (i.e., 0.9–1.1). The sample application can be evaluated visually by viewing the passage of a colored sample through the bed provided the column tube is transparent. More sophisticated technologies such as magnetic resonance imaging (MRI) have been used to follow zone broadening in situ during elution of a labelled sample (115).

When the column packing is acceptable it is often useful to run some well-defined proteins to measure the actual column performance in the desired separation range. For high resolution columns this is easily done by running a few samples containing mixtures of proteins. Some mixtures that we have found to be useful in the high and middle molecular weight ranges are given in Table 3.5.

The homogeneity of the bed may be checked by comparing the pressure drop over the bed at a certain fluid velocity to

\[ \frac{d \mu L}{d \mu L} \]

---

**Figure 3.9** Effect of sample volume on calculated plate count when checking the column packing. Sample volume in % of bed volume. Reprinted from Reference 114 by permission of Academic Press.
that calculated from the pressure drop equation below. A large deviation (e.g., an increase >50%) indicates nonuniform packing with a compressed region of beads in the column. A very low pressure drop is indicative of large void channels and void fraction, and peak symmetry should be checked. The ultimate test is to check the void fraction at different parts of the column to reveal inhomogeneities (see below).

### 3.4.4 Pressure Drop over Packed Beds

The backpressure generated when a liquid is flowing through a packed bed can be calculated from the formula given by Darcy or the more explicit expression derived by Hagen and Poiseuille (116). Originally, the relationship was utilized to obtain information on particle dimensions from the observed pressure drop (117). This procedure has also been used in liquid chromatography to estimate an apparent particle size (118). Many workers, including Blake, Kozeny, and Carman, have contributed to refining the equation given by Hagen and Poiseuille to the present formulation (116):

$$
\Delta P = \frac{\eta L}{d_p} \frac{(1 - \varepsilon)^2}{\varepsilon^3} \cdot k \cdot 36
$$

(3.27)

where $\Delta P$ is the pressure drop in Pa, $u_{nom}$ is the nominal liquid velocity (i.e., flow rate divided by the column cross-sectional area) in cm/min, $\eta$ is the viscosity of the solvent ($1.49 \times 10^{-5}$ min $\times$ N/m, H$_2$O, 25°C), $L$ is the bed height in cm, $d_p$ is the harmonic mean value of the particle size distribution in cm, $\varepsilon$ is the void fraction (i.e., $V_0/V_c$), and $k$ is the aspect factor. For spherical beads the aspect factor is close to 5 (116), and the last terms in Equation 3.27 are then reduced according to $k/36 \cdot (1 - \varepsilon)^2/\varepsilon^3 \approx 180(1 - \varepsilon)^2/\varepsilon^3$. This term is called the column resistance factor, and is typically 500–700 for spherical silica materials (118). This corresponds to a void fraction of 0.42–0.45. Semirigid materials with a void fraction of 30% (see Table 3.2) will have a column resistance factor of $\approx 2250$ (due to the denser packing). It is important to notice the influence of the viscosity temperature dependence on the generated pressure drop. The relative viscosity of water is 1.5 at 4°C, which yields a twofold increase in the pressure drop in a cold room when compared to ordinary room temperature.

The theoretical pressure drops of columns packed with materials of different particle sizes and void fractions can be inferred from Table 3.6. A large void fraction will lead to a low backpressure, but also to reduced resolution due to the relative decrease in bed pore volume (c.f. Eq. 3.16).

The pressure drop over beds packed with nonsolid supports always deviates from the linear relationship predicted by Equation 3.27 at sufficiently high fluid velocities. The steep increase in pressure noticed with softer materials is

| **TABLE 3.5 Solute Mixtures for Calibration of SEC Columns with Proteins** |
|------------------|------------------|-----------------|------------------|
| **Solute** | **Supplier** | **Molecular Mass** | **Radius from Equation 3.24 (Å)** | **Concentration$^b$ (mg/mL)** |
| **Mixture 1** |
| Cytidine | A | 243 | 0.1 |
| Bacitracin$^c$ | B | 1,422 | 10 |
| Cytochrome c | C | 12,327 | 18.7 |
| β-Lactoglobulin | C | 35,000 | 26.5 |
| Bovine serum albumin | B | 67,000 | 32.9 |
| Bovine gammaglobulin | D | 158,000 | 43.8 |
| Thyroglobulin | E | 669,000 | 85.0 |
| **Mixture 2** |
| Acetone | A | 5 | 1 |
| Myoglobin | B | 17,800 | 21.1 |
| Ovalbumin | E | 44,000 | 30.5 |
| Transferrin Apo | B | 77,000 | 34.5 |
| Aldolase | E | 158,000 | 48.1 |
| Ferritin | E | 440,000 | 61.0 |
| Blue Dextran 2000 | E | - | - |

$^a$Data from the suppliers.

$^b$Suggested to be detected at 0.2 AUFS, 280 nm. Adjustment according to instrumentation used and zone broadening will be necessary. Figures include weight of additives.

$^c$Freshly prepared.

Note: Suppliers: A, Merck (Darmstadt, Germany); B, Serva (Heidelberg, Germany); C, Sigma-Aldrich (St. Louis, MO, USA); D, Bio-Rad Laboratories (Hercules, CA, USA); E, GE Healthcare (Uppsala, Sweden).
due to compression of the beads and a subsequent reduction of the void fraction in a small zone near the column outlet (32, 119). The compression is due to the cumulative drag force caused by the flowing liquid. This force increases with increasing liquid velocity, viscosity of the liquid, and bed height (120). Thus, dividing a bed into shorter segments (i.e., stacked columns) will enable the use of relatively higher liquid velocities before the beads are compressed (121). The drag force is counteracted by the supporting force of the column walls (i.e., of narrow columns). Elastic materials are not destroyed by the compression, and the original performance of the column may be restored by repacking it (32). The homogeneity of the bed packed in a transparent column may be checked by running a colored sample that is excluded from the pore volume, such as Blue Dextran, and noting the velocity of the zone at different parts of the bed. Comparison of compressibility of different materials may be obtained by normalizing the pressure drop with respect to column length and particle size (as in Fig. 3.10). It is then important that the column diameter is sufficiently large to avoid supportive wall effects (e.g., a ratio of column to particle diameter of 1 × 10^3 seems to be necessary).

The matrix rigidity may be improved by increased cross-linking of the polymer (122, 123), or by allowing a larger matrix volume of the support (87). Increased matrix rigidity is thus achieved at the expense of pore volume. Therefore, high matrix rigidity is not an objective per se, but should be related to the backpressures that are generated by the modest fluid velocities that are applicable to the separation of high molecular weight solutes (cf. Section 3.6.3).

Gradual build-up of column pressure drop may be caused by migration of fines towards the column outlet. Fines may be present due to incomplete decantation (up to 10 decantations may be necessary to remove the major portion of fines) or it may be caused by bead fragments from sheared or fragile beads or due to mechanical crushing (due to excessive axial compression of silica materials).

**TABLE 3.6 Theoretical Pressure Drop, in kPa, Over Packed Beds of Various Void Fractions and Particle Sizes**

<table>
<thead>
<tr>
<th>Void Fraction</th>
<th>Particle Size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>0.50</td>
<td>134</td>
</tr>
<tr>
<td>0.45</td>
<td>223</td>
</tr>
<tr>
<td>0.40</td>
<td>377</td>
</tr>
<tr>
<td>0.35</td>
<td>661</td>
</tr>
<tr>
<td>0.30</td>
<td>1217</td>
</tr>
<tr>
<td>0.25</td>
<td>2414</td>
</tr>
<tr>
<td>0.20</td>
<td>5364</td>
</tr>
</tbody>
</table>

*Calculated, per cm bed height at a nominal eluent velocity of 1 cm/min from Equation 3.27.

**Note:** Conversion factors: 100 kPa = 1 bar = 14.5 psi. The theoretical pressure drop is obtained by multiplying the figure in the table with the column length (cm) and the nominal eluent velocity (cm/min). Nominal eluent velocity is calculated from flow rate divided by column cross-sectional area.

**Figure 3.10** Variation in column resistance factors as a function of eluent velocity for materials of different mechanical strengths (i.e., degree of crosslinking). Experimental data are normalized with respect to particle size, bed height, and initial void fraction of the beds. Supports: (a) Sepharose™ 6B, (b) Sepharose CL 6B, (c) Superose™ 6 prep grade, (d) Superose 6.

**3.5 SAMPLE AND BUFFER PREPARATION**

**3.5.1 Buffers and Additives**

The sample is preferably dissolved in the same buffer as is used for the size exclusion step, unless the procedure is a desalting step or buffer exchange. It is generally advisable to use aqueous buffer systems with buffering capacities in the pH range 6–8, as this will produce a good environment for many proteins, as well as cope with most SEC matrices. The acid dissociation constant, pK_a, of the buffering substance should be close (±0.5 pH) to the desired pH to yield the optimal buffering capacity of the solution. Traditionally, phosphate (pK_a = 7.2) and tris(hydroxymethyl)amino- methane (pK_a = 8.1) have been used at neutral pH. In cases when the buffering capacity of these substances is too low or the properties of the substance are incompatible with the sample (e.g., phosphate is known to inhibit certain enzymes)
(124), the biological buffers proposed by Good and colleagues may be more suitable (125). The universal citrate-phosphate-borate buffer described by Teorell and Stenhagen can be used to study the effect of pH on the separation in the pH range 2–12 with only a slight variation in ionic strength (124). However, borate may interact with glycopeptidates (126). When running a preparative purification or a desalting step, volatile buffer salts such as ammonium acetate or ammonium bicarbonate may be preferred as these are readily removed by freeze-drying. Suggestions for other volatile buffer systems covering the pH range 2–12 are found in the book by Perrin and Dempsey (124).

To avoid ionic interactions between the solute and the matrix, the ionic strength of the buffer is often increased to 0.05–0.50 M by the addition of a salt. Sodium chloride is used most frequently, but because the halides are very corrosive, these salts should be replaced with, for instance, sulfates, whenever stainless steel is in contact with the liquid. In some situations the salting-out effect of sulfate may create hydrophobic interactions with the matrix. In those cases, chaotropic ions such as perchlorate may be used to increase the ionic strength of the buffer (92). Interpretation of elution volume in terms of molecular weight from analysis at a high ionic strength must be made with care, because the conformation of proteins and the expansion of polymers may vary with variations in ionic strength.

Nonionic interactions have been reported for some types of matrices. These effects may be reduced by increasing pH, decreasing the ionic strength or by adding small amounts of detergents, ethyleneglycol or an organic modifier such as 1-propanol (1%) or acetonitrile to the buffer. SEC of lipophilic solutes has been carried out in 45% acetonitrile (127). A successful separation of membrane proteins on Sepharose CL-4B using a chloroform/methanol mixture as eluent has been reported (128).

Addition of detergents to the buffer is sometimes required to solubilize certain proteins, such as membrane proteins. This approach was used for the solubilization of integral membrane proteins in 0.1 M sodium dodecyl sulfate (SDS) followed by high performance SEC in 5–50 mM SDS (129).

Denaturing media such as 6 M guanidine hydrochloride are often used to break the hydrogen bonds that stabilize the tertiary structure of proteins and to transform them into random coils for subsequent molecular weight analysis, (see Section 3.2.3).

The use of 0.02% sodium azide in the buffer has proven to be useful to prevent microbial growth (sodium azide must be handled with care as it may form explosive insoluble salts with heavy metals and is believed to be a mutagen). Other preservatives that have been tested include 0.05% trichlorobutanol and 0.01% thiomersal, but they are less effective (10). Chlorhexidine 0.01% is claimed to be an efficient bacteriostatic agent. Cationic preservatives are not recommended as these may adsorb to residual negatively charged groups on the matrix at low ionic strength. If a bacteriostatic agent is unsuitable to use then the problem of bacterial growth may be reduced by using freshly prepared and sterile filtered (0.22-μm) buffer to continuously flush the column.

The choice of buffer, pH, ionic strength, and additives must be made with consideration of solvent–matrix interactions, the solubility and biochemical properties of the sample, and the limitations of the detection system used. Undesired effects may be caused by chelating or solubility properties of the buffer substance (e.g., borate–carbohydrate or phosphate–Ca²⁺ interactions) (124, 129), self-association of proteins (130) or high absorbance of buffer or additives at the wavelength used for the detection of the sample and interferences of these substances with chemical detection steps (c.f. Section 3.6.4) (124).

Some buffer systems commonly used in high performance SEC of proteins are given in Table 3.3. An extensive compilation of other buffer systems is provided by Perrin and Dempsey (124). It is advisable to prepare the buffer at the running temperature as the pKₐ value of the substance may vary considerably with temperature (e.g., −0.03 pH°C). Finally, filtering the buffer solution through a 0.45-μm filter before use will save the column from being blocked by undissolved substances.

### 3.5.2 Sample Load

The sample load, expressed as the amount of sample applied, is a function of sample concentration and sample volume. Whereas the constraint on the former is mostly due to the high viscosity of concentrated samples, the effect of the latter is governed by the dimensions of the chromatographic bed, the pore volume of the support, and zone broadening of the column. The sample load should in general be minimized in analytical SEC to ensure the highest attainable resolution, but should be maximized in preparative SEC to yield a maximal throughput of material.

As a general rule, the viscosity of the sample relative to the viscosity of the eluent should be less than 1.5. This corresponds to a maximal concentration of 70 mg/mL of a globular protein such as human serum albumin. This was experimentally verified in the present author’s laboratory on a column packed with Superose 6 prep grade, where a sample load of 100 mg/mL gave abnormally broad peaks and 50 mg/mL gave normal sharp peaks. Fernandez and co-workers were able to study viscosity effects in a packed bed with the aid of magnetic resonance imaging (MRI). Viscous fingering effects were clearly noticed on the chromatogram of BSA (as a slight shoulder on the rear part of the peak) at a concentration of 50 mg/mL. Viscosity effects were observed by MRI at concentrations as low as 10 mg/mL, but broadening of the zone due to column dispersion was found to reduce the effects (131).
Viscous fingering effects are caused by invasion of a less viscous solvent into the rear part of the more viscous sample zone, and such effects are more likely to occur for concentrated solutions of high molecular weight solutes. The effect is readily indicated by varying the concentration of the sample as demonstrated in Figure 3.11. It may be expected that viscosity effects are more pronounced in beds of small particle size as this will result in narrow void channels and less sample dilution (as experimentally found in the MRI experiments cited above). If the viscosity of the sample is very high then this may be compensated for by increasing the viscosity of the eluent by adding sucrose or dextran (10). However, this will require the use of a lower flow rate to maintain low zone broadening and a low backpressure.

The effect of sample volume on peak width, expressed as reduced plate height, was shown in Figure 3.9. The most important implication of this figure is the limited use of the inherent efficiency of materials of small particle size when large sample volumes are applied and, of course, the risk of erroneous plate counts when using a too large sample volume in column packing tests.

The figures for sample load are of course only tentative and, due to the nature of the sample, the support particle size, relative pore volume, and the desired separation, it may be experimentally found that other sample loads are applicable. Thus, small effects on the peak elution volume from the sample concentration have been noted in high precision analytical SEC (132). Special precautions are required for SEC of highly charged solutes. For instance, the retention volume of hydroxyethylcellulose increases at sample concentrations exceeding 1 mg/mL (133). However, the effect on the retention of proteins can be expected to be much more insensitive to concentration. Nevertheless, it is good practice to study the separation pattern at different sample loads to ascertain that the conditions used are appropriate. It was found that the maximal resolution was obtained at injection volumes of 2% and 6% of the column volume when a total sample volume of 0.5% and 5%, respectively, of the bed volume is to be processed per hour (53). The maximal resolution decreased substantially when the processing rate was increased. The exact optimum is of course dependent upon the initial resolution of the solutes and the requirements on final purity and recovery (see Section 3.2.2). In a study of the productivity of purification of IgG from transferrin on Superdex 200, it was found that the maximum load was between 2 and 3% of the bed volume for recovery of 99.5 and 95.0%, respectively, and a purity of 99.99% (17). Equation 3.17 indicates that a good injection device is as important as a large column volume (although for large injection volumes the injected zone will approach a square-wave plug) and that a change in temperature (e.g., transferring the separation method from room temperature to a cold room) will affect the optimal injection volume because the diffusivity will be reduced due to the increase in solvent viscosity (c.f. Eq. 3.9).

### 3.5.3 Calibration Substances

The choice of sample substances used for the determination of void volume $V_0$ and total liquid volume $V_L$ require special attention. The probe for the void volume must be large enough to be totally excluded from all pores of the SEC material, but not so large as to be subjected to secondary exclusion in the void volume or separation due to hydrodynamic chromatography (134). Such effects will lead to the appearance of a peak before the actual void volume. To avoid these effects, the radius of the probe should be less than 0.01 times the particle radius (i.e., $\sim 500 \text{ Å}$ for a 5-μm SEC particle). The solute must not be charged at the pH used, as this can cause ionic adsorption or repulsion effects. The traditionally used substance, Blue Dextran 2000, has an assigned molar mass of $\sim 2 \times 10^6$, which would correspond to a hydrodynamic radius of 350 Å (135). This substance is not monodisperse, and the low molecular weight part of the distribution may therefore permeate the matrix of a sufficiently porous support. However, the high molecular weight region is useful for the determination of void volumes of matrices with pore sizes of up to at least 1000 Å. In cases where the blue dye stuff of this probe shows adsorption to the support, it may be replaced by calf thymus DNA (87) or lyophilized *Escherichia coli* (13). In a recent work, the interstitial volume was estimated from the elution volume of human thrombocytes, fixed with formaldehyde (because Blue Dextran 2000 permeated the support under study) Sephacryl S-1000 (136). The calibration curve of this support was extended to $10^7$ by the use of intramolecularly cross-linked hemocyanin from *Helix pomatia* (136).

Sodium azide, sodium nitrate, cytidine, and glycylytyrosine have been used as markers of total liquid volume on highly porous SEC materials with detection by UV absorption. Glucose, sodium chloride, or deuterium oxide may be used with detection by refractive index. However, special problems arise when measuring the pore volume of a support of small pore size when the ionic strength of the buffer has

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**Figure 3.11** Disturbed elution profiles due to viscous fingering effects. Sample: 0.6 mL of native dextran.
to be kept low. Owing to the high surface area and low shielding effect, partitioning of solutes into the hydrated gel layer, adsorption, or ionic interactions may be more pronounced. In such cases the total volume is calculated from the geometric dimensions of the bed, and the distribution coefficient thus achieved is designed $K_{sv}$ (see Section 3.7.3). The difficulty of obtaining a correct estimate of the total liquid volume of some types of materials has been addressed (137). A method for assigning an experimental value of the total liquid volume relevant to macromolecular SEC separation, by extrapolation from a series of small solutes, has been presented by Dubin and colleagues (138).

If the column is to be calibrated for estimation of the total liquid volume relevant to macromolecular SEC separation, the total liquid volume of some types of materials has been addressed (137). A method for assigning an experimental value of the total liquid volume relevant to macromolecular SEC separation, by extrapolation from a series of small solutes, has been presented by Dubin and colleagues (138). If suitable references are not available, then the column may be calibrated by a secondary reference such as dextran, pullulan, PEG, or a similar well characterized polymer, and the data expressed in dextran radius, for example (see Section 3.7.3).

Finally, before applying the sample it is a good experimental practice to filter the sample solution through a 0.45-μm filter, unless the sample solution is perfectly clear. This will save the inlet filter from being partly blocked, with subsequent impaired sample application as a result. If impurities of the sample are found to clog the column a prepurification of the sample through a short column packed with the SEC material is recommended. The use of filtered buffer and sample solutions may preserve the column lifetime to over 1000 injections (139).

### 3.6 THE CHROMATOGRAPHIC SYSTEM

Some SEC purifications, such as desalting, may be run without the aid of a system, whereas other, such as analytical applications, may put high requirements on the system components. A complete system is generally composed of a pump, an injection device, a column, detector(s), a fraction collector, a computer for steering the system and data collection, and/or a recorder. The instrumentation may, with respect to pressure resistance, be divided into three categories: high pressure systems capable of overcoming pressure drops of several hundred bars, medium pressure systems working up to 50 bar, and low pressure systems used up to 1 bar. The main difference between the systems is the pumping principle, that is, a reciprocating pump with heads of stainless steel or titanium, a syringe pump with wetted parts of glass, and a tubing pump with inert tubing, respectively. For small columns the liquid transport may be made with the aid of a centrifuge, or pneumatically.

The choice of column dimensions is closely related to the application. Traditionally, columns for group separation are...
between the injector and the column has been used, but this is seldom necessary with modern injection devices.

3.6.2 Eluent Delivery

The eluent is contained in a reservoir that may be slightly heated (e.g., with the aid of a heating tape or an ordinary light bulb) to degas the eluent and avoid the creation of air bubbles in the chromatography bed when using the column for extensive periods of time. We have found the use of a degasser placed before the injection device both convenient and effective. This is not necessary when using column packing that generates backpressures above 10 bar, as small air bubbles will be dissolved in the eluent at this pressure.

The eluent is delivered from the reservoir either directly to the column or through a pump. In the first case the hydrostatic pressure from the reservoir to the column outlet must be sufficient to create a flow through the column that exceeds the flow rate used. The actual flow rate is then preferably controlled by a pump placed at the column outlet. This procedure may only be used when the flow rate is low and the bead size is large. A Marriott bottle is used as a reservoir to maintain a constant operating pressure over the bed (continuous degassing of the eluent is necessary when using a Marriott bottle). A more convenient and recommended way is to place the pump before the injection device. This will also facilitate the collection of fractions after the column. Whatever system is used, measurement of the actual flow rate is of utmost importance in analytical applications due to the possibility of leakage in the pump, connectors, valves, and so on, and the large effect of an erroneous flow rate on the calculated molecular mass from a calibrated SEC column (140). The most common way to measure the flow rate is by gravimetry, but it is also possible to use flow meters for continuous control (141). Even though high precision pumps are preferable for most work, the long-term flow constancy of peristaltic pumps equipped with Tygon tubing is satisfactory in many cases. An indirect way to control the reliability of the pump is to include an internal standard, such as a low molecular weight solute, with each run. This may also provide a way to compensate for any long term drift of the pump.

3.6.3 Optimal Fluid Velocity

In many cases it is convenient to discuss the flow used in the separation in terms of flow rate. However, it is important to realize that flow rate is only a measure of the volume of liquid delivered by the pump per unit time and that it is the fluid or liquid velocity, which is the actual distance of the mobile phase traveled along the axis of the packed bed per unit time, that will influence the separation. The fluid velocity depends upon the flow rate of the pump, column cross-sectional area, and the void fraction of the bed.

The maximum velocity of the liquid phase that is applicable to a SEC separation is restricted by both physical and physico-chemical considerations.

The flow of liquid through the column generates a backpressure as described by Equation 3.27. Significant deviations between the theoretical value of the pressure drop and the experimental one may indicate nonuniform packing (see Section 3.4.4). Abnormally high pressure drops over filters, frits, or connection tubings may be caused by clogging from precipitated sample or particulate materials. The column construction and materials, fittings, connections, pump type, and the mechanical rigidity of the packing material limit the maximum fluid velocity that can be used.

The useful velocity is in practice restricted by the low diffusion coefficients of macromolecules, which may lead to excessive zone broadening when too high liquid velocities are used (142). This is eliminated by ensuring that equilibrium between the mobile and stagnant phases is achieved. This can be done by decreasing the transfer distance of the solute (reducing the particle size of the support) and/or increasing the available transfer time (reducing the velocity of the liquid) and/or increasing the transfer speed (increasing the diffusivity, for example, by increasing the temperature).

The zone broadening due to nonequilibrium is expressed by the C term in the van Deemter equation (Eq. 3.4) and is illustrated in Figure 3.3. The optimal interstitial solvent velocity, yielding the minimum zone broadening, is found by differentiation of Equation 3.5 with respect to $u$, which results in Equation 3.28:

$$u_{opt} = \frac{D_m \cdot \gamma_s}{d_p} \cdot \sqrt{\frac{40}{\gamma_s \cdot \frac{V_0}{V_R} \left(1 - \frac{V_0}{V_R}\right) + \frac{60}{\gamma_s \cdot \frac{V_0}{V_R}}}}, \tag{3.28}$$

showing that the optimal velocity is proportional to the hindered diffusion of the solute and inversely proportional to the particle size of the support.

At $u_{opt}$, the major contribution to the zone broadening is the $A$ term in Equation 3.4. It seems practical to allow an interstitial velocity where the $A$ term is not solely dominating, for example, allowing an equally large contribution from the $C$ term. That “practical” velocity, $u_p$, is given by

$$u_p = \frac{D_m \cdot \gamma_s}{d_p} \cdot \frac{60 \cdot \lambda}{\frac{V_0}{V_R} \cdot \left(1 - \frac{V_0}{V_R}\right)}, \tag{3.29}$$

With $\lambda \approx 1$, $\gamma_s = K_D/4$ and $V_0/V_R(1 - V_0/V_R) \approx 0.23$, Equation 3.29 reduces to $u_p \approx 65K_D \cdot D_m/d_p$. Practical nominal velocities for various bead sizes and solutes are indicated in Table 3.7. Note that no single fluid velocity is optimal for the entire separation range. Thus, at low velocities, high molecular weight solutes will yield sharp peaks due to the large
transfer time (low C term), but small molecular weight solutes will yield broad peaks due to excessive axial zone broadening (large B term; c.f. Fig. 3.3). At high velocities, the conditions are opposite. However, by starting the elution at a low flow rate and increasing the flow rate during the run, a more optimal separation is achieved. In such an experiment it was found that the separation time could be decreased by a factor of two by increasing the flow rate during the run, as compared to a run at constant velocity (143). The velocity gradient to apply may be calculated from Equation 3.29.

### 3.6.4 Sample Detection

The most popular method to monitor the effluent for proteins is continuous measurement of the absorption in the ultraviolet range. Variable-wavelength detectors are often expensive, and the use of a single- or dual-wavelength detector is in most cases adequate. Detectors where the wavelength may be selected at 546, 280, 254, 214, or 206 nm (or other wavelengths) are available. It is thus possible to select the proper wavelength for the specific detection problem; for proteins, absorption at 280 nm is often utilized. For the detection of peptides not containing aromatic groups, lower wavelengths, below 220 nm, must be used. Care should be taken to avoid buffer solutions and additives that show appreciable absorption at the wavelength used for monitoring. Thus 0.03 M acetate or 0.2 M phosphate buffers, azide, merthiolate, and hibitane are not compatible with detection at 214 or 206 nm (144). Proteins also show different molar absorptivities at different wavelengths, and chromatograms at 280 and 254 nm cannot be expected to coincide. This can be used to increase the detection selectivity with the response ratio method (145). DNA absorbs at 360 nm, and the different absorptivity as compared to proteins is frequently used for quantification of DNA contamination in protein preparations.

Differences in spectroscopic properties of solutes may be fully utilized with the aid of a fast scanning detector, such as a diode array detector (DAD), for which a total spectrum is recorded in parts of a second (146). When running unknown samples, the risk of losing peaks may be reduced by feeding the signal to a two-channel recorder set at two different sensitivity ranges, unless signal handling is computerized.

The use of fluorescence monitoring is mainly applicable to compounds containing tryptophan or after chemical derivatization but, when applicable, the sensitivity and selectivity is substantially higher than with UV detection.

Refractive index (RI) detectors are frequently used for the detection of non-UV absorbing species such as carbohydrates and polymers (e.g., dextran, PEG). This detector is quite general, and will not only respond to variations in the solute concentration, but also to changes in the composition of the mobile phase, for example, salt peaks or salt depletion, and to temperature and pressure fluctuations. The sensitivity of the RI detector is in most cases inferior to that obtained by UV detection. It may also be noted that, depending upon the design of the flow cell, many UV detectors will respond to changes in the mobile phase composition of non-UV absorbing solutes, for example, a large change in sodium chloride concentration.

The combination of a low angle laser light scattering (LALLS) detector and an RI detector can be used to determine the molecular weight of the eluting solutes, provided that the refractive index increment of the solute is known (147). The technique, as applied to proteins, yields data in good agreement with results from sequence determinations (148). The improvements in detectors for online measurement of viscosity and light scattering provides robust detectors suitable for laboratory use. Viscosity detectors are more suitable for detection of low molecular mass solutes, whereas light scattering detectors are useful for detection of...
high molecular mass solutes. A multiple capillary detector yields information about the intrinsic viscosity of the sample (149). Molecular dimensions, that is, the viscosity average molar mass, $M_v$, may be calculated from the Mark–Houwink relation $\eta = K M_v^a$ where $K$ and $a$ are characteristics constants for the solute–solvent system used. Solute conformation may be inferred from the value of $a$; for rod-shaped molecules $a$ is $\sim 1.8$, and for flexible linear polymers $a$ is between 0.5 and 0.8 (46). The use of multiple-angle light scattering detectors (MALLS) provides means for determination of the weight average molecular mass of solutes, provided that the refractive index increment of the solute is known (150). MALLS have been used for size detection of proteins of molecular mass exceeding 6000 (150).

Absolute size determination may be achieved by mass spectrometry (MS), and the development of online LC-MS detectors where the flow rates commonly used in liquid chromatography (up to 1 mL/min) may be directly interfaced to the MS detector provides an interesting, though expensive, option for analytical SEC. One example was given by Nylander and co-workers, who identified cleavage products of neuropeptides by electrospray ionization mass spectrometry connected to a high performance SEC column, Superdex Peptide, 3.2 × 300 mm (151).

One advantage of using a size detector for analytical SEC is that the column is “merely” acting as a separating device. Thus, solute–matrix interactions, variability in eluent velocity, or temperature will not influence the result as long as the sample components are resolved. Furthermore, calibration of the column is not necessary. A review of modern methods for detection of macromolecules with special reference to polymer samples has been given by Striegel (152).

Electrochemical detection has been applied to column effluents, and the determination of thiol-containing proteins after SEC has been described (153). The use of a bromine detector, where bromine is allowed to react with the protein, and the amount of bromine consumed is determined, may provide an interesting general technique for protein determination (154).

In desalting procedures the registration of ionic strength is often of interest, and in these cases a conductivity detector equipped with a flow cell can be used. It is of course also possible to use an ion-selective electrode equipped with a flow cell for this purpose.

The use of flow-through detectors is mostly straightforward, although certain applications may reveal limitations in instrumental performance including slow electronic response to very fast analysis, refractive index effects in UV detection when changing mobile phase composition, and unbalanced photomultipliers resulting from large differences in absorption of buffers in sample and reference cells. To avoid the formation of air bubbles in the cell, the pressure inside the cell may be increased with the aid of narrow-bore tubing, such as 3 mm × 0.02 mm i.d., or a constant backpressure device operating at 50 psi connected to the outlet of the cell (145, 155).

In cases where a more specific assay is required, the effluent may be collected in fractions and a chemical reaction carried out subsequently. It is also in most cases possible to carry out this reaction on line after the column to obtain a continuous assay (156). The large potential of such methods may be exemplified by a method for the rapid diagnosis of myocardial infarction. The main components of serum were separated by fast SEC and the content of myoglobin (down to 10 μg/mL) selectively assayed by a post-column chemiluminescence reaction with luminal (157).

3.6.5 System Dispersion

The theoretical contribution to extra-column zone broadening due to the chromatography system may be calculated from the following (47, 49, 155):

$$\sigma_{\text{system}}^2 = \frac{V_{\text{inj}}^2}{K_{\text{inj}}} + \frac{d^4 \cdot F \cdot l}{12 \cdot D_m} + 2 \cdot \frac{V_{\text{cell}}^2}{V_{\text{cell}}},$$

where the first term is related to the injection of the sample volume, the second term is the contribution from tubings or connections with length $l$ and inner diameter $d$ at the flow rate $F$, $D_m$ is the diffusion coefficient of the solute, and the last term expresses the contribution from the flow cell volume, $V_{\text{cell}}$.

If the system is allowed to contribute 10% of the total zone broadening, a maximum of 40 μL sample, 15 cm tubing with 0.5 mm i.d., and a cell volume of 15 μL can be tolerated if a reduced plate height of 2 is to be maintained for a sample eluting in the void volume of a 300 × 10 mm i.d. column packed with a 10-μm material. For a 30-μm material packed in a 500 × 16 mm i.d. column, the corresponding figures are 300 μL sample, 30 cm tubing and 50 μL cell volume. Thus, it is very important to minimize extra-column broadening if the separation power of smaller particle SEC supports is to be fully utilized. It should also be noted that a minimum of connections should be used and tubings with different diameters should be avoided in order to prevent turbulent mixing of the sample zones. The effect of sample volume on peak width in high resolution SEC is treated more extensively in Section 3.2.2.

The risk of shear degradation of macromolecules when reducing the diameters of tubings or particle size while maintaining a high flow rate needs to be considered (158, 159). In order to avoid shear degradation, the use of tubings with an i.d. of 0.5 mm or larger and keeping the fluid velocity below 8 cm/min for 10-μm packing materials is recommended (159).

3.7 APPLICATIONS

Applications of gel filtration or aqueous SEC may be classified into three principal categories. The first is group
separation (also known as desalting or buffer exchange), where the protein of interest and impurities differ substantially (more than a decade) in molecular size and therefore the constraints regarding system and running parameters are relatively modest. The second is (protein) fractionation, where the size difference is smaller (down to a factor of two), and requires more optimization. The third is determination of molecular size where high resolution is needed to assure integrity of the characterized solute, and the influence of system effects must be minimized.

SEC has also been used in attempts to obtain information about matrix properties through a method called inverse size exclusion. This provides a valuable tool for obtaining apparent size-exclusion dimensions of soft materials for which standard methods are inapplicable. Other types of applications that are not traditional SEC include studies of the kinetics of protein aggregation (see Reference 162), and as a general approach for refolding of proteins (e.g., recovery of recombinant proteins produced as insoluble inclusion bodies) (24, 161).

The interaction of certain SEC supports with solutes may be used to separate substances. Of course this type of separation should not be classified as SEC. Other types of size-based separations are hydrodynamic chromatography, slalom chromatography, and field flow fractionation, and in some cases these may complement ordinary SEC for size separation of solutes. Some selected examples of typical applications of SEC of biomolecules will be given in this section.

3.7.1 Group Separation

3.7.1.1 Desalting Gel filtration, or SEC, has been used for desalting of protein solutions for more than 50 years (6). As the difference in molecular size between the protein and contaminating low molecular weight solutes is very large, the freedom to choose experimental conditions is very large, as long as a SEC material of suitable porosity is available. The porosity of the support is selected to exclude the solute to be desalted, the particle size is chosen to give a low surface area (to minimize adsorptive properties) and low backpressure rather than to yield columns of high efficiency. Because the protein is eluted in the void volume and the contaminating solutes have low molecular mass, the flow-sensitive dispersive effects of the column are minimal, and desalting may be carried out at high flow rates without impaired resolution (see Section 3.2.2). The buffer salts and additives should be volatile or the desalting should be carried out in distilled water. Desalting may be carried out on a small scale, as for the desalting of radioactive-labeled DNA in 1 mL Sephadex G-100 in a Pasteur pipette, or at a large scale, as for the desalting of large volumes of whey using a 2500-L column of Sephadex G-25 Coarse (17, 162). As the total pore volume is used for the separation, very large volumes of sample may be desalted in one step, as illustrated, by a now classical example, in Figure 3.12. In this application, 400 mL of a hemoglobin solution, corresponding to a sample load of 37% of the total bed volume, was desalted in one step on a column packed with Sephadex G-25. The figure also illustrates the square-wave shape of the peak when applying large sample volumes, and the small zone broadening (i.e., dilution factor) of the protein peak (in Fig. 3.12a the protein is diluted approximately nine times, whereas in Fig. 3.12b the protein is diluted only 10%). Note that the apparent effluent volume for the 400-mL sample is equal to the effluent volume of the 10-mL sample adjusted for the sample volumes (i.e., \( V_R = 380 - 10/2 = 375 \) mL and \( V_R = 575 - 400/2 = 375 \) mL).

Desalting on a micro/preparative scale (i.e., desalting of a 50-µL reduction/alkylation mixture) before sequence analysis using Sephadex G-25 prepacked into a 100 × 3.2 mm column has been presented by Hellman and co-workers (163). Formats for small scale group fractionation allowing applications of as little as 70 µL of sample in 96-well filter plate modules having excellent reproducibility and recovery can be found from several vendors (one example is given in Reference 166). The other extreme of desalting may be exemplified by the industrial desalting of 31 L of 6% albumin on a 125-L Sephadex G-25 Coarse column (165). The

![Figure 3.12](Image 369x185 to 572x299)

**Figure 3.12** Desalting of large sample volumes by SEC. Column: 850 × 40 mm i.d. column packed with Sephadex G-25. Sample: hemoglobin (open circles), NaCl (crosses). Sample volume: 10 mL (a), 400 mL (b). Note that the retention volume is not corrected for the sample volume. Reproduced from Reference 110 by kind permission of the author and publisher.
productivity was 33 g albumin per hour and liter of Sephadex, that is, more than 4 kg of albumin processed per hour at a sample volume of 25% of the bed volume. The productivity is roughly four times less than the maximum theoretical productivity, discussed in Section 3.2.2, which is due to the relatively low sample concentration used (51).

The procedure used for desalting may of course also be used for transferring the solute into another buffer system.

3.7.1.2 Group Fractionation In some cases the differences in size are sufficiently large to allow a fractionation to be run in “desalting mode.” One example was given by McClung and Gonzales, who separated plasmid DNA from RNA and proteins by using Superose 6 prep. Grade (166). Plasmid DNA was eluted in the void volume, which permitted them to purify 0.5 mg plasmid DNA in 20 min, and the procedure was found to be advantageous compared to cesium chloride gradient centrifugation.

Group fractionation using SEC is often used as a step in industrial downstream processing. In a process for purification of human influenza virus, Sepharose 4 Fast Flow was used as a step for removal of host cell impurities before the subsequent anion exchange step (167). The optimization of sample volume is shown in Figure 3.13, and a sample volume of 28% of the bed volume still gives good separation, as can be expected from the high pore volume of the material employed.

3.7.2 Fractionation

The most favorable situation is when it is possible to use a SEC support that will exclude the protein of interest and include the impurities or vice versa (as in group separation). In the normal case the difference in molecular size is too small for this approach and a successful separation will require the use of a SEC support with optimal properties, that is, a high pore volume, narrow pore size distribution, and a suitable pore size to elute the protein of interest at $0.2 < K_{av} < 0.4$ (c.f. Fig. 3.5). From theoretical calculations it was found that complete separation (e.g., with a resolution factor of 1.5) of molecules of spherical shape differing less than 30% in molecular mass may not be expected even for supports of maximum selectivity (45). As it is often desired to avoid additives in industrial applications, the adsorption properties of the support should be small. Impurities that show strong affinity for the SEC matrix may be removed by filtering the sample solution through a small bed of the SEC support before applying the sample on the column.

To favor high sample loading, the pore volume and selectivity of the matrix should be high and the cross-section of the column large enough to cope with the desired sample volume, and the strategy outlined in Section 3.2.2 should be applied. It is advantageous to use supports designed to facilitate the scale-up procedure from analytical to preparative runs. This requires that the SEC material is available in bulk quantities and that the packing procedure to obtain efficient columns is simple. In some cases the suppliers offer analytical columns and preparative grades of materials of essentially identical selectivity to facilitate scale-up of laboratory runs.

An example of a successful fractionation step is illustrated in Figure 3.14. This shows the intermediate purification of humanized IgG4 from a myeloma cell culture that has been captured by an affinity step, MabSelect™, and the monomeric form of IgG4 is separated from dimers and multimers by preparative SEC on Superdex 200 prep grade (168). Noted that the sample volume in this case (2.5% of the bed volume) is tenfold less than that possible for group separation, which is in good agreement with expectations from optimization theory as described in Section 3.2.2. An example of scale-up of a preparative SEC was given by the purification of the conjugate of IgE-ß-galactosidase in small-production scale, yielding amounts sufficient for diagnostic purposes (169). Owing to the complex sample, only partial purification of the conjugate was obtained. Preparative SEC was also used for intermediate purification of insulin-like growth factor-1 ($M_r = 7600$) from fusion peptide ($M_r = 14,500$) and uncleaved material using Superdex 75, as shown by Hartmanis and co-workers (170).
An example of small scale fractionation of proteins was given by Hennes and co-workers in a study of lipoproteins in hamsters (171). By using a micropreparative system (SMART™, GE Healthcare Life Sciences), they were able to fractionate the proteins from only 20 μL of serum and, thus, the hamsters did not have to be sacrificed in the study and samples could be continuously withdrawn during the entire study.

The throughput (i.e., the amount processed material per unit time) may be increased by applying a new sample at each time interval corresponding to $V_t - V_0$, as no material from the new sample is eluted during a time period corresponding to $V_0$. The gain in production rate of consecutive runs equals the void fraction, which for many materials is $\sim 35\%$ (see Table 3.3). The optimum sample volume and flow rate for a certain volume to be processed is given by Equation 3.17.

With the use of high precision pumps and controllers to govern motorized valves it is possible to use an automatic half-scale system ($700 \times 50$ mm i.d. column) with repetitive runs for preparation of substances for laboratory use. Such a system was used for repetitive fractionation of glucose oligomers, where 50 mL containing 10 g of a dextran mixture, with a weight average molecular mass of 1100, was processed per cycle on two $900 \times 65$ mm i.d. columns of Sephadex G-25 (17).

The application of SEC for refolding of proteins or dissolution of aggregates may be regarded as a special case of group separation combined with fractionation. Large sized aggregates are separated from the smaller sized denaturants, and the refolded proteins or monomeric forms of proteins are continuously purified from the remaining portion of the aggregate during the elution through the bed (24).

### 3.7.3 Analytical SEC

In analytical applications the resolution is of utmost importance. This is affected by the selectivity (i.e., peak-to-peak distance) and the zone broadening as discussed in Section 3.2.1. Equation 3.16 may be used to estimate the impact of various parameters on the resolution. It is advisable to check the column for efficiency and symmetry of a well defined solute and these values should be close to $2d_p$, for the plate height, at optimal flow rate, and $1.0 \pm 0.1$ for the peak symmetry at $10\%$ peak height (113).

The risk of sample overload must be considered when high resolution is desired. Some general aspects of sample volume and concentrations are given in Section 3.5.2. The applicability of these may be experimentally verified by first decreasing the concentration and then decreasing the sample volume and noting the width of the eluted zone. If this also decreases then the parameter(s) should be adjusted to the region where no effect on the zone is noted. Overload effects are most often noticed for samples eluting in or near the void volume, and viscous fingering may result in double or triple peaks, as illustrated in Figure 3.11. This effect is readily precluded by decreasing the sample concentration. To eliminate the influence of sample concentration on peak elution volume a procedure has been suggested where the sample is chromatographed at several concentrations and the elution volume taken as the intercept of a plot of concentration versus elution volume (132). However, this method is tedious and not necessary in most applications. As a general guide, a sample volume of $0.2\%$ of the column volume and a concentration of up to $30$ mg/mL for proteins, $6$ mg/mL for tRNA and $5$ mg/mL for noncharged dextran of high molecular mass can be applied.

Interactions between the matrix and the solute should be absent in analytical SEC. Such interactions may be influenced by parameters that do not ordinarily affect the size exclusion process and thus cannot be expected to have been controlled by the manufacturer. Interactions may also vary unpredictably from lot to lot. Interactions may be indicated by variations in elution volume or peak shape with concentration, temperature, ionic strength, organic modifier or chaotropic salts. However, the same effects may be caused by solute–solvent interactions (c.f. Section 3.2.3). A general procedure for testing matrix properties has been given by Pfannkoch and colleagues (87). One type of analytical SEC where interactions must be absent is in the measurement of equilibrium of protein association. An application of large zone analytical SEC showed that accurate data for the association constant of hemoglobin were obtained with Sephacryl S-200 HR, but Toyopearl HW-50 S was found to be unsuitable, as indicated by tailing peaks (160).

### 3.7.3.1 Process Analysis

SEC may be used in the analytical mode to monitor a purification process selectively, as...
illustrated in Figure 3.15. The purification of staphylococcal enterotoxin B was controlled by fast SEC using Superose 12 (172).

### 3.7.3.2 Determination of Molecular Mass

Analytical SEC is frequently used for the assay of molecular mass of proteins or molecular weight (i.e., mass) distributions (MWD) of hydrophilic macromolecules or polymers. Granath and Flodin were the first to describe the use of SEC for the analysis of MWDs of clinical dextrans (173). This application requires the calibration of the column with well-characterized solutes of shapes identical to the sample (e.g., narrow dextran fractions), unless a size detector is used. Hagel and colleagues used another approach, where the column was calibrated from running only one well characterized broad dextran sample through the column (174). The system was successfully used for evaluating a potential clinical effect on the glomerular barrier function as a result of pharmaceutical treatment (175). It must be noted that calibration of the column using the elution volume corresponding to the peak apex of a polymer requires that the molecular mass or molecular size corresponding to the peak apex is known (or the entire cumulative molar mass distribution is known as for the approach used by Hagel). Another way is to assign the value \((M_w \times M_n)^{1/2}\) to the peak and then adjusting the calibration so that proper values of \(M_w\), the weight average molecular mass, and \(M_n\), the number average molecular mass, are obtained. Such iterative procedures have been successfully applied to the calibration of columns for analytical SEC (73, 176).

Calibration of the column using the column-independent distribution coefficient \(K_D\) is recommended if the column is going to be used over a long period of time or the performance is to be compared with other materials. Calculation of \(K_D\) requires the determination of the void volume \(V_0\) and the total liquid volume of the bed \(V_t\), as well as the sample elution volume. If a proper probe for \(V_0\) is used, then the correct value for the void volume is obtained from the injection point to the peak apex. (Earlier, a practice of calculating the void volume from the commencement of the sample application to the inflection point of the ascending part of the peak was recommended. However, this will result in a value of \(K_D\) for the peak apex being close to but not equal to zero and is therefore not suitable.) If the probe is too large, then a too low value of \(V_0\) may be expected, due to secondary exclusion effects, and if the probe partially penetrates the support particles, a too high value of \(V_0\) can be obtained. Suitable probes for the determination of void volume and total liquid volume are discussed in Section 3.5.3. The total liquid volume may be determined from the peak apex of a small molecule anticipated to penetrate the entire pore volume. For supports with very small pores it may be difficult to find a suitable substance that will not show noticeable interaction with the large hydrated surface layer. In such

![Figure 3.15 Monitoring of a large scale purification process by analytical SEC. Column: Superose™ 12 HR 10/30. Sample: staphylococcal enterotoxin B in various stages of the purification process (172): (a) after initial purification of 400 L cell supernatant on S Sepharose™ FF, (b) after intermediate purification by gradient elution on S Sepharose™ FF, and (c) after final purification on Sephacryl S-200 HR. Courtesy of H. O. J. Johansson, N. T. Pettersson, and J. H. Berglöf of Pharmacia Biotech.](image-url)
cases the total volume is calculated from the geometric column volume \( V_c \), and the distribution coefficient is then designated \( K_{av} \) (from \( av = \text{available} \)). The two distribution coefficients can be related to each other with the formula

\[
K_D = K_{av}(1 + V_s/V_i),
\]

where \( V_s \) is the volume of the support matrix and \( V_i \) is the pore volume. \( K_{av} \), of a totally permeating solute is thus a measure of the relative pore volume of the support.

If there is no need to calibrate the column with the aid of \( K_D \) or \( K_{av} \) (e.g., for purpose of comparisons) then the elution volume, expressed by the first statistical moment of the peak, may serve as the dependent variable.

The injection point should be set when half the sample volume has been applied. However, when the sample volume is constant, the injection point may arbitrarily be set at the start of the sample application. Figure 3.13 illustrates a case where correction for sample volume has not been done, which may lead to erroneous conclusions regarding \( K_{av} \) unless, as in this case, the scientists are aware of the situation.

Thermostatting the column is preferred to ensure minimal variation in the calibration curve if the column is to be used over a long time period. It is also a good practice to check the efficiency and symmetry by elution of a test probe at regular intervals. When the column is not in use the flow rate may be reduced without impairing the calibration curve, but if the flow is switched off then the column must often be recalibrated. In this case precautions to avoid bacterial growth will be necessary. If it is properly handled an analytical column can be expected to have a lifetime of 1–5 years. An example of calibration of an analytical SEC column is given in Figure 3.16. Here, a mixture of seven reference proteins were injected and the elution volume converted to \( K_{av} \) for the calibration curve (177). The figure illustrates that the shape of the curve is fairly linear over the central portion of the fractionation range. Note that the calibration curve is, in practice, identical to the selectivity curve (the first is used for size determination of an unknown solute and the second for characterization of the SEC support). In some textbooks the calibration curve is plotted with the molecular weight being a function of the retention volume; however, from an analytical point of view it seems accurate to plot the retention volume as the dependent variable of the independent variable, solute dimension, or molecular mass, as for Figure 3.16.

This approach was used for estimation of protein molecular weight, as exemplified by the characterization of chick interferon by Phillips and Wood in 1964. They were able to assign a molecular weight of approximately 40,000 to the interferon by eluting the sample on a column of Sephadex G-10, calibrated with standard proteins (178). The obtained molecular weight was confirmed using an analytical ultracentrifuge. However, calibrating the column with standard proteins is only valid as long as the properties of the sample and standards are similar. In many cases, different shapes of proteins must be normalized by denaturation of the protein with SDS or quanidine hydrochloride (61). The size of an intact protein may be inferred from the hydrodynamic volume obtained using the concept of universal calibration of the column. These procedures are discussed in Section 3.2.3.
It is also possible to use the SEC column as a separation device only and to analyze the effluent for a size-related property. Thus, collected fractions may be characterized by chemical endgroup analysis, light scattering, osmometry, viscosimetry, mass spectrometry, and so on. The requirement on the resolvability of the column is high (collected fractions need to be as pure as possible), but the requirements on other parameters, such as flow rate and temperature stability, are low. Furthermore, the column does not have to be calibrated, and solute–matrix interactions will not influence the result, as long as components are resolved. The advances in online detectors suitable for characterization of solute size in column effluents has provided the user with many options (see Section 3.6.4).

### 3.7.3.3 Determination of Pore Dimensions

As the retention in ideal SEC is a function of pore size, approximations of the pore dimensions can be achieved by eluting molecules of well known sizes and applying established theory for SEC; this has been called inverse SEC or chromatographic porosimetry.

By measuring the entire selectivity curve with well characterized standards, estimations of the pore size, pore volume, and surface area may be obtained (179, 180). In the method of Freeman and colleagues, the data are obtained from the inflection point of the plot of \( K_w \) versus the hydrodynamic radius of the solute (179). The method of Halász and colleagues included an initial empirical calibration of the procedure to yield data in accordance with established methods (180). It has been reported that both methods give data in good agreement with absolute methods (181). However, the method of Halász was found to yield a broader pore size distribution (PSD) than that obtained by classical methods (182). The shortcomings of the method are due to an incorrect assumption of the relationship between available pore volume and solute size (Halász assumed that a solute will either be excluded from the pore or enter the pore and then have access to the entire pore volume, which is not correct; see Fig. 3.1) as reviewed by Knox and Scott (183). These authors mathematically derived the relationship between the pore size distribution curve and the SEC selectivity curve, assuming a cylindrical pore model, to give

\[
F(r) = \frac{1}{2} \left[ \frac{d^2K_D}{d(\ln R)^2} \right]_{R=r} - \frac{3}{2} \left[ \frac{dK_D}{d(\ln R)} \right]_{R=r} + K_D, \tag{3.32}
\]

where \( F(r) \) is the cumulative pore size distribution for pores of radius from 0 to \( r \) and \( K_D \) is the distribution coefficient for a solute of radius \( R \) (please note that \( K_{sv} \) cannot be used for this application!). The equation was found to yield data in excellent agreement with mercury porosimetry of rigid materials (183). However, the numerical calculation requires a very smooth calibration curve and may show instability. Thus, the authors recently recommended the calculation of pore size distribution from a simulation procedure (184).

An alternative direct calculation procedure based on pores being built up by the space between random solid spheres was used by Schou and colleagues (personal communication) for characterization of silica materials. However, the pore models used may be more or less realistic; for example, materials having cylindrical pores are probably very rare (the model is perhaps applicable to controlled porous glass) due to dissolution and redeposition of silica. Beads composed of perfect microspheres are probably also rare, and the pore model of polymer gels being composed of intersecting rods seems to yield inconsistent data (42). The shortcomings of the existing models for pore structure may be exemplified by the difficulty to assign an equation for hindered pore diffusion (see Section 3.2.2).

However, there seems to be a need for a simple method for characterization of apparent size exclusion dimensions of SEC supports for the purpose of comparisons of materials and for prediction of functional properties. A rough estimate of the pore size may be calculated from twice the solute size at the inflection point of the calibration curve (76). Another interesting observation that can be made from the theoretical calibration curves in reference 76 is that the linear part of the calibration curve intersects with \( \log R \) at a value close to the average pore radius of the cylindrical pore model used. However, the variability in experimental data may yield a high uncertainty to the estimate obtained. Hagel and co-workers showed that the procedure used by Jerabek (185) and supported by Knox and Ritchie (184), where the apparent pore size distribution is inferred by an iterative procedure to yield \( K_D \) values in agreement with the experimental data, may be a feasible approach (186). The data obtained may be used for functional predictions of size-exclusion behavior, and it was stressed that only an apparent pore size is obtained with inverse SEC (or chromatographic porosimetry) because different estimates are obtained from the same experimental data depending upon the pore model applied (186). The pore model generally applied is the one of cylindrical pore shape. The reason for this is that this is the model used for converting experimental data from classical methods such as nitrogen sorption and mercury porosimetry to pore size. It was shown that the selectivity curve of Superose 6 corresponded to a hypothetical support having cylindrical pores with a mean radius of 250–290 Å (186). This is slightly larger than the value found by applying Equation 3.32 due to the different shape of the apparent PSD curves (i.e., the latter method gave a very skewed PSD whereas a Gaussian distribution was assumed in the former method).

These methods are of course only applicable for obtaining operational information on the PSD provided that the reference data for the probe molecules are carefully selected to reduce disparities as revealed for data on proteins and dextrans (63). It must also be emphasized that an incorrect
application of theory (e.g., by neglecting the increasing exclusion from part of the pore volume for permeating solutes of increasing size), as proposed by Halasz and applied by a number of authors, will lead to erroneous results (187–189). In one work the mean pore diameters for Superose 6 and TSK SW 3000 were reported to be substantially less than 142 Å and 120 Å (189), which are unrealistically low (roughly half expected value) compared with other reports (76, 190). This, again, illustrates the importance of interpreting the data obtained and to compare these with information from other sources to validate the approach used. A review of methods for the characterization of pore size of chromatography supports has been provided by Hagel (76). A discussion of the various methods used for inverse SEC and data for apparent pore radii of some common materials for protein SEC has also been presented by Hagel and colleagues (186). An improved version of the method by Hagel and co-workers was used by DePhillips and Lenhoff to characterize the pore dimensions of ion-exchange materials (191). Methods for the determination of pore size distributions of SEC supports have been reviewed recently by Yao and Lenhof (192).

3.7.4 Non-SEC Separations

In mixed-mode separations, the elution of a solute is not due to size parameters only, but is affected by adsorption or affinity to or exclusion from the matrix or layers of solvents or solutes adsorbed to the surface of the matrix. These types of solute–matrix interactions provide the basis for many of the separation principles dealt with in detail in subsequent chapters. Because mixed-mode separations are sometimes noticed on supports designed for SEC, the subject will be discussed briefly here to exemplify types of non-SEC behavior.

Owing to its chemical nature and origin, the matrix may contain residual amounts of anionic groups, as in dextran and agarose supports (10, 88), or exposed anionic groups, as in silica-based materials (87). The crosslinking agent may introduce hydrophobic sites, as will the bounded organic layer of silica materials. The matrix may in rare cases also provide sites for biospecific interactions (13). These adsorptive properties of the matrices are highly undesirable in SEC and may be reduced by using a low pH to suppress ionization or a high ionic strength to decrease ionic interaction, adding detergents or modifiers to prevent hydrophobic interactions or simply by saturating the active sites with, for example, ovalbumin (13, 86), phospholipids (79), or basic peptides (193).

Interactions between the solute and the matrix may be taken advantage of, as shown for the initial purification of yeast cytochrome oxidase (194). More than 500 mL of an extract of submitochondrial particles from yeast was applied at high ionic strength (i.e., 30% w/w, of ammonium sulfate) to a column packed with Toyoperl (also called Fractogel). A highly purified and concentrated fraction of active enzyme was eluted by a gradient of decreasing salt content. The authors concluded that the very hydrophobic protein was probably adsorbed due to hydrogen bonding and that the interaction provides a favorable alternative to salt fractionation for the initial purification of yeast cytochrome oxidase. This separation would be classified as hydrophobic interaction chromatography (see Chapter 6).

Separations that are influenced by solute–matrix interactions should of course not be referred to as size exclusion chromatography. The retention volumes of solutes may in a non-SEC mode vary substantially with changes in the mobile phase composition, as shown in Figure 3.17. The general trend in this figure is that proteins having an isoelectric point above the pH of the buffer are being retained due to ionic interaction with the silanol groups of the matrix at low ionic strength, whereas acidic proteins (being negatively charged) are subjected to ion exclusion at these conditions. High ionic strength reduces these ionic effects. However, as many solutes and matrix surfaces possess both ionic and

Figure 3.17 The effect of ionic strength on the retention volumes of native proteins of various isoelectric points (I_p). Column: TSK-G3000 SW. Buffer: 0.01 M sodium phosphate at pH 7.0, containing 0–0.5 M ammonium acetate. Proteins: (a) L-tyrosine, (b) vitamin B12, (c) cytochrome c (I_p = 10.3), (d) ribonuclease (I_p = 9.3), (e) α-lactalbumin, (f) chymotrypsinogen (I_p = 9.2–9.6), (g) soybean trypsin inhibitor (I_p = 4.2–4.5), (h) ovalbumin (I_p = 4.7), (i) serum albumin (I_p = 4.7–4.9), (j) transferrin (I_p = 5.5), (k) serum albumin dimer (I_p = 4.7–4.9), (l) apoferritin (I_p = 4.6–5.0), (m) β-galactosidase, (n) thyroglobulin (I_p = 4.5). Reproduced from Reference 63 with kind permission of the authors and publisher.
hydrophobic sites, the optimum composition of the mobile phase will balance the different effects (e.g., at high ionic strength the retention volume may start to increase due to hydrophobic interaction).

Mixed-mode separations may lead to unexpected results, such as the concentration of a solute zone upon passage through the column (88), or the separation of proteins according to their isoelectric points (195). A separation of ribonuclease A and cytochrome c was accomplished by utilizing cationic effects at low ionic strength (196). Purification of cytochrome c was achieved by adsorption of cytochrome c at high ionic strength with subsequent elution with a decreasing salt gradient (197). Several publications where mixed-mode separation has been utilized or may be suspected to influence the separation have been published (195–200). It has also been pointed out that such effects may result in poor resolution compared to separations based on SEC (201).

Naturally, this information is very valuable when selecting materials and conditions for which separations based on SEC are desired.

Size exclusion chromatography is, by definition, a method for separating molecules of different size due to their different permeation of a porous matrix. This distinguishes SEC from some other size-separating techniques such as hydrodynamic chromatography, slalom chromatography, and field flow fractionation.

Hydrodynamic chromatography may be carried out in a column packed with nonporous beads (21, 134). The separation takes place in the void volume, and the solutes are eluted according to decreasing size due to the fact that large solutes will, statistically, spend more time in the faster flowing center portion of the parabolic flow profile of the solvent. The advantage is that fast separations may be carried out, but disadvantages such as shearing of large solutes and low peak capacity seem to limit the applicability of hydrodynamic chromatography.

Slalom chromatography was introduced as a result of the observation that elongated stiff molecules such as DNA could be trapped in the void volume of a column and eluted at a low flow rate (22). The phenomenon is probably related to the relaxation time of the solutes. Results published for separation of DNA show remarkable results, because the resolution increases with increased flow rate (202). Because the separation takes place in the void volume, the separating volume is small, and the method is not suitable for preparative applications. Also, the technique is mainly applicable to stiff rod-shaped solutes of appreciable size, and as the size of solutes decreases, hydrodynamic chromatography effects will start to influence the result.

The third technique, field-flow fractionation (FFF), explored by Giddings and co-workers shows some resemblance to hydrodynamic chromatography in that the solutes are carried forward due to the distribution of solutes across a parabolic flow profile in a thin separation channel. The differential distribution of sample components is caused by a force field applied perpendicular to the flow (23). An adoption of this technique, called asymmetrical flow field-flow fractionation, where solutes in the size range of 20–5000 Å are separated according to their diffusion coefficient, has been used for analytical separation of monomers from multimers of monoclonal antibodies (203). The authors found that FFF was advantageous compared to SEC with respect to peak capacity, selectivity, separation time, and dynamic separation range for analytical applications. However, the peak capacity of FFF for completely resolved peaks may be estimated to 10 (the reported value was 15 at the resolution factor of 1), which is less than that expected for a SEC column operating at optimal conditions (i.e., 13; see Section 3.2.2). The maximum selectivity as functionally expressed by the complete separation of monomer and dimer of a globular protein is of the same order as that expected for SEC columns, and the separation time, in the cited case 6.3 min, may be matched by using SEC materials of small particle size (e.g., 10-cm-long column packed with 2-μm supports operated at 120 cm/h, which will still yield high resolution in the molecular mass range investigated, i.e., 156,000; see Table 3.7). Thus, although analytical SEC, at its best, still provides a good alternative for fast separation of proteins, FFF may be seen as a complementary technique, especially for the separation of very large solutes (exceeding 100 Å). However, FFF is not suitable for separation of low molecular weight solutes (e.g., peptides) or for preparative size separations.

3.8 SYMBOLS USED IN SEC

As a result of the many different approaches taken to develop liquid chromatography as a separation technique and also to characterize chromatography supports, the nomenclature in liquid chromatography has, for some parameters, turned out to be inconsistent. For example, in reversed-phase chromatography (RPC) the total liquid volume is not denoted $V_0$ but $V_m$. This is unfortunate, and to avoid ambiguities the designation $V_m$ for the mobile-phase volume in RPC has been suggested instead of $V_0$ (204). A review including a detailed discussion of the, sometimes contradictory, proposals for nomenclature in liquid chromatography issued by IUPAC and ASTM has been given by Ettr (204). The proposal of “steric exclusion chromatography” for SEC made by one ASTM Committee (205) has not yet found broad acceptance by workers in this field of chromatography, as judged from the vast literature on the subject. The most recent suggestion for nomenclature in liquid chromatography seems to offer a reasonable compilation of designations that may be accepted by the workers in the field (206). This nomenclature has been adopted throughout this revision. The symbol for relative molecular mass, $M_r$, is used throughout the chapter. It may be noted that molecular weight is sometimes referred to as...
TABLE 3.8 Symbols Used in SEC

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Parameter</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_b$</td>
<td>Retention (elution volume)</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$V_b$</td>
<td>Void volume</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$V_i$</td>
<td>Intraparticle pore volume$^b$</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$V_s$</td>
<td>Support volume$^b$</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$V_L$</td>
<td>Total liquid volume</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$V_c$</td>
<td>Geometric column bed volume</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$V_o/V_c$</td>
<td>Void fraction</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$V_c/V_0$</td>
<td>Bed permeability</td>
<td>IUPAC-74</td>
</tr>
<tr>
<td>$K_d$</td>
<td>Distribution coefficient</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$K_{iv}$</td>
<td>Gel-phase distribution coefficient</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$e$</td>
<td>Void fraction, $V_o/V_c$</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$L$</td>
<td>Column length, bed height</td>
<td>ASTM</td>
</tr>
<tr>
<td>$A_c$</td>
<td>Column cross-sectional area</td>
<td>ASTM</td>
</tr>
<tr>
<td>$d_p$</td>
<td>Average particle diameter of support</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$\Delta p$</td>
<td>Pressure drop</td>
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<tr>
<td>$\eta$</td>
<td>Viscosity of solvent</td>
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</tr>
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<td>$F$</td>
<td>Volumetric flow rate</td>
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<td>Interstitial fluid velocity, $F/A_c \cdot 1/e$</td>
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<td>$u_{nom}$</td>
<td>Nominal fluid velocity, $F/A_c$</td>
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</tr>
<tr>
<td>$N$</td>
<td>Number of theoretical plates in column</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$H$</td>
<td>Height equivalent to a theoretical plate, $L/N$</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$h$</td>
<td>Reduced plate height, $H/d_p$</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$w_b$</td>
<td>Peak width at base ($4\sigma$ for a Gaussian peak)</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$w_h$</td>
<td>Peak width at half peak height</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$R_e$</td>
<td>Resolution factor</td>
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</tr>
<tr>
<td>$A_s$</td>
<td>Asymmetry factor, $b/a$</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$n_{ea}$</td>
<td>Peak capacity factor at a resolution of $R_e$</td>
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</tr>
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<td>$M_r$</td>
<td>Relative molecular mass of solute</td>
<td>SIS 016174</td>
</tr>
<tr>
<td>$D$</td>
<td>Diffusion coefficient of solute in free solution</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$D_m$</td>
<td>Diffusion coefficient of solute in mobile phase</td>
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</tr>
<tr>
<td>$D_s$</td>
<td>Diffusion coefficient of solute in support pores</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$\gamma_m$</td>
<td>Obstruction factor in extra particle space, $D_m/D$</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$\gamma_s$</td>
<td>Obstruction factor in support, $D_s/D$</td>
<td>IUPAC-93</td>
</tr>
<tr>
<td>$V_h$</td>
<td>Hydrodynamic volume of solute</td>
<td>ASTM</td>
</tr>
<tr>
<td>$R_h$</td>
<td>Hydrodynamic viscosity radius of solute, $R_h$</td>
<td>ASTM</td>
</tr>
<tr>
<td>$R_0$</td>
<td>Stokes radius of solute</td>
<td>ASTM</td>
</tr>
</tbody>
</table>


$^b$The symbol for the stationary phase as defined by IUPAC-93 does not include the solid matrix. However, in SEC no stationary phase, as defined for adsorption techniques, exists and it is convenient to let $V_s$ denote the solid support conceptually being responsible for the separation. The matrix volume has also been referred to by $V_c$.

MW, which may be regarded as an abbreviation for molecular weight and not a symbol. Unfortunately, the nomenclature suggestion from IUPAC is not complete and some of the symbols used still lack formal acceptance. Thus, the designation $V_s$ for solid matrix volume is not yet supported, but until a formal proposal is made, the use of $V_s$ as earlier suggested will remain, especially since this practice is not ambiguous in SEC. The symbols given in Table 3.8 are believed to represent an acceptable compilation of designations used today in size exclusion chromatography (SEC).

3.9 ACKNOWLEDGMENTS

As this is intended to be my last scientific contribution to the field of separation science I feel that it is appropriate to give an acknowledgment to some key persons at Pharmacia, where it all once started. I will always be grateful to Rune Andersson who in 1973 gave me the first task to develop chromatographic methods for the separation of pharmaceutical compounds prior to analysis, as well as to Adolf Berggren for his support and thrust in the young student (at that time). I am greatly indebted to Kirsti Granath for her guidance and mentorship in the area of characterization of polymers with the aid of SEC. In her laboratory I came to meet Torvald Andersson, the uncrowned “king of column packing,” and we spent a memorable and rewarding time improving our understanding of separation science and developing new materials. This chapter is dedicated to Rune, Adolf, Kirsti, and Torvald, and all young scientists who will not merely note the who but who will seek the why to be able to advance science by establishing the how.

Trade names mentioned in this chapter are a property of their respective owners.

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4

ION EXCHANGE CHROMATOGRAPHY

EVERT KARLSSON

Department of Biochemistry and Organic Chemistry, Uppsala University, Box 576, 751 23 Uppsala, Sweden

IRWIN HIRSH

GE Healthcare Bio-Sciences AB, 751 84 Uppsala, Sweden

*Present address: Novo Nordisk A/S, Nybrovej 80, 2820 Gentofte, Denmark

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4.1 INTRODUCTION

Ion exchange chromatography (IEC) has been in common use for more than 50 years for the separation and purification of proteins. There is therefore a great deal of literature documenting the widespread experience with IEC. It is traditionally one of the most utilized protein separation techniques. In a survey in 1997 comprising 134 purification protocols, in which 426 chromatographic steps were used, IEC accounted for 40%, gel filtration for 18%, and affinity chromatography for 29%, which includes immobilized metal affinity and dye affinity chromatography (1). However, data from a survey in 2000–2003 using the SciFinder™ (ACS, 2004) web tool showed that the use of IEC is in decline and accounts for ≏14% of chromatographic methods, and affinity (AC) and size exclusion chromatography (SEC) are the most utilized methods (Fig. 4.1). Market data from GE Healthcare Bio-Sciences (2003) show that, for industrial applications, IEC was the leading technique (45% of all chromatographic steps). It will be revealing to see if in the future industrial trends follow the literature trends.

The practice of IEC in laboratory experiments and industrial processes differs greatly. At the laboratory scale, everything is smaller. The volumes to be processed are small and columns larger than 1L are rare. However, in industrial separations, columns of 1000 L may be used routinely to process tens of thousands of liters of cell culture or fermentation broth. These large volume applications necessitate very high flow rates and media that must withstand high pressures. The cost of buffer and salts, as well as their proper disposal, are also important considerations when working at large scales. Mixing of a sample in a laboratory is hardly ever considered to be a problem. However, in industry it is sometimes a serious problem, and the mixer may require cooling. In both cases the generation of fines must be avoided when handling chromatographic media.

IEC has high resolving power, high protein binding capacity, and versatility (there are several types of ion exchangers). Also, the composition of the buffer and pH can be varied, and it is easy to use. Although IEC is an established technique in most biochemical laboratories, the fundamental mechanisms behind protein binding to charged surfaces are not fully understood. Several models have been proposed [see review by Ståhlberg (2)]. The following is a brief discussion of the more common models.

In the stoichiometric model of Boardman and Partridge (3), a number of charged groups of the protein bind to the same number of oppositely charged groups of an ion exchanger, and counterions are released both from the protein and the ion exchanger. The charges are localized to special points on the protein molecule, and the number of charged groups binding to the ion exchanger is called the Z-value, which is determined as described in Section 4.3.5.
This model assumes that one group on the protein interacts with just one group on the ion exchanger. There are many exceptions from such one–one interactions; for example, Thr 8 of the acetylcholinesterase inhibitor fasciculin interacts with both Val 71 and Asp 276 of the enzyme (4).

In the slab model (5), both the protein and the ion exchanger are regarded as planar surfaces surrounded by a diffuse layer of charges in contact with a salt solution. Because of thermal motion, counterions are not bound to ions of opposite charge, but are instead distributed in a diffuse layer close to the surface, the diffuse double layer. In the modified form of the slab model, the theory of charge regulation (6), proteins are assumed to be spheres with charges distributed over the surface, and half of the sphere comes into contact with the ion exchanger. When two surfaces of opposite electric charge approach each other, the electric field from one surface penetrates the intervening solution and reaches the second surface. The electric potential on both surfaces changes, which leads to changes in the charge density. The ion exchangers have a higher charge density than proteins and thus a stronger electric field. Therefore, the field of the ion exchanger will have a larger effect on the electric field of the protein and the net charge of the protein will not be constant, but a function of the distance between the protein and the surface of the ion exchanger.

In 1850, Thompson, in England, carried out the first experiments on ion exchange. He observed that the percolate from soil saturated with ammonium sulfate and washed with water did not contain ammonium sulfate, but calcium sulfate (gypsum). The ion exchange processes were for a long time thereafter studied in soil and minerals such as zeolites. Adams and Holmes (1936) synthesized ion exchangers by condensation of phenolsulfonic acid and formaldehyde (cation exchangers) and phenylendiamine and formaldehyde (anion exchangers). These experiments can be regarded as the beginning of modern IEC (7).

The first use of an ion exchanger in protein chemistry may have been for the removal of pectinmethyl esterase from a preparation of pectinpolygalactonurase. The contaminant was adsorbed to a polystyrene cation-exchanger (Amberlite IR-100, sulfonic acid type) (8). Some years later, the cation exchanger Amberlite IRC-50 was used successfully for the chromatographic separation of a number of basic proteins (9), such as cytochrome c, ribonuclease, and lysozyme. This resin is a polymer of methacrylic acid cross-linked with divinylbenzene, and has a $pK_a$ of 6.5 (at ionic strength 0.1 M), and is therefore suited for basic proteins (see Section 4.4.2).

Note that herein the words resin, gel, matrix, and media are used interchangeably and that many of the discussions may also apply to membrane chromatography and continuous bed (monoliths) if they utilize ion exchangers. The most notable exceptions are discussions related to the limitations of diffusion.

Polystyrene resins are not well suited for protein chromatography due to their low protein binding capacity and very hydrophobic matrices, which can bind proteins very strongly or irreversibly (see Section 4.4.1).

The introduction of ion exchangers with hydrophilic and macroporous matrices in the middle of the 1950s by Sober and Peterson (10–12) constitutes the great leap forward that extended the use of IEC to most proteins. They synthesized carboxymethyl (CM) and diethylaminoethyl (DEAE) derivatives of cellulose. Adsorption of proteins to the hydrophilic base matrix by forces other than electrostatic is low, and the macroporous structure renders ion exchange
groups in the interior of the particles accessible to proteins, resulting in a high capacity. Their work was preceded by several reports on the use of derivatized cellulose as ion exchangers, but without fully realizing the potential of the method (13–16). Since then, a number of chromatographic media, in particular beaded ones, have been commercialized. These include gels based on crosslinked dextrans, crosslinked agarose, and synthetic hydrophilic polymers.

In multi-step industrial separations the first chromatographic step, often called the “capture step,” is usually a two-step on/off procedure. Proteins of interest are adsorbed from a large volume sample and desorbed within a relatively small volume of new buffer. Two-step procedures can give a relatively high degree of purification and they can decrease the sample volume considerably, which facilitates further purification. Batchwise techniques (see Section 4.7.7) or columns designed to capture proteins from a large sample are used (see Section 4.7.6).

To avoid the time and expense of clarification and buffer exchange of large samples, expanded bed ion exchangers have been developed (see Section 4.4.12). During sample application, debris and insoluble material pass through the expanded bed, while proteins of interest are adsorbed to the ligand.

In this chapter we discuss the different ion exchangers, the interactions between proteins and ion exchangers and their matrices, and the influence of pH and various salts on protein separation. Some aspects of the recovery and storage of proteins will also be briefly discussed. Hydroxyapatite chromatography will also be treated in this chapter, as chromatography of basic proteins on hydroxyapatite depends on ionic interactions. The chapter concludes with some examples of applications on both laboratory and industrial scales.

4.2 THE ION EXCHANGE PROCESS

4.2.1 Factors Influencing Protein Binding and Functional Sites of Ion Exchangers

The basis for IEC is the electrostatic attraction between proteins in solution and charged groups of the ion exchanger. The strength of the interaction depends upon the charge of the proteins and the ion exchangers, the dielectric constant of the medium, and competition from other ions for the charged groups of the ion exchanger and protein. When the concentration of competing ions is low, the proteins adsorb to the ion exchanger. When it is high, the proteins are desorbed. The most common technique in IEC is adsorption of target proteins from a buffer of low ionic strength and desorption with a more concentrated buffer (see Section 4.7.2).

Most IEC of proteins is carried out in aqueous solutions, but in some cases an organic solvent is added to the buffer in order to decrease hydrophobic interactions with the matrix. The addition of organic solvents will also serve to increase the electrostatic interaction, which according to Coulomb’s law is proportional to $1/D$ ($D =$ the dielectric constant of the medium). The dielectric constant of water is 80 and for many organic solvents $\approx 20$.

The high $D$ value of water is able to overcome the electrostatic interaction between the ions in many salt crystals, and this is why so many salts are soluble in water. For the $D$ value effect to be valid there must be a sufficiently thick layer of solvent molecules between the two charged groups.

If there is direct contact, as between the active site of an enzyme and its substrate, the contact is due to a steric fit between the molecules that results in several types of interactions: electrostatic, hydrogen bonding, and hydrophobic. This type of direct contact is exploited in affinity elution, where a charged substrate or inhibitor is used to elute a protein from an ion exchanger (see Section 4.8.2.4).

In chromatography, substances alternate between being bound to the stationary phase and moving in the mobile phase. The stationary phase is a column, thin-layer plate, paper, and the mobile phase a buffer, a gas flow. A substance that spends more time in the stationary phase moves slower. Substances that do not bind to the adsorbent are constantly in the mobile phase. They pass through un retarded and are, strictly speaking, not submitted to chromatography, but filtered through the column. This happens when the ion strength is so high that it eliminates the electrostatic attraction to the ion exchanger and with molecules with no charge or a charge of the same sign as the ion exchanger. Separation according to differences in net charge is illustrated in Figure 4.2.

Sober, Peterson and colleagues (12) fractionated serum proteins on the anion exchanger DEAE cellulose (positively charged). Each fraction was then analyzed by moving boundary (free) electrophoreses, wherein electrophoretic mobility depends on the net charge. Most, but not all of the proteins eluted in order from the ion exchange column according to the strength of their overall net negative charge.

Factors other than the net charge that can influence IEC of protein include the following:

- charge distribution on the protein surface
- nature of the particular ions in the solvent
- nonelectrostatic interactions with the ion exchanger, hydrophobic interactions, and hydrogen bonding
- temperature
- additives such as organic solvents.

The effect of charge distribution, hydrogen bonding, and hydrophobic interactions can be seen from chromatography of snake neurotoxins and their derivatives. The neurotoxins are proteins of 7–8 kDa. Some differ only by a single amino-acid mutation and the derivatives by a modification
of the same group, for instance an amino group, in different positions of the molecule. The separation can therefore be attributed to a single factor. The same factors also influence the separation of proteins differing much from each other, but in these cases it is more difficult to know to what extent they affect the separation.

Ion exchangers have as a functional site, a positively (anion exchangers) or a negatively (cation exchangers) charged group to exploit the electrostatic interaction between the protein and the ion exchanger. Dipolar ion exchangers (see Section 4.4.10) have both positive and negative groups. Some adsorbents are designed to exploit several separation parameters. In multimodal ion exchangers (see Section 4.4.13) ionic forces, hydrophobic interactions, and hydrogen bonding are exploited for adsorption of proteins. Other ion exchangers designed to utilize only electrostatic interactions may also be multimodal due to hydrogen bonding (see Section 4.5 and Fig. 4.7). The functional site of hydroxyapatite contains both positive (Ca$^{2+}$) and negative (PO$_4^{3-}$) charges. Ca$^{2+}$ interacts with phosphate groups on proteins, forms complexes with carboxyls and hydroxyl groups on carbohydrates and carbohydrate moieties on glycoproteins, and PO$_4^{3-}$ forms ionic bonds with positive groups on basic proteins (see Section 4.10).

4.2.2 The pH Parameter; Protein Binding at pH $\approx pI$

pH is one of the most important of the parameters affecting protein binding, as it determines the charge on both the protein and the ion exchanger. The net charge of a protein varies with pH, and binding should occur when the net charge is of opposite sign to that of the ion exchanger. At pH values far away from the pI, proteins bind strongly and do not desorb with low ionic strength buffers. This is true in many cases, but there are also exceptions. Several proteins bind at pI, or even when the net charge is of the same sign as that of the ion exchanger (17–19). Such binding may depend on an asymmetric charge distribution with a cluster of positive or negative charges in one domain of the molecule and/or on the Donnan effect.

The Donnan effect occurs when the pH in the micro environment of the ion exchange groups is significantly different than that of the bulk buffer solution. Negative ion exchange groups in cation exchanger can attract protons and repel hydroxyl ions. As a result, the pH in the matrix can be one unit lower than in the surrounding buffer. In anion exchangers the two ions move in opposite directions and can result in a one unit pH increase in the micro environment. If a protein is adsorbed at pH 5, it may be exposed to pH 4 and may therefore be denatured and inactivated.

An ion exchanger is normally used in conditions where most of its functional groups are charged. Weak cation exchangers are usually used at pH $\geq pK_a$ and weak anion exchangers at pH $\leq pK_a$. Strong ion exchangers have $pK_a$ values outside the pH range at which it is usual to work with proteins, and changes in the pH will not change the charge of the ion exchanger.

4.2.3 Competition between Ions and Proteins for Sites on an Ion Exchanger

Proteins must compete with other ions in solution for the charged groups on the ion exchanger. When the concentration of competing ions is low, protein binding will occur between several charged groups on the proteins and oppositely charged groups on the ion exchanger. On dipolar ion exchangers, proteins interact with both negative and positive groups (see Section 4.4.14). The number of charged groups interacting with the ion exchanger is called the Z-value. At higher concentrations of competing ions, the proteins with the weakest interaction will be displaced first.
There is no general rule as to what salt concentration is needed to displace a protein with a certain net charge from an ion exchanger. However, most proteins are eluted at salt concentration lower than 1 M. The type of ion (from the salt) is also an important factor. Some ions displace proteins more efficiently than others, and those that interact specifically with charged groups on the proteins are more efficient displacers (see Section 4.8.2.4). Ions can also affect the selectivity, resolution, and elution order from the column (see Section 4.6.2).

4.2.4 Hydrophobic Interaction and Hydrogen Bonding

In addition to Coulombic forces (charge–charge interactions), other types of interactions may contribute to the binding of proteins to an ion exchanger. Hydrophobic interactions can occur, especially on ion exchangers with hydrophobic matrices such as polystyrene-based matrices and some HPLC ion exchangers (see Section 4.5.1, Fig. 4.7). Similarly, in some cases, hydrogen bonds are formed (see Section 4.5). In such cases the separation depends on a combination of electrostatic and hydrophobic interactions or hydrogen bonding. These not electrostatic forces may be crucial for obtaining a separation of two similar proteins. Multimodal ion exchangers (Section 4.4.13) are designed to take advantage of such nonelectrostatic forces.

4.3 CHARGE PROPERTIES OF PROTEINS

4.3.1 Charged Groups in Proteins

Eight of the 21 (selenocysteine included) amino acids of proteins have side chains that are weak acids or bases (Table 4.1). Several belong to the most common amino-acid residues of proteins. Thus, nearly all proteins have both positive and negative charges at pH values commonly used in IEC. Side chains that fulfil special roles in proteins, such as active sites, can have pK\textsubscript{a} values that deviate from the standard values shown in the table (see Section 4.3.6). The amide groups of asparagine and glutamine for all practical purposes are neither basic nor acidic, although at a pH of \(~0.5\) they do accept a proton.

Most charged groups reside on the protein surface. The main exceptions are when internal metal ions in metalloproteins use histidines and cysteines (e.g., in Cu, Zn, and Fe proteins) or glutamates and aspartates (mainly in Ca and Fe proteins) as ligands to the metal. In these cases, the site of the proton is occupied by the metal, which can be displaced at a low pH. It should be noted that metal proteins do not form a special case. Nearly one-third of all enzymes belong to this category. Only rarely have internal salt bridges been observed. Other prosthetic groups may also bind to the peptide chain via a charged amino-acid side chain; for example, pyridoxal phosphate forms covalent bonds with lysine residues. The N-terminal-amino and the C-terminal-carboxyl groups provide further charged groups.

Post-translational modifications can change the charge of proteins through the following mechanisms:

- addition of siatic acid residues to glycoproteins
- methylation of lysine and histidine
- acylation of the N-terminus by acetic or formic acid
- cyclization of an N-terminal glutamate to pyroglutamate
- amidation or esterification of the C-terminal α-carboxylate.

### Table 4.1 Charged Amino-Acid Side Chains (20)

<table>
<thead>
<tr>
<th>Group</th>
<th>Structure</th>
<th>pK\textsubscript{a}(^a)</th>
<th>Occurrence in Proteins (%)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>Carboxylate</td>
<td>12.0 (12.5)</td>
<td>5.7</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Carboxylate</td>
<td>3.9–4.0 (3.7)</td>
<td>5.3</td>
</tr>
<tr>
<td>Cysteine(^c)</td>
<td>Thiol</td>
<td>9.0–9.5 (10.6)</td>
<td>1.7</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Carboxylate</td>
<td>4.3–4.5 (4.3)</td>
<td>6.2</td>
</tr>
<tr>
<td>Histidine</td>
<td>Imidazole</td>
<td>6.0–7.0 (6.1)</td>
<td>2.2</td>
</tr>
<tr>
<td>Lysine</td>
<td>ε-amino</td>
<td>10.4–11.1 (10.7)</td>
<td>5.7</td>
</tr>
<tr>
<td>Selenocysteine(^d)</td>
<td>Phenol</td>
<td>&lt;9 to &gt;11 (10.4)</td>
<td>3.2</td>
</tr>
<tr>
<td>Tyrosine(^e)</td>
<td>Amino</td>
<td>6.8–8.0</td>
<td>3.2</td>
</tr>
<tr>
<td>α-amino</td>
<td>Carboxylate</td>
<td>3.5–4.3</td>
<td>3.2</td>
</tr>
</tbody>
</table>

\(^a\)First column pK\textsubscript{a} in proteins, within parentheses pK\textsubscript{a} of free amino acids.

\(^b\)In sequences of 1021 unrelated proteins.

\(^c\)Cysteine + cystine.

\(^d\)S in cysteine + cystine replaced by Se, occurs only in a few proteins, for example, in glutathione peroxidases (remove H\textsubscript{2}O\textsubscript{2} and lipid and phospholipid peroxides) and iodothyronine deiodinase [converts thyroxine (T\textsubscript{4}) to its active form, thyronine (T\textsubscript{3})]. The pK\textsubscript{a} is slightly lower than that of cysteine.

\(^e\)Tyrosine can have pK\textsubscript{a} < 9 if exposed to solvent, or >11 in the interior of molecule (see Section 4.3.6).
Many modifications are reversible and serve to temporarily regulate the function of the proteins. Incomplete or variable post-translational modifications show up as heterogeneity in chromatography (20). A further source of charge heterogeneity is covalent modification, which occurs during handling of proteins, such as nicking (hydrolysis of a peptide bond) of the peptide chain and loss of amide groups. Manyzymogens of proteolytic enzymes are converted into active enzymes by hydrolysis of specific peptide bonds.

In vitro chemical modifications also give rise to heterogeneity. Even if the different isomers formed have the same net charge, it may still be possible to separate them by IEC (see Section 4.3.4).

4.3.2 Protein Titration Properties

Most proteins are acidic, with pI < 7 (22, 23). Therefore, a first and often successful separation approach is to use an anion exchanger with a low ionic strength buffer (≤0.05 M) and slightly basic conditions (≈pH 8). If the protein does not bind, the next best strategy is often a cation exchanger with a low ionic strength buffer at ≈pH 6.

The weak acids and bases in proteins titrate over a wide pH range. Their net charge and consequently their electrophoretic mobility vary with pH, as shown in Figure 4.3. As the net charge varies with pH, it can be expected that the proteins will elute from an ion exchange column in different orders at different pH values. Therefore, if a poor result is obtained at one pH it is worth trying either a different pH with the same ion exchanger as discussed in Section 4.3.3, or alternatively an ion exchanger of the opposite charge.

Basic proteins with pI values greater than ≈9.5 are rarely encountered, but do occur. Examples are mitochondrial cytochrome c (pI = 10.1–10.2) and hen egg white lysozyme (pI = 11.3–11.6). A large number of protein neurotoxins from snakes, sea anemones, and scorpions are also basic. Very acidic proteins with a pI below 5 are also found. Their acidity may be due to aspartates, as with pepsin (pI = 2.9–3.1) or post-translationally added groups, as with sialoglycoproteins.

4.3.3 Effect on Separation of Change of pH

It is often possible to further purify proteins from each other by re-running the chromatographic step at another pH, as is demonstrated in the following, where two components are co-eluted at pH 5.0, but were separated at pH 3.7 (24).

A Trichoderma reesei culture filtrate was desalted on Sephadex G-25 equilibrated with 0.01 M ammonium acetate, pH 5.0 (the starting buffer), and applied to a column (2.6 × 30 cm) of DEAE Sepharose CL-6B, equilibrated with starting buffer. The column was eluted with a 1000-mL linear gradient (0.01–0.5 M) of pH 5.0 ammonium acetate at a flow rate of 40 mL/h. Three different activities, designated A, B, and C, were separated. Component C, the most acidic, was collected, lyophilized, and dissolved in 0.05 M ammonium acetate pH 3.7 and re-run on the same column, but at this new pH. The column was then eluted with a 700-mL linear gradient (0.05–0.3 M) of pH 3.7 ammonium acetate. Component C was thereby resolved into two separate cellobiohydrolases.
4.3.4 Influence of Charge Distribution on Separation

To study the role of the number of amino groups, a neurotoxin from the cobra *Naja naja siamensis* was acetylated with $^3$H-acetic anhydride (25). The toxin, which binds to nicotinic acetylcholine receptors, is a small basic protein of 71 amino-acid residues (five lysine residues, a free N-terminus, and three carboxyls) in a single peptide chain crosslinked by five disulfide bridges (Fig. 4.4a).

An amount of reagent corresponding to 1/12 of the total amino groups produced a mixture of mostly monoacetyl derivatives. The derivatized protein was dissolved in 0.02 M ammonium acetate pH 6.7 and applied to a column (2.0 × 30.5 cm) of the cation exchanger Bio-Rex 70 equilibrated in 0.20 M ammonium acetate pH 6.50.

The column was first eluted with 0.05 M ammonium acetate (pH 6.5), then with a concave gradient of 0.05 M to 1.4 M ammonium acetate (pH 6.5). The degree of acetylation of the 12 peaks produced (Fig. 4.4b) was determined from the ratio of radioactivity: $A_{280}$:

- peaks 11, 12 = triacetyl
- peaks 8–10 = diacetyl
- peaks 3–7 = monoacetyl labeled on lysine
- peak 2 = N-terminus labeling
- peak 1 = native toxin with no label.

The five lysine derivatives (peaks 3–7) have the same net charge but different chromatographic contact regions (see Section 4.3.5). This shows that differences in net charge are not the only factor that determines the separation by IEC. Different distribution of charged groups can have a large effect: peak 7 eluted 300 mL ahead of peak 3, or about three column volumes.

Derivative 2 with five lysines ($pK_a = 10.4–11.1$, Table 4.1), is more cationic than derivatives 3–7, with four lysines and one α-amino group ($pK_a = 6.8–8.0$). The separation depends both on a difference in net charge and on different chromatographic contact sites. These two factors also determine the group separation into tri-, di-, and monoacetyl derivatives and the native toxin.

Other examples of separation by exploiting a differential charge distribution on molecules of the same net charge can be found in References 28–31. Separation due to differences in charge distribution is probably very common.

4.3.5 The Chromatographic Contact Region and the $Z$-value

The chromatographic contact region (32) is the region of the protein surface that determines the chromatographic behavior. The contact region in IEC consists of charged amino acids and noncharged amino acids that bind to the ion exchanger by hydrogen bonding or hydrophobic interactions. They are distributed over a large part of protein surface and even on opposite sides of the molecule (Fig. 4.4a and fig. 3 in Reference 28). A mutation or modification of an amino acid in the contact region will change the chromatographic behavior, but a region with a high concentration of charged groups can determine the chromatography, as for instance in β-lactoglobulin (Section 4.2.2). Chromatographic contact regions in ion exchange, reversed-phase, and immunoaffinity chromatography have been determined for lysozyme variants (33).
The Z-value is the number of contact sites between a protein and an ion exchanger (3, 32) and are distributed over a large part of the protein surface. They cannot all bind simultaneously to an ion exchanger. A protein molecule is in constant Brownian motion and exposes different sites to an adsorbent, and, depending on how a protein is oriented when binding to an ion exchanger, different Z-values are obtained. An experimental value is an average of these values.

The Z-values can be determined by isocratic or gradient elution and calculated from the slope of the following plots:

Isocratic elution (3): \( \log k' = Z \log 1/C + \log K \)
Gradient elution (7): \( \log k' = 2Z \log 1/C + \log K \) (monovalent ions)
\( \log k' = 3/2Z \log 1/C + \log K \) (divalent ions)

where \( k' \) is the capacity factor \( [(t_c - t_0)/t_0, t_c] = \) retention time (elution volume) for the component, \( t_0 = \) retention time for a nonretarded component, \( C = \) molar concentration of the displacer ion, and \( K = \) constant.

As the Z-value increases, a protein is bound more strongly and becomes more difficult to elute. Boardman and Partridge (3) showed that the retention of cytochrome c on Amberlite IRC-50 increased rapidly with increasing Z-value. The Z-values are normally small and depend on the degree of ionization of the protein (pH), conformation of the protein, the nature of the displacer, temperature, and flow rate. The Z-values in the pH interval 4–8 have been measured for the following (17):

- soybean trypsin inhibitor (pI 4.6)
- ovalbumin (pI 4.7)
- b-lactoglobulin (pI 5.2)
- conalbumin (pI 6.0–6.6).

The Z-values varied between 1 and 5, reached a maximum at pH 7, and remained constant or decreased slightly at pH 8. When the net charge was zero (pH = pI) Z-values for all four proteins were in the range 1–3.5. Binding at zero net charge may depend on a cluster of anionic groups due to asymmetric charge distribution. Z-values were higher in Mg\(^{2+}\) than in Na\(^+\) (Reference 17, fig. 2). The highest value for albumin was in KCl and for hemoglobin in NaCl. Human serum albumin has an unusually high value of 8.5–11.9 (34).

Soybean trypsin inhibitor has 31 anionic groups (one C-terminal-carboxyl, 17 Asp, 13 Glu) and 22 cationic groups (one a-amino, 2 His, 10 Lys, 9 Arg) and its net charge at pH 8 is −11.5 (assuming no charge on the histidine residues and pH about pKa of the a-amino group). The Z-values at an ionic strength of 0.5 M were 2.8 in NaCl and 4.1 in MgCl\(_2\) (17). Thus, only three or four of the 31 anionic groups could bind simultaneously to the ion exchanger. That more amino acids are in contact with the ion exchanger in the presence of MgCl\(_2\) indicates a change in the protein structure. A larger protein does not necessarily have more contact points with an ion exchanger. Soybean trypsin inhibitor (20 kDa) had a Z-value of 3.3 and ovalbumin (43.5 kDa) 2.4 (18). However, there are also cases when a large protein is more difficult to elute, probably due to a higher Z-value. The monomer of a cobra neurotoxin (~8 kDa) eluted at pH 5.2 from the cellulose exchanger CM-52 in 0.2 M ammonium acetate and the dimers in two resolved peaks in about 0.3 M (E. Karlsson, unpublished observation).

Z-value decreases with increasing load: for IgG at pH 10 and 0.1 M NaCl the value was 3.6 at 1 mg/mL and 2.5 at 15 mg/mL (35). Because of steric reasons, all molecules are not able to bind to the same number of sites and therefore bind with different strengths, so several peaks can be obtained in the chromatography (see Section 4.9.4, “False Peaks”).

The equation \( \log k' \) versus \( \log 1/C \) is derived assuming that all chromatographic contact sites are charged amino acids, according to the stoichoimetric model of Boardman and Partridge (see Section 4.1). However, contact sites can also include sites for hydrophobic interactions and hydrogen bonding. Different ions can also change the protein structure and the elution behavior from an ion exchanger (see Section 4.6.2). Straight-line plots are often obtained at low ionic strength (3, 36, 37), but also nonstraight-line plots (38). The temperature dependence has been determined for some proteins (39, 40). For cytochrome b\(_5\), the Z-value between 4°C and 36.1°C increased from 3.0 to 3.6 (39). At 25.2°C and flow rates of 0.2 mL/min to 1 mL/min, the Z-value was 3.4, and at 0.1 mL/min it was 3.2. The value obtained at the lower flow rate was considered to be due to the protein having sufficient time to reach true equilibrium.

### 4.3.6 Factors Influencing Protein Charge

Protein charge depends on the dissociation constants and pKa\(_s\) of the weak acids and bases on its surface. The pKa of an acid is defined as the pH of 50% titration or protonation. An acidic group provides a net charge of −0.5 at pH = pKa, and a basic group provides a net charge +0.5 at its pKa value. At one pH unit from the pKa, a charged group is titrated to 91%, as calculated from the Henderson–Hasselbalch equation. Thus, acidic and basic groups provide 0.1 charges one unit above and below their pKa\(_s\) values, respectively. By convention, acidic dissociation constants, pKa\(_s\) values, are reported.

The pKa\(_s\) values of side chains of amino-acid residues in proteins are usually different than those of free amino acids (Table 4.1). Many factors can modify the pKa\(_s\) of the side chains, such as the influence from the neighboring groups and the position in the tertiary structure. Deviations from standard pKa\(_s\) values can be seen for side chains that fulfill special roles in proteins. Tyrosine residues have different pKa\(_s\) values depending on whether they are in the interior of the molecule or exposed to the solvent. In a neurotoxin a tyrosine that reacts very slowly with tetranitromethane and
is located in the interior of the molecule has a $pK_a$ of 11.6 (41). In another toxin, a tyrosine that is fully exposed to the solvent and reactive has a $pK_a$ of 9.7 (42). Pepsin has two aspartates in its active site that are connected via hydrogen bonds and the two carboxyls interact closely. As a result of this interaction, one of the carboxyls (Asp 32) has the unusually low $pK_a$ of 1.5. The other carboxyl (Asp 215) has a $pK_a$ of 4.7. The situation resembles ionization of maleic acid, which has $pK_a$ values of 1.9 and 6.2 (Ref. 20, p. 430).

A salt bridge between Asp 70 and His 31 in T4 lysozyme alters the $pK_a$ to 0.5 and 9.8, respectively. Here the surface charge properties changed after the very drastic conformational change from folded to unfolded states. Even less drastic conformational changes can influence the surface charge and hence the chromatographic behavior.

Structural changes can be brought about by changes in temperature or the addition of cosmotropic and chaotropic ions (see Section 4.6.2), organic solvents, polyethylene glycol (PEG), urea, detergents, or substrates or inhibitors that bind to the active sites of the proteins. The latter is exploited in affinity elution (see Section 4.8.2.4).

### 4.4 THE STATIONARY PHASE—THE ION EXCHANGERS

Ion exchangers consist of a matrix with either acidic or basic groups on the attached ligand. The basic ion exchangers are called anion exchangers and contain positive groups. Cation exchangers are acidic and contain negative groups. A large number of ion exchangers are available. A list of ion exchangers is not included as such a list would be very long, repetitive, and incomplete. Several ion exchangers are rather similar, differing only by the degree of substitution, and new ion exchangers are introduced constantly.

All manufactures of ion exchangers should be able to provide information about the type of resin used as the base matrix (to which the functional group, ion exchanger, is attached), capacity, and particle size/distribution of their products. A comprehensive list of manufactures and their ion exchange products would be rather large and require continuous updating. The authors suggest searching the Internet for manufacturer or supplier name.

#### 4.4.1 Matrices

The matrices can be roughly divided into hydrophilic and hydrophobic. The first type of matrix is usually best suited for protein chromatography, because the interaction with the matrix due to hydrophobic forces is weak. Proteins are often irreversibly adsorbed (or denatured in the process of desorption) to hydrophobic matrices, such as polystyrene, and such ion exchangers are of only limited use for protein chromatography. However, the classification of matrices into hydrophilic and hydrophobic is not a strict one. On many ion exchangers, hydrophobic interactions and hydrogen bonding to the base matrix or linker arm occur.

The particle size, shape, porosity, mechanical and chemical stability, and ease of packing and unpacking are all important factors when developing a separation method. Most ion exchangers consist of small globular particles. Analytical HPLC separations usually utilize the smallest rigid particles with a narrow size distribution to maximize resolution. Particle diameters of 5 μm or less are not uncommon. Low pressure chromatography applications, often preparative and industrial separations, can use larger particles with a large size distribution and diameters between 30 and 300 μm. Cellulose ion exchangers have particles in fibrous form. The continuous-bed ion exchangers have no particles.

Most ion exchangers are macroporous, that is, the particles are porous with a large pore size. Proteins and other molecules can diffuse into the particles and bind to the ion exchange groups in the interior. This gives a higher protein binding capacity. However, only a small fraction of the ion exchange groups can participate in the protein binding (see Table 4.3).

Nonporous ion exchangers have, as the name implies, no pores. The ion exchange process occurs only at the surface of these particles and the proteins diffuse only a short distance to and from the binding sites. This gives a better resolution, but the capacity is low. Nonporous ion exchangers are therefore best suited for analytical applications.

For chromatography at high flow velocities the particles must be mechanically resistant. This is achieved either by increased crosslinking or by coating a hydrophilic layer with ion exchange groups on a mechanically resistant support, such as polystyrene beads.

The different matrices have different chemical stability. Silica ion exchangers will dissolve at alkaline pH, and prolonged exposure of agarose and dextran ion exchangers to acidic environments can reduce bead rigidity. It is essential to always check the manufacturer’s product information regarding solvent and pH stability before even beginning to develop a method, especially when cleaning with acids or bases.

The most common functional groups are straight-chain ligands such as sulfopropyl. Surface extended ion exchangers (Section 4.4.9) have tendrils with ion exchange groups tethered to the base matrix. A third type has functional groups obtained by polymerization of the matrix, for instance crosslinked methacrylic acid. Matrices can be composed of different materials:

- hydrophobic or partly hydrophobic polymethacrylate polymers, such as polystyrene beads coated with a hydrophilic layer to which ion exchange groups are coupled, and hydroxylated vinyl polymers
- hydrophilic polymers, such as cellulose, dextran, agarose, and polyacrylamide
- silica.
**4.4.2 Functional Groups and Acid–Base Properties**

Cation exchangers are acids. Anion exchangers are bases. Most ion exchangers have functional groups with a single positive or negative charge with the exception of phosphate (as with hydroxyapatite). Ion exchangers are usually classified as weak or strong. The name refers to the pKₐ values of their functional groups, and indicates nothing regarding their ability to bind proteins. At pH values far from the pKₐ, protein binding can be equally strong to either a weak or a strong ion exchanger. Often, for convenience, especially for capture steps, one begins with a strong ion exchanger, but when higher resolution and capacity are required it may be necessary to screen a large array of ligands to produce suitable results.

Examples of strong and weak ion exchanger functional groups and their approximate pKₐ values are shown in Table 4.2. Precise values are, however, difficult to determine. Titration curves show that some ion exchangers do not have a single pKₐ value. DEAE ion exchangers with a high degree of substitution contain a significant amount, up to 30%, of so-called tandem groups formed by further derivatization of an already coupled DEAE group. This leads to the formation of two additional kinds of charged groups, one a quaternary amine with a pKₐ of ~9, the other a DEAE group for which the pKₐ has been lowered to about pH 6 under the influence of the nearby quaternary nitrogen, potentially via hydrogen bonding, and the resulting structure creates a weak base that does not so easily bind a second proton.

Note that pKₐ depends on salt concentration and for DEAE cellulose is 6.9 (in water), 9.1 in 0.1 M and 9.8 in saturated NaCl (~6.2 M). For Bio-Rex 70 it is 7.5 in 0.01 M, 6.5 in 0.1 M and 6.0 in 1 M NaCl. DEAE, when not fully charged at pH < pKₐ, is likely to have a different selectivity than Q (homogeneous quaternary amine) ion exchangers (see Section 4.7.2.2).

Weak cation exchangers have a lower functional pH limit. They are normally not used at pH values where a significant part of the groups have lost their charge. As a rule of thumb, the pKₐ is suggested as the lower limit. Strong cation exchangers, such as sulfoethyl, can be used at lower pH values. Similarly weak anion exchangers have their pKₐ as an upper limit of usefulness. Quaternary amines, strong anion exchangers, have no upper limit as they do not lose their charge, regardless of pH.

The pH interval over which an IEC is carried out can be restricted by reasons other than the charge properties of the ion exchanger. One obvious restriction is the pH stability range of the protein. However, even at pH values where the proteins are stable, unwanted effects can occur and proteins may adsorb irreversibly (see Section 4.5.2).

**4.4.3 Capacity and Porosity**

The total ionic or total capacity is determined by titration and is in the range 100–500 mol/mL. This corresponds to a concentration of ion-exchanging groups of 0.1–0.5 M. Sometimes, the degree of substitution is expressed in mol/g. However, ion exchangers are not used in dry form, so the degree of substitution is usually calculated in mol/mL.

The total capacity or available capacity is the amount of protein that can be bound per mL and depends upon ligand
density, the size of the protein, and the porosity of the media. This capacity is also called the static capacity.

**Dynamic capacity** takes into consideration the fact that the capacity for beaded media is generally diffusion-limited. Thus, capacity depends on flow rate and bed height, as well as the factors mentioned for static capacity. Flow rates and bed height determine the residence time (contact time) of a protein on a column (see Table 4.4) and thus the time available to diffuse into the adsorbent and interact with available ion exchange ligands. At higher flow rates, when interaction time is reduced, the dynamic capacity is lower than the static capacity. Pore size can also greatly affect the ability of the protein to diffuse into the adsorbent and interact with the ligand. Base matrix design and selection requires one to balance capacity (i.e., pore size and particle size) with mechanical and chemical stability under the desired operating conditions.

The available capacity is determined at low ionic strength, often 0.01–0.05 M, pH 5 for cation exchangers and pH 8 for anion exchangers. Manufacturers often present total capacities at low ionic strength for model proteins, such as albumin or hemoglobin. When developing a purification process for a nonmodel protein one must be aware that total binding capacities can be drastically different. Differences in capacity can arise due to flow velocity, ionic strength, buffer type, competition for the ion exchange ligand from other charged solutes in the milieu, and the simple fact that the protein of interest has different physicochemical properties than the model protein.

The size of the protein will also have a significant effect on the available capacity. The pore spaces may limit the access of proteins to functional sites within the matrix. Table 4.3 shows the available capacity of a cellulose cation ion exchanger for proteins of different molecular mass. Owing to steric factors, only some of the ionic groups are available to bind with proteins. The available capacity will therefore decrease with increasing molecular mass. The number of functional groups that can possibly bind to a protein is maximal when binding capacity (available capacity) is achieved.

Even if the total ionic capacity for two media is roughly the same, one media may show a greater binding capacity simply because it has the greatest percentage of charged groups available for binding of proteins. When the residence time is short, diffusion into the pore space becomes the limiting factor for capacity. A simple way to increase the available ligand and thus the dynamic binding capacity when residence time (flow velocity/bend height) on the column is limited is to use a smaller bead size. A smaller bead will increase the surface area to volume ratio and thus amount of ligand available for binding. Dynamic capacity is usually a very important economic consideration in industrial processes when a large volume sample is applied at a high flow rate. However, decreasing the bead size is not always an option in such processes due to cost- or process-related issues, and alternate solutions may be needed to maximize throughput.

After loading the column, target protein will be detected in the effluent. This is called a breakthrough point and used as a measure of the dynamic capacity. The factor QB10% is often used for defining the acceptable breakthrough point (how much target protein can be lost in the flow-through). This factor defines the amount of target protein bound to the ion exchanger when the protein concentration in the effluent is 10% of that of the sample being loaded. More stringent conditions like 5% or 1% may be necessary due to economic factors. Often, in laboratory-scale processes, the binding capacity is not limiting and the breakthrough point is never reached.

As discussed above, the binding of proteins to the ion exchange ligand is not instantaneous and requires time for diffusion. The dynamic capacity is therefore dependent upon the residence time on the column. Longer beds or lower flow

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Weight, kDa</th>
<th>Maximum Load</th>
<th>% Occupied Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/mL</td>
<td>µmol/mL</td>
<td></td>
</tr>
<tr>
<td>Chicken egg white lysozyme</td>
<td>14.3</td>
<td>130</td>
<td>9.0</td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td>45</td>
<td>70</td>
<td>1.55</td>
</tr>
<tr>
<td>Phosphoglycerate mutase</td>
<td>60</td>
<td>40</td>
<td>0.67</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>82</td>
<td>35</td>
<td>0.43</td>
</tr>
<tr>
<td>Enolase</td>
<td>88</td>
<td>48</td>
<td>0.55</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>140</td>
<td>21</td>
<td>0.15</td>
</tr>
<tr>
<td>Glyceraldehyde phosphate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>145</td>
<td>26</td>
<td>0.18</td>
</tr>
<tr>
<td>Aldolase</td>
<td>160</td>
<td>22</td>
<td>0.14</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>228</td>
<td>12.5</td>
<td>0.057</td>
</tr>
</tbody>
</table>

*Percentage of ionic groups involved in protein binding at maximum load, assuming an average Z of 5 and one–one type of binding. Total ionic capacity of CM 52 is 190 µmol COOH per mL (manufacturer’s booklet).
velocities can also be used to increase residence time and thus achieve greater dynamic binding capacity (see Table 4.4).

### 4.4.4 Polystyrene Ion Exchangers

This classical group of ion exchangers is made by polymerization of styrene with divinylbenzene (DVB) as crosslinker in the presence of a catalyst such as benzoylperoxide (Fig. 4.5). Ion exchange groups are then substituted into the matrix. Dowex is a well known example of these exchangers. This media has a low protein capacity and may bind proteins almost irreversibly due to hydrophobic interactions. It is consequently not used for protein chromatography, but has for many years been important in chromatography of small molecules, such as for desalting of water and for separation of peptides and amino acids, as in the Moore and Stein amino-acid analysis.

Polystyrene beads have a high mechanical stability, but in order to make this matrix suitable for protein separations a high porosity and a high protein binding capacity had to be designed into the matrix. Such media consist of macroporous polystyrene-divinylbenzene beads coated with a hydrophilic layer to which the ion exchange groups are coupled. The result is an ion exchanger that can be used for protein chromatography at high operating pressures. Several commercial versions of this media are available.

### 4.4.5 Methacrylate Ion Exchangers

Bio-Rex 70 (older name Amberlite IRC-50) is a copolymer of methacrylic acid and 5% divinylbenzene and has 11.6 mM of carboxylic groups per gram. As the actual capacity is 10 mmol/g, the aromatic constituent accounts for about 14% of the resin. In the packed chromatographic bed this capacity corresponds to 3.5 mM/mL of 3.5 M concentration of carboxylates. The pKₐ is ~6.0 in 1 M NaCl and increases to 7.5 in 0.01 M. The particles aggregate when the pH is lowered to 5.7. At this pH proteins may be adsorbed irreversibly to the

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**TABLE 4.4 Effect of Residence Time on Lysozyme Dynamic Binding Capacity** with SP Sepharose XL.

<table>
<thead>
<tr>
<th>Bed Height, cm</th>
<th>Flow Velocity, cm/h</th>
<th>Capacity, mg/mL resin</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>100</td>
<td>228</td>
</tr>
<tr>
<td>5.5</td>
<td>300</td>
<td>201</td>
</tr>
<tr>
<td>5.5</td>
<td>450</td>
<td>177</td>
</tr>
<tr>
<td>10.2</td>
<td>100</td>
<td>249</td>
</tr>
<tr>
<td>10.2</td>
<td>300</td>
<td>242</td>
</tr>
<tr>
<td>10.2</td>
<td>450</td>
<td>219</td>
</tr>
<tr>
<td>19.7</td>
<td>100</td>
<td>265</td>
</tr>
<tr>
<td>19.7</td>
<td>300</td>
<td>244</td>
</tr>
<tr>
<td>19.7</td>
<td>450</td>
<td>221</td>
</tr>
</tbody>
</table>

*Capacity measured at QB₁₀₀ (protein concentration in effluent = 10% of that in the sample). See Section 4.7.6.5 for a discussion of flow velocity (cm/h) and flow volume (mL/min).

Source: Data kindly provided by Anders Ljunglöf, GE Healthcare Life Sciences.

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*Figure 4.5 Covalent structures of the methacrylate ion exchanger Bio-Rex 70 (Amberlite IRC-50) (top) and polystyrene-based ion exchangers (bottom).*
resin, probably due to hydrogen bonding. Bio-Rex 70 has often been used close to neutral pH (that, is near its pKₐ) and it therefore forms a strong 3.5 M buffer. The equilibrium conditions of the resin and the ionic strength of the buffer mainly determine the pH of the effluent. The acrylic acid ion exchangers are best suited for chromatography of small basic proteins. They were used for the first purification of proteins by IEC (see Section 4.1). They have good resolving power for basic proteins and have proved of value for the fractionation of the collection of basic proteins that make up the snake neurotoxins. Hydrogen bonding to the carbonyl and carboxylic acid groups and hydrophobic interaction with the matrix may contribute to the separation. See Figure 4.5 for the molecular structure of a methacrylic acid ion exchanger.

4.4.6 Cellulose Ion Exchangers

In the mid-1950s, Sober and Peterson (10–12) introduced the cellulose ion exchangers for proteins. This constituted a major breakthrough in protein chromatography. Their work was preceded by several reports on the use of derivatized cellulose as ion exchangers (13–16).

Cellulose has a macroporous structure, and the ion exchange groups inside the matrix are thus more accessible to large macromolecules. Microfibrillar cellulose binds four times its own weight of water. Its capacity for protein binding is high and its interaction with proteins due to hydrophobic or other nonionic interactions is low.

The cellulose ion exchangers are prepared from strongly alkaline (mercerized) cellulose by derivatization. For instance, carboxymethyl groups are introduced by reaction with chloroacetic acid and diethylaminoethyl groups by reaction with 2-chlorotriethylamine. A typical degree of substitution is 1 mM/g cellulose, which corresponds to 200 μmol/mL bed volume. It is mostly amorphous regions in the otherwise fibrous structure that are derivatized by this treatment. However, under alkaline conditions the bundles of polyglucan chains open up.

The cellulose ion exchangers exist in several different physical forms. The fibrous cellulosates are those that are obtained directly from the derivatization process. They contain fibers of polyglucan chains mixed with amorphous regions. The microgranular ion exchangers are obtained by partial acid hydrolysis of cellulose, a treatment that removes most of the amorphous regions and creates large void spaces. Crosslinking and derivatization stabilize the ordered structures. Microgranular adsorbents have a more uniform distribution of charges. There are also commercially available cellulose ion exchangers in beaded form.

4.4.7 Dextran and Agarose Ion Exchangers

The beaded forms of dextran and agarose gels, originally prepared for gel filtration, have been derivatized to produce ion exchangers for protein chromatography.

The dextran-based ion exchangers are derived from Sephadex. The designations A and C denote anion and cation exchanger, respectively, while the numbers 25 and 50 refer to the starting gel, which is Sephadex G-25 or G-50. Both have considerably enlarged pore sizes compared to the underivatized gels, because the repulsion of similarly charged groups expands the gel. This results in a swelling of the gel in liquid that is dependent on pH, concentration of buffer ions, and degree of crosslinking.

Swelling is more prominent for the less crosslinked A-50 and C-50; it decreases when the ion exchanging groups are neutralized and is largest at low ionic strength. A column of DEAE Sephadex A-50 or CM Sephadex C-50 will shrink to half its volume when the ionic strength is increased from 0.01 M to 0.5 M.

Agarose ion exchangers are derived from crosslinked agarose, which is derived from seaweeds. These gels are more porous than the dextran gels, and volume changes with ionic strength or pH are insignificant (for the structure of agarose gels see Figs. 2.3 and 2.4). The modern varieties of agarose media are produced by a more efficient crosslinking procedure than their predecessors. Modern agarose beads are much more rigid and well suited for large scale work and other applications that require higher flow velocities. Agarose-based ion exchangers are useful for chromatography of larger proteins, as they are more macroporous. Their degree of substitution is less than that of the dextran-based ion exchangers; however, it is the pore size here that is considered the limiting factor for capacity, not the degree of substitution (see Table 4.3).

4.4.8 Perfusion Chromatography

Media for perfusion chromatography have large pores (diameter 6000–8000 Å, through-pores), that allow flow through the particles that are interconnected with 800–1500 Å diffusive-pores. Sample molecules pass through the interior as well as around the particles. In conventional chromatography molecules passing through the column are carried inside the media by diffusion. By increasing the flow rate, the time for diffusion decreases, the molecules have less chance to interact with the media, and capacity and resolution decreases. In perfusion chromatography molecules are transported to the interior by the large through-pores and then diffuse into the diffusive-pores, which should be short (<1 μm) to minimize diffusion time. The greatest advantage of perfusion chromatography occurs at high flow rates where mass transport by intraparticle convection exceeds transport by diffusion. Peak spreading due to diffusion is therefore smaller than in conventional chromatography. For proteins, flow rates of ~1000 cm/h are used without scarifying the resolution. Columns for perfusing chromatography have as matrices styrene-divinyl polymers that have a high chemical and mechanical stability. For ion exchange the hydrophobic matrix is tightly coated with a hydrophilic layer of charged
groups. The perfusion media are marketed under the name Poros™. A review on perfusion chromatography is found in Reference 45.

4.4.9 Surface Extended Ion Exchangers/Tentacle Ion Exchangers

By coating the surface of the matrix with polymeric chains one can increase the ligand density, alter the dielectric constant of surrounding solution, and decrease the probability of proteins interacting with the matrix by nonionic forces (46). The Sepharose XL and Capto™ Q and Capto S media from GE Healthcare are coated with dextran and Fractogel EMD Tentacle media, from Merck, with methacrylate derivatives. The ion exchanger can then either be coupled to the matrix, the polymeric chain, or both. Such arrangements introduce the possibility for interactions with the polymer chain extending from the surface, which may be beneficial or detrimental.

The transport mechanism into the pores of such media is different. In conventional media, intraparticle transport mainly takes place according to a pore diffusion mechanism. Media with surface extenders present a more complex surface of charged ligand over which the protein must diffuse. Elucidation of the mechanism controlling the particle influx across this surface is an active area of research (37, 47–49).

Because of these different transport mechanisms it is not accurate to transfer methods developed on standard media onto surface extended media and expect comparable results. One may find significant changes in pH values for maximum capacity and selectivity when changing between these two types of media.

4.4.10 High Performance Ion Exchangers

In high or medium pressure chromatography, rigid ion exchange beads of small and uniform size are used. In general the particle is 3–10 μm in diameter and is based on silica matrices coated with a hydrophilic layer of ion exchange groups.

The silica-based supports have very high pressure tolerances in packed beds. However, they are unstable at alkaline pH, and pH > 8 should be avoided whenever possible. The manufacturers’ operating and cleaning instructions should be the first source for stability information.

A number of media are based on crosslinked noncarbohydrate polymers. Mono™ Q and Mono S (GE Healthcare) have a charged hydrophilic surface layer attached to a macroporous polystyrene polymer matrix. The media are monodisperse with a bead size of 10 μm and are stable in aqueous solutions with a pH range 2–14. This stability enables a wide range of options for operation and cleaning.

Some ion exchangers have ion exchange groups on vinyl polymers made less hydrophobic by substitution of hydroxyl groups. They are stable between pH 2 and 12, and can be regenerated by washing with 0.1–0.5 M NaOH. They are produced by Tosoh Biosciences and distributed by several suppliers such as BioRad and Merck.

However, polystyrene-based ion exchangers coated with a hydrophilic layer and hydroxylated vinyl polymer-based ion exchangers still retain some hydrophobicity, and their separations can depend on electrostatic as well as hydrophobic interactions and, in the case of the hydroxylated polymer, also hydrogen bonding. This multimodal character (see Section 4.4.13) may constitute an advantage (Section 4.5.1).

Most HPLC ion exchangers used for protein purification are porous, with more than 95% of the surface area inside the particles. A large pore size (>30 nm) allows most protein to enter. A pore diameter of 30–50 nm is recommended for proteins in the molecular weight range 30,000–100,000. For larger proteins the pore size should increase to 80–100 nm. The large number of ion exchangers available may be confusing when trying to select a chromatographic support. However, the choice may not be critical, because organic and silica-based stationary phases with the same ionic groups, porosity, and particle sizes have about the same chromatographic behavior.

The high performance ion exchangers allow considerably increased resolution compared to conventional ion exchangers. Drawbacks include a higher cost, especially as scales increase due to the engineering needed to run systems at higher pressures. In practice, the HPLC adsorbents are not used for crude extracts because of the risk of fouling the column. Removal of bulk protein or other fouling particulates or viscosity-increasing macromolecules like DNA is recommended before HPLC is used. Often it is an excellent final step as it offers a high resolution, sample sizes are generally smaller, and thus engineering costs are reduced, and most column fouling material have already been removed by previous steps.

HPLC media are often used in analytical chromatography where only minor quantities of protein are at hand. Many analyses with modern instruments require only small amounts of proteins: N-terminus amino-acid analysis for sequencing of the first 20–30 amino acids requires one nanomole or less. Here the high operating pressures and resolution allow for very high throughput, as measured in the number of samples that can be analyzed per day.

A very large number of other ion exchangers for HPLC and conventional chromatography based on organic polymers are commercially available. For reviews see Boschetti (50) and Freiser and Gooding (silica-based HPLC ion exchangers) (51).

4.4.11 Nonporous and Continuous-Bed Ion Exchangers

Adsorption to porous chromatographic particles requires that solutes diffuse across a film of liquid at the outer surface of the adsorbent and then through a pool of liquid in the pores
of the matrix. Chromatographic media used for preparative applications, particularly for large scale industrial production, generally have highly porous particles exhibiting a high binding capacity. The mass transport into and out of the pores, however, is a main cause of zone spreading (wider peaks), especially with slowly diffusing proteins and other large molecules. The rate of solute transport determines both the flow at which a column can be operated and the zone spreading (see Section 3.2.1.2). There are various approaches to limiting this problem. One is to use adsorbent particles without pores, nonporous ion exchangers, where all binding occurs at the surface of the particles (52). Very small, well packed, particles will diminish the problem of mass transfer through the liquid pool, but will create high backpressures and a bed that can be easily fouled; however, they have limited use in preparative work due to low binding capacity. However, such media are most useful for analytical separations and micropreparative applications focused on resolution, not capacity. Recovery of low amounts of proteins (μg or less) is high. Although the particles are small (<3 μm), surface areas are still not great, and the maximum sample load is small, only 5–10% of that of porous ion exchangers.

Commercial suppliers of chromatography media often offer a compromise between the porous high capacity diffusion-limited properties and those characteristics that enhance mass transport. Depending upon the type of application the priority can be high capacity for an initial product capture step with high sample loads or high resolution (low band broadening) for final product polishing or analysis. Media are also manufactured with a range of pore sizes designed to allow convective flow through the particles as well as providing a large surface area for binding.

Another solution to the problem of diffusion across a film is the use of continuous bed ion exchangers in which the matrix is a continuous bed. Rather than a bed packed with particulate material, continuous beds have been prepared by copolymerization of \( N,N' \)-methylenebisacrylamide and acrylic acid (cation exchanger) or \( N \)-allyldimethylamine (anion exchanger) in high salt and in the presence of a catalyst. The hydrophobic gel material polymerizes to form narrow channels in a foam-like material. Stacked membranes exhibit similar properties.

During chromatography with continuous bed ion exchangers, peak width is constant, or can even decrease, when the flow rate is increased; that is, resolution increases with flow rate (50–52). The diffusion distances can be even smaller than in solid-particle ion exchangers. Maximum binding capacity with these media is still limited compared to porous particulate ion exchangers, although the mechanical and chemical stability is good. The advantages are most favorable for separation of very large, slowly diffusing molecules and small particles. For a review on the development of separation methods see Reference 53.

### 4.4.12 Expanded Bed Ion Exchangers

Adsorbents and columns specially designed for purification of proteins from unclarified feedstock in expanded beds were introduced to the market in the early 1990s. This technique is based on the formation of a stable turbulent free bed of adsorbent particles when applying an upward flow through the bed. The particles will migrate up the column to a height where there is a balance between particle sedimentation velocity and upward flow velocity. A defined size range and density of the adsorbent particles allow the formation of a stable bed with well defined and consistent hydrodynamic properties. The adsorption characteristics are similar to those of packed beds of standard chromatography media, but with a lower theoretical plate count. To achieve high operating flow velocities without overexpansion of the bed, particle density is high. Particles usually have a dense core material, like quartz or iron, which is coated with the matrix to which the ligand is bound. Typically, the bed expands ~2–3 times the settled bed height, so long columns, usually with adjustable adaptors, are necessary. Such a process is called expanded bed adsorption (EBA) chromatography.

Expansion of the adsorbent bed creates a distance between the adsorbent particles. This greatly increases the void volume of the bed and thus lowers the theoretical plate count, but also allows unhindered passage of cells, cell debris, and other particulates during application of crude feed to the column and can therefore eliminate the need for prechromatographic unit operations to clarify feedstock (e.g., centrifugation and filtration). EBA is often used as a capture step and has a better efficiency and capacity than a batch technique. Although this technique has been successfully implemented in industrial processes and is particularly appreciated when there are smaller batches of multiple entities being purified, there appears to be a general reluctance within the industry to acquire the technical expertise necessary to examine and develop very large scale processes with EBA (personal observation).

For a comprehensive overview of this technique the reader is referred to Reference 54.

### 4.4.13 Multimodal Ion Exchangers

Ion exchangers can be used both for separating and concentrating proteins. Proteins normally bind to an ion exchanger at a low ionic strength and as a result samples must often be diluted or the buffer changed via dialysis or diafiltration. With large sample volumes this may not be feasible for economic or logistical reasons. Therefore ligands with multimodal functionality suitable for ion exchange at high ionic strength have been developed and commercialized. Adsorption to these ligands is due to a combination of electrostatic, hydrophobic interactions and hydrogen bonding.
A large number of ligands for multimodal ion exchangers were tested at the R&D laboratories of GE Healthcare Life Sciences, Uppsala, Sweden, with respect to protein binding capacity and recovery. The best anion exchangers had, as positively charged groups, primary or secondary amines or a combination of both, with hydrogen donor groups in the vicinity. Carboxylic cation exchangers with aromatic groups were optimal for the capture of proteins at high salt concentrations. An amide group on the α-carbon and the position of the aromatic group relative to the carboxyl were important (55, 56). See Figure 4.6 for the ligand structure of multimodal ion exchangers.

Proteins could be adsorbed from solutions of high conductivity (28 mS/cm), which is the typical conductivity of fermentation broth and corresponds to the conductivity of ≈0.25 M NaCl. Desorption was achieved by changing the pH or salt concentration or both.

4.4.14 Dipolar Ion Exchangers

Porath and co-workers prepared ion exchangers with dipolar ligands, such as β-alanine, sulfanilic acid, and arginine (57, 58). The electric field potential decreases faster around dipoles than around unit charges, which should facilitate desorption. Scopes has suggested that dipolar ion exchangers should be useful for affinity elution (43). Both negative and positive groups of proteins interact with a dipolar ion exchanger and the contact regions would be different than on an ion exchanger with only negative or positive charges. Consequently, the selectivity should be different. Dipolar ion exchangers have not been extensively utilized, but they do offer a different selectivity than an ion exchanger with only one type of charged groups, and should be kept in mind when conventional ion exchangers are not sufficient.

4.4.15 Liposome Ion Exchangers

Liposomes immobilized in the pores of carrier gels containing anionic or cationic phospholipids have been used for IEC and offer a possibility for studying biological processes. For instance, separation of ribonuclease A, cytochrome c, and lysozyme on phosphatidylserine liposomes was better than on Mono S (59).

Dipolar and liposome ion exchangers are not commercially available.

4.5 NONIONIC INTERACTIONS

4.5.1 Hydrogen Bonding and Hydrophobic Interactions

Some snake venom toxins that differ only by one noncharged amino-acid mutation can be separated due to nonionic interactions. The nature of the matrix separation may depend on hydrogen bonding or hydrophobic interactions. Iso-toxins differing only by an Ile/Ser (60) or Pro/Ser (61) mutation were separated on Bio-Rex 70. In each case the Ser variant eluted later because the OH-group of the side chain of serine could form a hydrogen bond with the matrix.

Chromatography of fasciculins (inhibitors of acetylcholine esterase) shows the effect of hydrogen bonding or hydrophobic interactions depending on the matrix of the ion exchanger. The two fasciculins differ only by a single amino acid at position 47 (Tyr or Asn). Asn 47 in fasciculin 2 can form three hydrogen bonds with Bio-Rex 70, one via the carbonyl and two via the amide hydrogens, and fasciculin 1 can form only one via the OH-group of Tyr 47. Consequently, fasciculin 2 elutes after fasciculin 1 (Ref. 62, fig. 1B). Tyr 47 is more hydrophobic than Asn 47, and fasciculin 1 with Tyr 47 elutes after fasciculin 2 both from the cation exchanger BioGel TSK SP with a hydrophobic polyvinyl matrix and from the reversed-phase column C8 (unpublished observation).

The hydrophobic effects are seen in chromatography of derivatives of fasciculin 2 obtained by modification with nor-leucine methylester and a water-soluble carbodiimide (30, Fig. 4.7). A hydrophobic group, –NH(COCH3)–CH2CH2CH2CH3, was substituted on the carboxyls and on one of the tyrosines, probably tyrosine-47, which is exposed to the exterior of the molecule and thus more reactive. Fasciculin has five arginines, four lysines, and an N-terminal α-amino group with a charge of about +0.5 at neutral pH.
(pKₐ = 6.8–8.0, Table 4.1) and five carboxyls. The net charge is +4.5. Modification of a carboxyl removed one negative charge.

FAS2, fasciculin 2, charge, +4.5
A–E, derivatives of one carboxyl charge, +5.5
F, tyrosine derivative, probably of Tyr 47, charge +4.5
G–K, di-derivatives of one carboxyl and one tyrosine, more hydrophobic than A–E, charge +5.5

Derivatives G–K and F have increased hydrophobicity. G–K elutes after A–E, even if they have the same charge. F with a charge of +4.5 elutes from a cation exchanger after A–E with a charge of +5.5. The separation within the groups A–E and G–K depends on differences in charge distribution as discussed in Section 4.3.4.

4.5.2 Adsorption under Extreme Conditions

At low pH or even at very high ionic strength, proteins may adsorb very strongly to ion exchangers. At pH 3–3.5 a phospholipase A₂ from cobra venom adsorbed irreversibly to CM-cellulose (E. Karlsson, unpublished observation) and cellulase to CM Sephadex C-50 (G. Pettersson, personal communication). This is likely due to increased hydrogen bonding.

Heinitz et al. (63) studied retention as function of ionic strength on a weak anion exchanger (polyethyleneimine coated on silica). At low ionic strength the retention was high; increasing the ionic strength decreased and eventually eliminated the electrostatic attraction and the protein eluted at the breakthrough. Further increase of the ionic strength increased the retention due to increasing hydrophobic interaction. For α-chymotrypsinogen a similar hydrophobic effect was observed at 1.3 M Na₂SO₄ and with NaCl at 2.5 M (64).

Proteins can adsorb to DEAE cellulose from ammonium sulfate of 40–90% saturation (saturated 4 M, room temperature) and even to unsubstituted cellulose under high ammonium sulfate concentrations (65).

4.6 THE MOBILE PHASE: BUFFERS AND SALTS

4.6.1 Selection of Buffers

Normally, the concentration of buffer salts during protein adsorption is low, ~10–50 mM. Proteins adsorbed at a salt concentration too far below desorption concentration can be difficult to desorb and some denaturation can occur. A suitable adsorption concentration can be determined by simple testtube experiments (see Section 4.7.2.2). The residence time on the column can also affect elution. Proteins adsorbed to a column for prolonged periods may be more difficult to elute than proteins desorbed shortly after adsorption. After a prolonged stay, proteins can be more difficult to elute from the column (S. Hjertén, personal communication). A
protein alternates between being bound to the ion exchanger and free in the solution. The free proteins should predominantly be those with lowest Z-values, that is, the least strongly bound. The free proteins should rebind predominantly with the highest affinity, that is, with highest Z-values, and the bound proteins would be slowly more difficult to elute.

It is evident that a buffer for ion exchange should not irreversibly inactivate the proteins. However, sometimes a particular ion or substance is required to maintain protein activity or solubility that interferes with detection at 280 nm or with activity assays. In general one should strive to utilize buffers with the following characteristics:

- sufficient buffering capacity
- ions that facilitate good selectivity
- salts that are easily removed
- low costs for both the raw material and disposal of used solutions.

The first three points will be discussed in some detail below.

### 4.6.1.1 Buffer Capacity

In a short paragraph, Peterson and Sober (66) write that sufficient buffer capacity is needed to eliminate pH disturbances that might result from interaction with an incompletely titrated adsorbent (not equilibrated with the starting buffer?) or from the adsorption process itself. If not properly buffered, resolution may be impaired, especially at the early part of the chromatogram when the concentration of the buffer is low (43, 67). On this basis, buffers with high capacity and with buffering ions that do not bind to the ion exchanger are recommended (43, 68). Additionally, it is recommended that the pKₐ of the buffering species should not be more than 0.5 units, or preferably less, from the working pH (maximum buffer capacity at pH = pKₐ ± 0.5).

The recommendation that the buffering ion should not bind to the ion exchanger is less valid, considering the frequent use with good results of phosphate buffers on positively charged DEAE ion exchangers. Buffering ions that bind to an ion exchanger will only slowly change the equilibrium of the ion exchanger.

A positive buffering ion such as Tris (pKₐ = 8.2) with Cl⁻ as counterion is suggested with anion exchangers (see below). For a cation exchanger, negatively charged buffering ions such as phosphate, carbonate, acetate, or MES (morpholino-ethane sulfonate) can be used with the counterions Na⁺ or K⁺. If pH changes occur, they are likely to be small and not abrupt. An example of minor pH changes in the initial part of the chromatogram and the apparently small effect on resolution is shown Reference 50 (fig. 4).

Minor increases in pH can often be noted in anion exchange chromatograms when a salt gradient is used for elution with phosphate buffer. This is likely due to the release of HPO₄²⁻ ions bound to the exchanger. Their release will shift the buffer equilibrium to the left (see following equation):

\[
\text{H}_2\text{PO}_4^- + \text{H}_2\text{O} \leftrightarrow \text{HPO}_4^{2-} + \text{H}_3\text{O}^+
\]

thereby reducing the concentration of protons in solution and thus increasing the pH. A slight increase in pH can make proteins more anionic and increase binding affinity to anion exchangers.

pH gradients can be formed on ion exchangers, and pH changes may occur depending upon the equilibration conditions of the resin.

Bio-Rex 70 has a very high buffer capacity (see Section 4.4.5), and when ammonium acetate is used, the pH of the effluent is determined by the equilibrium conditions of the resin. The pH of the effluent varied from 6.8 to 5.9 in Figure 4.4a on Bio-Rex 70 equilibrated with 0.20 M AmOAc and eluted with a gradient of 0.05 to 1.40 M AmOAc (both at pH 6.5). When the concentration of the eluent was <0.20 M, the equilibrium concentration, the pH increased as the resin released ammonium ions and took up protons. When the eluent concentration was >0.20 M, the pH decreased as the ion exchange between the resin and the eluant was reversed.

Thus, IEC on Bio-Rex 70 in AmOAc close to neutral pH utilizes both pH changes and differences in charge. The pH changes are probably smaller with buffers with better buffering capacity than AmOAc at neutral pH and on an ion exchanger with lower buffering capacity than Bio-Rex 70. Use of two buffers of different pH values to form a pH gradient is a common practice.

To see if pH changes occur during adsorption to an ion exchanger with a lower buffer capacity than Bio-Rex 70, a sample of muscarinic toxins, freeze-dried from ammonium acetate, was dissolved in 10 mM AmOAc (pH 6.9). The pH had to be adjusted to 6.9 because of low amounts of acetic acid left after lyophilization (see Section 4.9.2). The sample was adsorbed to a narrow zone at the top of a column of SP-Sephadex C-25 equilibrated with 10 mM AmOAc pH 6.9. Counterions were released, such as CH₃COO⁻, from the positive groups of the proteins and NH₄⁺ from the negative groups of the ion exchanger, causing a small increase in the AmOAc concentration, but the zone of increased concentration migrated away from the adsorbed proteins when elution continued, and no pH change was detected in the effluent.

Buffers with very low buffer capacity have been used. For instance, ammonium bicarbonate (69, 70) (pKₐ values 9.25 NH₄⁺, 6.37 H₂CO₃, and 10.25 HCO₃⁻) and ammonium acetate (28–30, 42, 62, 63, 71–73) (pKₐ values 9.25 and 4.76 acetate) have been used in the isolation of a very large number of neurotoxins and neurotoxin derivatives.

Use of the two buffers contradicts two of the emphasized guidelines for selecting buffers; they have very low buffer capacity (working pH far from pKₐ) and the buffering ions
bind to the ion exchanger, but were nevertheless effective and the above “rules” should therefore be considered as suggestions only. A large variety of buffers can be used and there are no hard and fast rules as to what buffer to use. This is a good illustration of one of the great advantages of IEC—versatility.

4.6.1.3 Buffers

4.6.1.3.1 Volatile/Nonvolatile None of the recommended buffers is volatile, and the isolated substances cannot be recovered directly by freeze-drying and must therefore first be desalted. This can be a serious drawback, especially when working with very dilute and low volume solutions. It can be difficult to remove nonvolatile salts completely by desalting. From glycine- and histidine-containing buffers, nanomolar amounts interfere with amino-acid analysis, and low amounts of Tris can esterify aspartic and glutamic acids during acid hydrolysis for amino-acid analysis (D. Eaker, personal communication).

One of us (E.K.) has extensive experience of isolation of neurotoxins from snakes and sea anemones using ammonium acetate buffers close to normal pH. Toxins can serve as specific markers for subtypes of receptors (74) or potassium ion channels and as lead substance for the development of drugs to combat severe pain (74) and autoimmune diseases such as multiple sclerosis, type 1 diabetes, and rheumatoid arthritis (65). In one study on the fractionation of Dendroaspis angusticeps (green mamba) venom, 15 peaks were obtained, of which at least 12 contained toxins with unique pharmacological properties (62). If the elution buffer were nonvolatile, it would hardly be possible to recover all of them for further analyses. As with any purification process the elimination of additional operations will decrease the work load and increase overall yield. It is therefore best to create a method that allows one to freeze-dry directly without a detour to desalting. With low amounts of material, 10–20 μg, much of the sample is likely to be lost in the desalting. Much to the author’s delight a serendipitous discovery was made when changing from nonvolatile sodium phosphate to volatile ammonium acetate. Two toxins co-eluting in sodium phosphate were well separated in ammonium acetate.

4.6.1.3.2 Temperature and Salt Effects on Buffers During freeze-drying or even when moving from a room-temperature environment to a cold room, the ability of buffers to perform as desired can be greatly affected. This is in large part due to the temperature dependence of pK_a (66). Tris buffer pH adjusted at room temperature is ~0.05 units of pH lower in a cold room at +4°C (dpK_a for Tris is ~0.028/deg). For a phosphate buffer dpK_a/dT = −0.0028 and Na_3HPO_4 has a solubility of only 15 g/L or 0.09 M at 0°C.

Sodium phosphate buffer (0.20 M, pH 7) is a mixture of 0.12 M Na_3HPO_4 and 0.08 M NaH_2PO_4. In a cold room some Na_3HPO_4 is likely to precipitate out of solution. This will not only hinder flow through a column, but because the buffer now has an excess of NaH_2PO_4 it will also reduce the pH (see Section 4.6.1.1). Phosphate buffers to be used in a cold room should be prepared as potassium salts to avoid precipitation. It may also be wise to adjust the pH with KOH instead of NaOH. If the buffer for gradient elution is prepared by addition of a neutral salt, such as NaCl, often to a concentration of 1 M, the pH will change because of the large increase in ionic strength.

4.6.1.3.3 Other Impacts of Buffer Selection In large scale applications the cost of the buffer salts is important when selecting a buffer, and phosphate buffers are often used. Economics are rarely considered in laboratory-scale experiments and only need to be considered if the ultimate goal is a large scale process. A further consideration when choosing buffers is the possibility of exploiting the special effects of some buffer salts (see Section 4.6.2).

The possibility that a particular buffer species will interfere with subsequent studies should also be taken into account. For example, buffers containing histidine, glycine, or Tris may interfere with amino-acid analysis, even after dialysis leaving only nanomolar concentrations. Tris will esterify aspartic acid and glutamic acid during acid hydrolysis for amino-acid analysis (D. Eaker, personal communication).

4.6.2 Effect of Ions on Resolution and Selectivity

Lewith (67) and Hofmeister (75) (1888) ordered anions and cations in a series after measuring the concentrations needed
to precipitate proteins. Lewith was a student of Hofmeister, so the series is therefore called after Hofmeister. Ions have different effects on the water layer that surrounds proteins and hence on the secondary and tertiary structure of proteins. Kosmotropic ions bind water strongly, minimize the surface area, and stabilize the protein structure. Chaotropic ions bind water weakly, maximize the surface area, and destabilize the structure. The effect of anions is greater than that of cations. The chaotropic effect increases according to the Hofmeister series:

\[
\text{Anions: } \text{SO}_{4}^{2-} > \text{PO}_{4}^{3-} > \text{HPO}_{4}^{2-} > \text{CH}_{3}\text{COO}^{-} > \text{HCO}_{3}^{-} > \text{Cl}^{-} > \text{NO}_{3}^{-} > \text{ClO}_{4}^{-} > I^{-} > \text{SCN}^{-}
\]

Ions before chloride are called kosmotropes, those after chloride chaotropes. Chloride in the range 0.1–0.7 M has little effect on water structure and protein stability (79–79).

\[
\text{Cations: } \text{NH}_4^+ > \text{K}^+ > \text{Na}^+ > \text{Li}^+ > \text{Ba}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+} > \text{guanidium}
\]

The hydrophobic interaction follows the Hofmeister series, strongest for fluoride, sulfate, and phosphate, and then in decreasing order.

The retention of proteins depends on the ionic strength, pH, nature of the buffer ions, and the protein structure. The retention of small molecules from a cation exchanger increases in the order (80)

\[
\text{Ba}^{2+} < \text{Ca}^{2+} < \text{Mg}^{2+} < \text{NH}_4^+ < \text{K}^+ < \text{Na}^+ < \text{Li}^+
\]

and from an anion exchanger,

\[
\text{MOPS} < \text{sulphate} < \text{oxalate} < \text{bromide} < \text{chloride} < \text{formate} < \text{fluoride} < \text{acetate} (80)
\]

\(\text{MOPS} = 3-(\text{morpholino}) \text{propanesulfonic acid}.)

Gooding and Schmuck (81) studied the displacement by chlorides of lysozyme, chymotrypsinogen, \(\alpha\)-trypsinogen, ribonuclease A, and cytochrome \(c\) from the cation exchanger SynChropak CM 300 equilibrated with 0.02 M Tris-HCl (pH 7). The order of elution agreed with the displacement power of cations for small molecules with one exception: \(\text{NH}_4^+\) was a better displacer of chymotrypsinogen than \(\text{K}^+\).

The five proteins were also eluted by salt gradients from the same cation exchanger: five peaks were obtained with \(\text{BaCl}_2\) and Na-acetate, four with \(\text{Na}_2\text{SO}_4\), three with KCl, and four with NaCl. The resolution was better with Na-acetate than with BaCl\(_2\), which compressed the peaks.

Gooding and Schmuck (82), in one experiment, eluted cytochrome \(c\) and lysozyme, and in a second trypsin and chymotrypsin, from the cation exchanger SynChropak CM 300. The resolution was better with Na-acetate and ammonium acetate than with KCl, NaCl, or NH\(_4\)Cl (Table 4.1). Neither chloride nor acetate binds to the ion exchanger, but the resolution was better with NH\(_4\)-acetate than with NH\(_4\)Cl. This indicates that nondisplacing ions that do not compete with proteins for the charged groups of the ion exchanger can also influence separation.

Yao and Hjerten (83) chromatographed several proteins on the anion exchanger QAE-agarose in Tris buffer at pH 8 with carboxylates or chloride as displacers. Carboxylates, particularly acetate, gave a better resolution than chloride.

Kopaciewicz and Regnier (84) showed that citrate was a better displacer than chloride of ovalbumin, but not of soybean trypsin inhibitor. Carbonic anhydrase, ovalbumin, and soybean trypsin inhibitor were separated on anion exchangers, but the peaks were compressed when displaced by NH\(_4\)Cl and MgCl\(_2\), and the two salts were classified as strong displacers, intermediate displacers sodium acetate and sodium citrate spread out the peaks and improved the separation. Using a strong displacer can be advantageous with strongly bound proteins. Elution with MgCl\(_2\) instead of NaCl improved the yield of ferritin considerably.

Six proteins (85) were eluted from the anion exchanger Mono Q at pH 7 with gradients of NaBr, NaI, NaCl, Na-acetate, and NaClO\(_4\). Alpha-lactalbumin eluted ahead of ovalbumin in NaCl and NaBr gradients, but they eluted together in NaI, and in reverse order in Na-acetate. Elution with the chaotropic ion ClO\(_4\) gave a poor resolution compared to elution with the other salts.

The above examples show that both displacing and non-displacing ions can change both resolution and selectivity.

An ion that binds to a specific site is likely to change the chromatography; for instance, phosphate binds to a histidine in the active site of ribonuclease A, which then elutes earlier from a cation exchanger than with chloride (D. Eaker, personal communication). In immobilized metal affinity chromatography (IMAC), metals are bound to an adsorbent, and proteins with exposed amino acids with affinity for the immobilized metal are bound (86, 87). For instance, recombinant proteins are often synthesized with a tag of a histidine peptide and the protein is adsorbed to an IMAC column with Cu\(^{2+}\) or Ni\(^{2+}\). Whether the addition of any of these ions to a buffer will change the chromatography of histidine-containing proteins has not been investigated.

The effect of different ions on the resolution and selectivity in IEC is rarely investigated. One reason for this is that the result is difficult to predict and would require time-consuming several experiments. Acetate buffers can be a good first choice, and ammonium acetate is volatile, which facilitates recovery. However, as the outcome depends on the protein structure, there is certainly no buffer, which is always the best choice. One selects a buffer and if the result is not satisfactory, chromatography at another pH is tried or the purification continues by using another separation method.
4.6.3 Membrane Proteins, Detergents, Enzyme Inhibitors, and other Additives

Membrane proteins are, as the name implies, bound to membranes. Intrinsic membrane proteins such as receptors for neurotransmitters and hormones are tightly bound by hydrophobic forces. They can be separated from membranes by agents that disrupt the membrane structure such as detergents, organic solvents, or chaotropic ions. Peripheral membrane proteins, such as acetylcholinesterase from erythrocytes, are loosely bound. They can be dissociated from membranes by relatively mild methods such as salt solutions, metal chelates, and pH changes; this leaves the membrane intact.

4.6.3.1 Detergents Nonionic detergents (e.g., Triton) and ionic detergents (e.g., SDS) are used for solubilization of membrane proteins. SDS dissociates noncovalent protein complexes and is commonly used for the determination of molecular mass by electrophoresis (e.g., PAGE). After reduction with, for instance β-mercaptoethanol, the molecular mass of subunits can also be determined. After solubilization with nonionic detergents, many membrane proteins are able to be purified by chromatographic methods.

4.6.3.2 Enzyme Inhibitors After adsorption of proteins to a column, lipids can be removed by elution with a nonionic detergent. As large amounts of proteolytic enzymes are released when membranes are solubilized, one should add enzyme inhibitors before solubilization. An awareness of the proteases present will minimize the expense and possible hazards associated with inhibitor addition. A chelator, such as EDTA, will inhibit metalloproteases, p-methylsulfonylfluoride (PMSF) serine proteases, and iodoacetate or iodoacetamide with SH-groups in the active site such as papain. One may also be able to find prepackaged enzyme inhibitor cocktails. If the scale of work is small they are likely to be very convenient, effective, and affordable. Inhibitors that interfere with the function of the enzyme to be purified or produce an undesirable side reaction with one of the buffer components should not be used. EDTA would make necessary cofactors like divalent cations unavailable.

4.6.3.3 Additives Additives are often used to promote chromatographic separations. Organic solvents such as methanol and acetonitrile are added to decrease the dielectric constant of the solution and increase the solubility of the hydrophobic proteins. The adsorption of proteins to polymeric resins might be partly due to a decreased dielectric constant in the hydrophobic microenvironment of the charged groups on the matrix. In some applications with HPLC ion exchangers, 10–20% acetonitrile is added to the buffer to decrease the hydrophobic interaction with the matrix.

Zwitterions such as betaine and taurine can improve resolution by decreasing aggregate formation and strong binding to ion exchangers. Urea and chaotropic ions are used to increase solubility. A neutral polymer, such as PEG, competes with protein for water molecules. The retention of different proteins increases to a different degree. Milby and colleagues (88) showed that myoglobin and hemoglobin were much better separated in PEG than in the same buffer without PEG.

4.7 EXPERIMENTAL PLANNING AND PREPARATION

4.7.1 General Aspects

IEC may be carried out in a variety of ways according to the properties of the sample and the objective of the separation. Its high capacity and ability to adsorb and concentrate proteins from dilute solution make it particularly useful in early stages of a separation scheme when both the sample volume and the protein mass are large. Batch adsorption IEC can serve as a simple scavenging step that removes charged impurities, such as DNA, and facilitates further chromatographic separations.

4.7.1.1 The Three Phases of Purification When designing a procedure for purification from a crude sample it is useful to think of chromatographic separations as comprising three distinct phases: capture, intermediate purification, and polishing. Each may contain one or more operations, and chromatography is often one.

In the capture step the goal is a rapid isolation from the gross impurities, such as proteases, and a concentration to reduce the working volume. Intermediate purification should further remove protein impurities and increase overall product purity. The focus of the polishing phase is resolution. Trace impurities, often product-related impurities with characteristics similar to the target molecule, must be removed to achieve a predefined level of purity. A combination of purification steps that achieves the defined purity with the fewest steps should be the goal.

IEC, in combination with other techniques, is well suited for any step in the three phases of a process. As mentioned in Section 4.1 it is an excellent technique because of its versatility, selectivity, and high capacity. As a capture step one can use an ion exchanger coupled to a large bead size that allows for high flow velocities and is unlikely to clog. Often, it is also desirable to be able to clean the column and bed with NaOH at fairly high concentrations as it is excellent for the removal of most of the soils commonly remaining after a capture step.

During intermediate purification, where resolution is more important, a smaller bead can be used and a greater emphasis needs to be placed on optimization of the binding and elution conditions.
In an IEC polishing step, selectivity in combination with a very small bead size (~5–50 μm), which gives a high number of theoretical plates, will result in excellent resolution. Polishing steps, analytical separations, and other small scale applications where speed or high resolution are the main goals can be achieved on HPLC columns with small particles and a narrow size distribution. Alternatively, continuous-bed-type ion exchangers can be explored.

4.7.2.2 Platform Processes A clear biochemical understanding of the target molecule or class of molecules and the contaminants greatly enhances the development of robust purification processes. When repeatedly purifying a class of biomolecule (e.g., antibodies), a platform process based upon the capture—intermediate purification—polishing paradigm for a downstream purification process is often used (also known as a generic or template process). Such an approach is extremely useful, especially for industrial applications, where one common expression system may be used for recombinant production of many protein variants. In such a case the impurity profile should already be well understood. This, in combination with a ready downstream purification template, will significantly speed up the development of the purification process as well as facilitate implementation of the manufacturing process.

4.7.2 Choice of Ion Exchanger and Buffer

4.7.2.1 Binding of the Target The net charge of a protein, positive or negative, depends on the pH, so the selection of buffer type and cation or anion exchanger will also be governed by pH. Normally it is best to choose conditions where the protein of interest is adsorbed to the ion exchanger. The electrophoretic titration curves of Figure 4.2 can provide a guide for choosing both the ion exchanger and pH, although they are usually not investigated because they are complicated to make and require a reference and a means of visualizing the target protein that may be unavailable at early stages of development.

4.7.2.2 Choice of pH and Ion Exchanger The choice can be made by simple test tube experiments that screen several ion exchangers and the protein of interest across the pH range. In a test tube the ion exchanger (~1–1.5 mL) is equilibrated with the starting buffer and mixed for 1–2 min with the sample equilibrated in the same buffer. After centrifugation, the supernatant is assayed for the protein to determine the level of binding. At first one should try at slightly acid and alkaline conditions (e.g., pH 5 and 8). If the protein is only partially adsorbed the conditions can be used for isocratic elution, and the retention can be calculated from the distribution coefficient (see Section 4.3.5).

Desorption conditions making use of increases in ionic strength or changes in pH can be examined in the same manner. For high throughput screening, microtiter plates, and hopefully robotics, can be used instead of test tubes. From this first screening one can then move to a packed column for optimization and capacity studies.

An alternative method is to apply a small sample at low ionic strength to an anion exchanger at pH 8 or a cation exchanger at pH 5. If the compounds of interest adsorb to the ion exchanger they are displaced by a salt gradient.

GE Healthcare has developed a high throughput process to speed up development of a new separation process (HTPD, high throughput process development, see Chapter 20). Conditions are evaluated in parallel in PreDictor™ 96-well filter plates pre-filled with a chromatographic medium. Many different parameters can be explored, and optimal conditions may be obtained rapidly. The experiments are also supported by the Assist software to simplify the design of experiments and analysis of data. Correlation of results in the plates with results in columns is reported to be excellent, and scale-up of optimal conditions is simplified.

With sensitive proteins the ionic strength to be used should be close to the ionic strength needed for desorption. Proteins too strongly adsorbed can be difficult to desorb or become inactivated. Several peaks, arising from denaturation, can be obtained. The recovery should be checked accordingly. A prolonged residence time on the column may also make proteins more difficult to elute.

There is often little to be gained from choosing a weak as opposed to a strong ion exchanger, especially if the weak ion exchanger is fully charged. Kopaciewcz and Regnier (84) chromatographed carbonic anhydrase, ovalbumin, and soybean trypsin inhibitor on the strong anion exchanger Mono Q and on the weak anion exchanger Synchropak WAX, and the resolution was about the same at pH 8 and 7 and better on the strong ion exchanger at pH 6. Phosphocellulose shows specific interactions with enzymes binding phosphate-containing substrates (88–90).

4.7.2.3 Negative or Flow-Through Chromatography
An alternative strategy in which the protein of interest passes unretarded through the column, while many other proteins and contaminants are adsorbed, is often called negative or flow-through chromatography (see Section 4.8.2.3). A high degree of purification can be obtained with this method, but no volume reduction. The unretarded fraction, strictly speaking, is not submitted to chromatography (Section 4.2.1) and must often be further purified or at least concentrated.

4.7.2.4 Anion and Cation Exchangers Mixed or in Series The use of anion and cation exchangers in series or a mixture of the two is an established technique for deionizing water. This technique has found limited application in protein purification (91–94). Stringham and Regnier (93) isolated IgG1 from bovine serum on a mixed-bed ion
exchanger at low ionic strength near the isoelectric point of IgG to preclude its binding to both anion and cation exchanger while most of the other proteins were bound. Horváth (92) considered that mixed-bed ion exchangers should have great use in large scale chromatography A dipolar ion exchanger (Section 4.4.14) can be regarded as a mixture of an anion and a cation exchanger. Cytosolic proteins have been isolated by chromatography on anion and cation exchangers in series (94). Recently, mixed-bed ion exchangers were recommended for isolation of peptides and phosphopeptides in proteomics (95).

If extreme pH values are to be used, strong ion exchangers are essential. At pH values below 5, cation exchangers to be fully ionized should be sulfonated or phosphorylated, and at pH values above 9 anion exchangers containing quaternary amines should be used.

4.7.2.5 Mixed Binding Modes In some cases, mechanisms other than ion exchange will influence separation. It has already been mentioned (see Section 4.5.1) that hydrogen bonding and other nonionic forces can enhance resolution. Proteins adsorb at high ionic strength to multimodal ion exchangers by hydrophobic forces (Section 4.4.13).

4.7.2.6 Porosity The porosity of the matrix can influence the choice of ion exchanger. Larger protein molecules, in general, require media with larger pore sizes (for an exception see surface extended media, which have a different transport mechanism; Section 4.4.9). The manufacturer normally provides exclusion limit data. Ion exchangers with smaller pore sizes are usually better suited for smaller proteins. If the porosity is large enough, a further increase will not add value.

4.7.2.7 Practical and Economic Considerations Buffers are discussed in Section 4.6 and some practical aspects are added here. In HPLC a blank gradient should be run before applying a sample to check for UV absorbing material in the buffers. Low grade phosphate can contain material that can interfere with monitoring in the lower UV range. The contaminants can be removed as described by Karkas and colleagues (96). Halides can corrode stainless steel, especially in combination with low pH values, and systems with stainless steel components should be washed thoroughly with water after use with salts that contain halides and or designed out when designing equipment utilized in production.

If the sample size is large and fouling of the chromatographic column is expected during the capture stage, a simple and inexpensive ion exchange material that can be discarded after use should be used; in batch mode a possible alternative is the use of EBA or precolumn chromatographic steps for clarification. The final choice between adsorbents with similar porosity, capacity, and hydrophilicity is probably best made according to economy and convenience. To facilitate operations on a larger scale, one must also consider the base matrix effects on process logistics. The ease of packing/unpacking an acceptable column, usable flow rates, as well as the chemical resistance of the matrix to buffers, sample, and cleaning/sanitizing agents can greatly affect overall process economy.

4.7.3 Column Dimensions

The amount of ion exchanger needed for an industrial separation can be calculated from the dynamic binding capacity (see Section 4.4.3) and an understanding of the variation in output between bioreactor/fermentation batches. At the laboratory scale, calculations are rarely made, as the amount of target and contaminating proteins, which influence the protein binding capacity, are often not well characterized and the columns have excess capacity. This excess capacity in small scale applications is hardly a serious disadvantage, except perhaps to economy.

The column length in gradient elution has a relatively small effect on resolution. Columns 5 cm in length have ~75% of the resolving power of columns five times longer (97, 98). For adsorptive techniques like IEC, maximal resolution comes primarily through the selectivity of the ligand and the proper choice of buffers and gradients. Efficiency factors such as increased theoretical plate counts, through the use of smaller bead sizes and longer columns, plays a significant, but smaller role in increasing resolution and is often used in final purification steps (polishing).

Columns to be used for isocratic elution must be substantially longer to gain resolving power. In this type of elution the buffer remains unchanged and the proteins migrate on the column simultaneously. Their resolution will increase as the square root of the column length just as in gel filtration chromatography.

4.7.4 Equilibration of the Ion Exchanger

An ion exchanger will always have counterions at pH values where it is charged. The purpose of the equilibration procedure is to ensure that the ion exchanger is in equilibrium with the counterions.

Regeneration is often needed to remove fouling material adsorbed to the ion exchanger following extended use or a “dirty” sample. It is recommended to use the regeneration procedures recommended by the manufacturer (see also Section 4.7.8). Note that DEAE ion exchangers are unstable in the free base form and should be converted to the salt form if the adsorbent has been washed with NaOH, for instance by washing with 1 M sodium acetate (pH3, adjusted with HCl) (see Section 4.7.8).

When equilibration is performed on a Büchner-type or glass filter, one should wash with the starting buffer until the pH and conductivity of the effluent are the same as that
of the starting buffer. Equilibration may also include a change in pH. To facilitate a more rapid equilibration, washing should start with the final buffer if it has a higher buffering capacity than the starting buffer. In such a case a more rapid pH change (titration) is facilitated by washing with a stronger base or acid. Once the desired pH is measured the ion exchanger is washed with the first buffer until it is equilibrated.

When equilibration is performed in a beaker, add the starting buffer in 10–20× volume excess and adjust the pH of the slurry with acid or base (be careful not to create fines by grinding the media, as often happens with the use of magnetic stirrer bars). After settling and decantation, a new portion of the buffer is added and the pH is adjusted if necessary. With Bio-Rex 70, which is a 3.5 M buffer, the procedure has to be repeated until the pH is less than 0.1 units from the desired pH, and after pH adjustment the resin is equilibrated.

For cleaning, if ammonium acetate buffers are to be used, ammonium hydroxide is added until the slurry is strongly alkaline. After settling and decantation the resin is neutralized with acetic acid. After a new decantation, ammonium acetate of the desired concentration is added and the pH adjusted with acetic acid or ammonium hydroxide. The procedure is repeated, usually twice or three times, until the resin is equilibrated, that is, until the pH does not change on addition of a new portion of ammonium acetate. After elution with the final buffer, ion exchangers can require several bed volumes of the starting buffer before they are re-equilibrated (50).

Even if the final and starting buffers have the same pH, at the breakthrough of the starting buffer pH is different. For instance, SP-Sephadex C-25 or BioGel SP were eluted with 1.0 M ammonium acetate (pH 6.8) and then re-equilibrated with 10 mM ammonium acetate (pH 6.8). At the breakthrough of the 10 mM buffer, the pH of the effluent was 7.5. This is probably due to release of NH₄⁺ and uptake of H⁺ from the solution, leading to an increase in pH.

pH changes also occur during gradient elution. For isolation of neurotoxins on Bio-Rex 70 equilibrated with 0.20 M ammonium acetate, for example at pH 7.3, and during elution with a gradient from 0.03 to 1.0 M AmOAc, the pH of the effluent changed from 7.8 to 6.5.

pH changes also occur when re-equilibrating anion exchangers. However, in this case the ion exchanger will release anions and take up hydroxyl ions, resulting in a pH decrease.

### 4.7.5 Preparation of Sample

Ideally, the ionic conditions of the sample should match those of the starting buffer. This is often achieved by dissolving it in the starting buffer and checking the pH. However, proteins are ions, and the ionic strength is therefore often higher than that of the starting buffer. If the ionic strength of the sample is too great it may not allow proper binding to the column, and the target protein may pass through unretracted by the column. It is therefore critical to always retain and test such fractions for target proteins.

To enable binding the sample can be diluted. However, the low ionic strength normally required for adsorption (<50 mM) might lead to inactivation or solubility problems. For instance, immunoglobulins are not soluble at an ionic strength of 10 mM. Such solubility problems can be addressed through the inclusion of additives such as glycerol, which increase stability but do not significantly interfere with binding. Another option to be considered is the selection of salt-tolerant multimodal ion exchangers.

To avoid clogging of the column and reduce fouling of the media it is important that the sample, if necessary, is clarified by centrifugation or filtration or some other means before application. However, one may be able to avoid such additional operations with the use of EBA (see Section 4.4.12), as it was developed to handle large volume samples containing particles and thereby avoid time-consuming clarification procedures. Batch methods in which the media is discarded after use may also be considered.

### 4.7.6 Large Scale Ion Exchange

IEC has a high loading capacity and an ability to concentrate proteins from dilute solutions, and is therefore well suited to the purification of large quantities of proteins. Although process-scale IEC is routinely carried out with columns in the cubic meter range, even a standard laboratory column with a volume of 500 mL can be sufficient to concentrate and purify gram quantities of protein from tens of liters of a dilute sample. In practical terms, large-scale IEC differs mainly due to economic and throughput constraints, and the increased use of step, as opposed to continuous-gradient, elution procedures. Otherwise the same general procedures and principles are used as on a smaller scale.

#### 4.7.6.1 Scaling Up

It is generally desirable to be able to plan a large scale procedure at a smaller scale as it is cheaper, faster, and requires less starting material. Once sufficient data are generated from trial runs, and guided by previous experience and general principles, controlled and systematically varied conditions of sample loading and elution can be applied to find the conditions that give the desired result in terms of resolution, yield, and time. The use of experimental design guided by DoE (design of experiment) software can greatly reduce the number of experiments needed, rather than varying one parameter at a time, to define operating conditions producing acceptable yields and purity.

Once this is done on a small scale, the conditions can be scaled up by application of a few simple scaling factors. As a rule of thumb it is recommended to scale up production by orders of magnitude (1 → 10 → 100 → 1000), and an ideal column diameter to bed height ratio of 5:1. However,
these guides may not be practical within a given working environment, and engineering work-arounds must be developed (i.e., modified column distribution systems for ratios skewed from the ideal, such as 1.8-m diameter, 20-cm bed height).

Levison and colleagues (99) and Cooper and colleagues (100) discuss practical aspects of large scale protein purification, including a comparison of different column formats.

4.7.6.2 Constant Bed Height Column volume is increased proportionally to the sample volume (assuming equal concentrations of solutes). It is advisable to determine this increase in volume by choosing a column with a larger diameter to avoid excessively the long separation times and high backpressures associated with great bed heights. It is therefore generally desirable to use short, wide columns for ion exchange, and very large columns should have end pieces that are specially designed to spread the incoming liquid flow evenly over a relatively large bed area. The multiple inlet design (100) is suitable.

Additionally, elution volumes and residence time (and thus dynamic binding capacity) will change from the small scale conditions if bed height is increased under these conditions. Such changes can produce significant problems with an industrial process if anything more than a simple on/off capture step is desired.

4.7.6.3 Constant Contact Time Alternatively, if the flow properties of the media allow, one can scale up by increasing both bed height and column diameter and simply maintain a constant contact time. Such a practice is common in large scale HPLC applications, but challenging with low pressure applications where relatively small increases in backpressure, due to increased bed height, can destroy bed integrity.

4.7.6.4 Effects on Elution Volume When the linear flow rate is kept constant, the volumetric flow rate will increase by the same factor as the increase in the cross-sectional area of the column. If the large column is longer or shorter than the small scale version, elution times will be increased or decreased accordingly. Eluent volume will increase with the column volume. This applies equally to stepwise elution schemes and to continuous gradient elution, and to all steps in the elution sequence.

Supporting functions such as buffer mixing, packing, and cleaning often take longer to perform at large scales; otherwise the time to run the chromatographic unit operation should be the same as on a small scale.

4.7.6.5 Conversion Formulas: Flow and Contact Time To convert between linear and volumetric flow velocities, the following formulas should be applied:

Flow velocities to flow volumes:
\[(\text{cm/h}) \times \pi r^2 = \text{cm}^3/\text{h} \text{ or mL/h}\]

Flow volumes to flow velocities:
\[\text{mL/h} = \text{cm}^3/\text{h} = (\text{cm}^3/\text{h}) \times 1/\pi r^2\]

To convert mL/h into mL/min simply divide by 60. \(\pi \approx 3.14\), and \(r\) is the radius of the column in cm.

Contact time = flow velocity (cm/h)/column length (cm)

4.7.6.6 Effects of Changes in Scale It is important to note that these scaling factors only apply if the sample loading, elution conditions, and the ion exchanger are the same in the two scales. If the sample loading, mg protein/mL ion exchanger, is changed in the scale up then the results will be more difficult to predict, particularly at high loading capacities. Changes in the elution scheme will have the same effects on the separation as they would have had without the change of scale. The need to use the same ion exchange media means that it is inadvisable to optimize the separation using high performance or surface extended ion exchangers if subsequent large scale separations will use standard ion exchangers.

The choice of ion exchanger for large scale work follows the same principles as for small scale separations, but with two additional factors. Care should be taken to choose a gel matrix that will withstand high linear flow velocities in large volume columns and that is chemically stable to cleaning (usually with 0.5–1.0 M NaOH) and can thus be reused. However, when batch adsorption is used to concentrate and simplify the sample (see below) there is no need for high physical stability, and less sophisticated, less expensive media such as DEAE-cellulose may suffice.

4.7.6.7 Sample Conditioning Removal of particulate matter is especially important when large sample volumes are to be applied. Normal flow filtration depth filters with various porosities are available for this purpose. Larger sample volumes might be better suited for clarified through centrifugation or tangential-flow membrane filtration. In the case of large sample volumes, it may be difficult or impossible to ensure that the sample is exactly equilibrated to the starting conditions for elution. In this case the sample ionic strength can be adjusted by dilution if necessary, and the pH adjusted to a value that will give acceptable binding (often in-line for sophisticated large scale processing).

The choice of buffer systems for large scale work will naturally be influenced by cost considerations, but the need for adequate buffering capacity remain and should not be sacrificed for small cost savings. Chemical compatibility of buffering systems with pumping systems, seals, tubing, and columns should also be carefully considered when designing a process.
4.7.7 Batchwise Procedures

Although batchwise procedures are not, strictly speaking, chromatographic, they are nonetheless well worth considering as an alternative to column operations, in particular in connection with large scale applications. Batchwise adsorption and desorption are also very useful for crude or viscous samples independent of scale.

In batch procedures the sample is gently mixed with the ion exchanger in conditions that promote binding of the components of interest. Avoid any stirring conditions that can grind the media, like magnetic stirrers, generating fines that might later prevent a high flow rate through the column.

The time taken for binding depends on the sample and the ratio of volumes between the ion exchanger and the sample. As a guideline, an hour is sufficient for protein solutions. After binding, unbound components are washed away on a filter funnel under the same buffer conditions; the bound proteins are then desorbed by a stepwise increase in salt concentration or by a change in pH. Elution can be performed either on the funnel or after the ion exchanger has been packed in a column. If the volume of eluting buffer is kept small, a considerable increase in concentration of the protein can be achieved, particularly if a column is used. Large scale operations may require more complex handling due to the scale of work; however, the overall procedure is the same.

After recovering the protein, the ion exchanger may be regenerated for further use providing it is not heavily contaminated with particulate, lipid, or some other material. If this is the case it is often best to simply discard the media. However, if one wants to recover the ion exchanger, procedures for cleaning and regeneration as provided by manufacturers should be followed (see Section 4.7.8). Again, try to avoid the generation of fines.

The choice of sophisticated matrices to achieve the desired separation may be excessive if not actually needed. Inexpensive bulk ion exchangers that can be added dry, such as those based on cellulose or dextran, are often an excellent choice if the flow properties and chemical resistance are acceptable.

4.7.8 Regeneration, Cleaning, and Storage of Adsorbents

After each use, ion exchangers usually have some material bound, often denatured or precipitated proteins. Depending on the source of the sample cell culture additives, host cell proteins, lipids, endotoxin, or nucleic acids can be present, and the contaminating material should be identified either chemically or through expert knowledge of the source material applied to the column.

It is important to remove this material after the ion exchanger has been used to avoid contamination of future separations. Remaining material can also limit capacity by blocking adsorption and impairing column performance in terms of resolution and flow properties. Blank runs, in which only process buffers, but no sample, are applied to the column are useful in determining the presence/absence of contaminants after a cleaning protocol. Contaminants often manifest as peaks in blank runs or anomalous peaks in routine separations. During development, media samples taken from the column at the point of sample application, where contaminants tend to build up, can be analyzed to identify soils that may remain after various cleaning and regeneration protocols are applied.

Regeneration procedures vary according to the stability of the matrix and the functional group. Manufacturers generally describe how their products should be handled in their product literature. Only a few comments are presented here.

Agarose ion exchangers are normally regenerated by exposure to high salt, followed by high molar concentrations (0.5–1.0) of NaOH. Cellulose ion exchangers are washed with high salt and lower molar concentrations (~0.1 M) of NaOH. Severely fouled exchangers are often regenerated under the same conditions, but with longer exposure times, or at higher temperatures or a combination thereof.

Exposure of dextran and agarose to pH below 3 should be avoided. They can be washed with 1 M sodium acetate (pH 3, adjusted with HCl or other strong acid), 0.5 M NaOH, and 1 M Na acetate (pH 3). Trisacryl-based adsorbents are stable in pH 1–13.

Nonionic detergents or ethanol can remove lipoproteins and lipids. Polystyrene divinylbenzene-based matrices can withstand high concentrations of alkali (2 M), as well as cleaning with strong acids. Silica-based adsorbents are unstable to alkali, and pH above 8 should be avoided. Recommendations for the care of HPLC media have been published elsewhere (51–53). Manufacturers also give instructions on how to clean and store chromatographic media.

Adsorbents are best transferred to 15–20% ethanol or 10 mM NaOH for storage. To avoid stagnation and minimize the chance for microbial growth during long term storage, routine recirculation or agitation regimes should be implemented. Volumes of 20% ethanol above a certain amount (as defined by local regulatory authorities) may be considered explosion hazards and therefore must be stored in explosion containment areas.

4.8 CHROMATOGRAPHIC TECHNIQUES

4.8.1 Sample Application

In column procedures the sample solution is applied to the column by pumping. The proper flow rate for adsorption depends on several factors. In general it can be quite high if the protein of interest is far away from desorption conditions in the starting buffer. However, for samples that are viscous it may be necessary to reduce the flow rate during the
application or to dilute the sample. For protein solutions more concentrated than \( \sim 30 \, \text{mg/mL} \) this can be a concern, although only experience of the particular sample and experimental set-up can give definite answers.

To facilitate scale-up parameters, flow rates are most often expressed by the dimension length per time, most commonly \( \text{cm/h} \). Consequently the maximal volume flow rate increases as the square of the column diameter, given that other factors are constant. If scale up is based on constant contact time, bed height must be provided along with linear flow velocities in order to ensure proper scale up.

Dextran ion exchangers can only be run at about 15 cm/h, whereas more rigid media like the polystyrene divinylbenzene media and silica-based media can be run at hundreds of \( \text{cm/h} \). Often, one can speed up a process by running equilibration steps (where the target protein is not present on the column) at much higher rates as long as the pressure effects do not disturb the packed bed or exceed the ratings of the column.

Temperature changes will influence the maximal possible flow rates. When changing to cold-room temperatures, due to the increased viscosity, one must often decrease the flow to ensure that the maximum operating pressure of the column is not exceeded. Resolution can also be lower in a cold room. Conversely, improved resolution will be obtained above room temperature (95). In general, flow rate is a parameter that should be checked if maximum resolution is of importance, and the packed bed should be tested to ensure it is stable under the desired flows with the sample, especially when increasing column size beyond \( \sim 100 \, \text{mm} \), where there is a significant loss of wall support (the frictional force of the wall on the beads that is counter to the drag force of the flow).

During sample application it is sometimes possible to see protein bands on the column with the naked eye. The banding is due to protein–protein displacement. The proteins that bind most strongly to the ion exchanger are found closest to the top of the column.

After application the column is washed with starting buffer. The proteins that do not adsorb are recovered in a breakthrough peak. Normally, washing continues until the detector signal, often UV absorption, from the eluate decreases to an appropriate low value. Two to five column volumes should be sufficient and in the best case a single column volume suffices. The retained proteins are then eluted by a change in buffer conditions.

### 4.8.2 Elution Techniques

The proteins that adsorb to the ion exchanger are eluted from the column either by an increase in ionic strength, by inclusion of new ionic species, by a change in pH, or some combination thereof, as with the multimodal ligands (see Section 4.4.13). The increase in ionic strength is achieved either by adding a nonbuffering salt, such as NaCl, to the starting buffer or by using a more concentrated buffer. One should note that the addition of high concentrations of non-buffering salts can affect the pH.

If a pH change is used, elution from anion exchangers should be by a decrease in pH, to make the adsorbed proteins less negative. Conversely, elution from cation exchangers is affected by an increase in pH to make the proteins less positively charged. pH gradients have been used to separate Fab fragments and other proteins with differences in isoelectric points as small as 0.1 units (101). One alternative mode for separation of proteins with very similar isoelectric points is to establish the pH gradient on the column as with chromatofocusing. Another alternative is to run two consecutive IECs at different pHs. Two proteins co-eluting at one pH are less likely to co-elute at another pH (Fig. 4.10).

The elution buffer can be changed step by step or continuously; these are called stepwise and gradient elution, respectively. If the chromatography is being run with a constant buffer composition it is called isocratic elution. Displacement due to a specific interaction of the protein with an ionic species added to the elution buffer is the basis for affinity elution. In displacement chromatography (see Section 4.8.2.5), a general eluting agent is added to the buffer that displaces all proteins, which will move ahead of the displacer in the column.

In some cases, if the proper buffer conditions can be found, the target molecule can serve as the displacement species. This is a particularly useful technique for concentrating high value targets present in large volumes but in low concentrations, such as IgGs in milk whey. At high flow rates the capacity of the column can be filled quickly, even though a significant amount of target might remain in the flow-through fraction. This strategy can be economically viable, especially if the flow-through can be further processed to yield other valuable products.

To make the best use of the resolving power of ion exchange, a protein should be submitted to chromatography in such a way that it elutes retarded by the column. In chromatography a component alternates between the mobile and stationary phase. According to this definition, a substance eluting in a breakthrough peak or in an unretarded peak has not been submitted to chromatography, as it is always in the mobile phase when moving through the column. It has only been filtered through the column.

#### 4.8.2.1 Isocratic and Stepwise Elution

Isocratic elution is often used in cases where the sample and its binding properties are well known and the same kind of sample is run repeatedly (i.e., in analytical separations). To achieve proper reproducibility, great care should be taken to ensure that the column is properly equilibrated and the sample ionic composition does not vary. A well known example of isocratic elution in an analytical context is the Moore and Stein method for amino acid analysis.
In isocratic elution all components move simultaneously, so only sample volumes much smaller than the total bed volume can be applied. The resolution (see Section 4.9.3) is proportional to the square root of the column length. The peaks widen later in the chromatogram and finally become so wide that they can hardly be detected. After several bed volumes of elution buffer (the practical upper limit is 3 to 10 bed volumes) (59), the chromatography is interrupted.

Stepwise elution is a serial application of several isocratic elutions. At each step, only about a single total bed volume of eluant is passed through the column. Stepwise elution is often used for recovering a protein in a concentrated form in the breakthrough peak of the displacing buffer. Here it is best if the elution conditions for the desired component are known. The application conditions are then chosen so that the desired protein stays bound to the column while contaminants, often bulk proteins, pass through. After washing, the salt concentration or pH is changed so that the protein of interest is eluted in a breakthrough peak.

Stepwise elution can give a few disagreeable surprises, some of them illustrated in Figure 4.8. A retarded peak (#3) is eluted with one buffer. If the change to the next buffer is made too early, the breakthrough of this buffer will catch up with the trailing material and push it out as a breakthrough peak. Furthermore, if the capacity of the column is inadequate, the second component might also be found in the first breakthrough peak (also true for gradient elution).

For each step that produces a breakthrough peak, the resolving power might not be adequate, resulting in several components co-eluting. Only peaks eluting after the breakthrough of the displacing buffer indicate chromatographic homogeneity. Another potential problem with stepwise elution that usually arises with a change in equipment is that the volume of the mixing chamber or bubble trap in the chromatographic system is a significant fraction of the total column volume, and with dilution of the current "step" with the previous "step," the resulting buffer applied is insufficient to create the desired elution conditions. This can be avoided by running the system in “bypass” mode or adequately washing out the system between “step” applications. If the system “dead volume” is sufficiently small compared to the total column volume, this issue may not arise. Comparison of the conductivity traces (after the column) between the two devices is an excellent tool for diagnosing such problems.

Peaks eluting at the breakthrough of a displacing buffer have a typical shape, a sharp front and a tailing end as in peaks 2 and 6 in Figure 4.8a. It is usually more difficult to choose the right conditions for stepwise elution. If the buffer is too weak the protein might elute in a broad retarded peak; if it is too strong it might elute together with several other components. In general one should avoid stepwise elution with an unknown sample and instead use gradients. From the information obtained with gradient elution one should be able to construct a useful step elution program.

### 4.8.2.2 Gradient Elution

In gradient elution, the concentration (more rarely pH) of the eluting buffer is changed continuously. At one concentration the least strongly adsorbed protein is desorbed; at somewhat higher concentration, the second protein, and so on. Gradient elution has the separation obtained in isocratic elution, but because of the continuously increasing elution power the peaks do not become much broader as the gradient develops. Tailing due to nonlinear adsorption isotherms is also diminished. Normally, a single substance is not recovered in several peaks (for an exception, see Section 4.9.4), and a maximal use of the resolving capacity of the column is obtained.

Gradients are obtained by mixing starting and final buffers so that the percentage final buffer pumped into the column is changed continuously. Modern chromatographic systems can be programmed to give any conceivable gradient. However, simpler gradient mixers can easily be constructed by connecting two cylinders by a tube (see Fig. 4.9). One cylinder (F), equipped with a stirrer, contains the final buffer of concentration \( C_f \), and the other cylinder a starting buffer of concentration \( C_s \). The eluting buffer is continuously withdrawn from the stirred chamber. The concentration of the eluent column is inadequate, the second component might also be found in the first breakthrough peak (also true for gradient elution).

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Peaks eluting at the breakthrough of a displacing buffer have a typical shape, a sharp front and a tailing end as in peaks 2 and 6 in Figure 4.8a. It is usually more difficult to choose the right conditions for stepwise elution. If the buffer is too weak the protein might elute in a broad retarded peak; if it is too strong it might elute together with several other components. In general one should avoid stepwise elution with an unknown sample and instead use gradients. From the information obtained with gradient elution one should be able to construct a useful step elution program.

#### 4.8.2.2 Gradient Elution

In gradient elution, the concentration (more rarely pH) of the eluting buffer is changed continuously. At one concentration the least strongly adsorbed protein is desorbed; at somewhat higher concentration, the second protein, and so on. Gradient elution has the separation obtained in isocratic elution, but because of the continuously increasing elution power the peaks do not become much broader as the gradient develops. Tailing due to nonlinear adsorption isotherms is also diminished. Normally, a single substance is not recovered in several peaks (for an exception, see Section 4.9.4), and a maximal use of the resolving capacity of the column is obtained.

Gradients are obtained by mixing starting and final buffers so that the percentage final buffer pumped into the column is gradually increased. Modern chromatographic systems can be programmed to give any conceivable gradient. However, simpler gradient mixers can easily be constructed by connecting two cylinders by a tube (see Fig. 4.9). One cylinder (F), equipped with a stirrer, contains the final buffer of concentration \( C_f \), and the other cylinder a starting buffer of concentration \( C_s \). The eluting buffer is continuously withdrawn from the stirred chamber. The concentration of the eluent
After $v$ mL has been taken from a total of $V$ mL is described by the equation (102)

$$C_v = C_f - (C_f - C_s) \times (1 - v/V)^{A_f/A_s}$$

Here $A_f$ and $A_s$ are the cross-sectional areas of the two cylinders. A linear gradient is obtained for $A_f/A_s = 1$, concave for $A_f/A_s < 1$, and convex for $A_f/A_s > 1$. The concave gradients with $A_f/A_s$ of 1/3 or 1/4 are very steep at the end of the gradient. The concentration has increased by 50% after 88% and 94%, respectively, of the gradient.

Linear gradients are recommended as a first choice. If the resolution is not satisfactory, changing the gradient volume or the concentration of the final buffer changes the slope of the gradient. With more sophisticated gradient devices such as process control software programs, the gradient can be made shallower in areas where the peaks are less well resolved. Similarly, concentration ranges without proteins eluting can be passed by a steep part of the gradient. However, one should not try to solve resolution problems only by gradient tinkering, but rather by carrying out the ion exchange at another pH (Fig. 4.11).

### 4.8.2.3 Negative or Flow-Through Chromatography

A high degree of purification can be obtained with IEC if the proteins of interest pass unretarded through the column while unwanted species are adsorbed. This method is sometimes referred to as negative or flow-through chromatography. As remarked in Section 4.8.2.1, the unretarded fraction has not been submitted to any chromatography. To purify it further, the sample can be chromatographed, perhaps on the same ion exchanger at another pH where the proteins of interest adsorb and are then eluted with a salt gradient.

Section 4.11.1 gives an example of negative chromatography. Creatine kinase eluted unretarded from the cation exchanger Mono S at pH 6.0 and was then chromatographed on Mono Q at pH 8.0, conditions that promoted binding of the target protein. Another example of negative chromatography with a Q anion exchanger is shown in Section 4.11.3. In this example the target molecule (a virus) passes unretarded in the void volume, while contaminants remain bound (later removed by a salt gradient).

Negative chromatography is commonly used to remove host-cell DNA and endotoxin from recombinant proteins. This is often called a scavenging step for final purity. As an initial clean-up step, negative chromatography can be extremely useful in reducing many types of ionic species capable of fouling, and will increase capacity and yield. This step is performed in a column or in batch mode at a pH where the foulants bind to the resin, but the target protein passes through unretarded. An inexpensive disposable anion exchange resin is often used if the concentrations of foulants very high or a good cleaning procedure is difficult to develop.

### 4.8.2.4 Affinity Elution

In affinity elution, adsorption is nonspecific, but desorption is specific. In affinity chromatography, adsorption to an immobilized ligand is specific, and the desorption is either specific (elution with a buffer containing free ligand) or nonspecific (change of pH or salt concentration).

In most affinity elution experiments the adsorbent is an ion exchanger. For instance, if a protein is adsorbed to a cation exchanger, the column is eluted with a specific ligand with a negative charge, that is, a charge of the same sign as the ion exchanger. The binding of the ligand reduces the net charge of the protein and weakens the electrostatic interaction between the protein and the ion exchanger. When the proper elution conditions are selected the electrostatic interactions are decreased so much that the protein is eluted from the column. The binding of the ligand can also produce conformational changes that facilitate desorption.

Scopes has studied affinity elution of glycolytic and other enzymes extensively to determine the proper pH conditions and suitable ligands for affinity elution (43, 103–105). The enzymes have negatively charged substrates and, after adsorption to the cation exchanger, they were eluted with the
substrate. For instance, fructose-1,6-bisphosphate aldolase and fructose-1,6-bisphosphatase were eluted with fructose-1,6-bisphosphate. The tetrameric enzymes bind four substrates each with a charge of $-4$, and the charge was reduced by 16 units, which was sufficient to elute the enzymes.

When the target protein is adsorbed to an ion exchanger other proteins also adsorb. To minimize this the column can be pre-eluted with a dummy ligand with about the same charge as the affinity ligand. Citrate and EDTA have been used as dummy ligands on cation exchangers.

Affinity elution ligands are effective because of their ability to affect the partition coefficients of the target molecules (proteins) they are intended to displace. The partition coefficient ($\alpha$) is the amount of protein bound to the resin as fraction of the total (43, 106–110).

- When $\alpha = 1$, all of the molecules are adsorbed and do not move.
- When $\alpha = 0$, none of the molecules is adsorbed to the column.
- When $0 < \alpha < 1$, the molecules elute after $1/(1-\alpha)$ column volumes of buffer have passed through the column after application (43).

Scopes studied affinity elution extensively to determine the proper pH conditions and suitability of a ligand for affinity elution (43).

The partition coefficient depends on pH and ionic strength. An increase of pH or ionic strength will weaken the binding to a cation exchanger and decrease $\alpha$; on an anion exchanger the effect is opposite. At $\alpha = 0.95$, the protein migrates at a rate that is $1/20$th of the buffer; at $\alpha = 0.5$, this rate is half the rate of the buffer (43, 104). To weaken binding to the ion exchanger, the column can be pre-eluted with a buffer of another pH or higher ionic strength. This will decrease $\alpha$, facilitate desorption, and facilitate the desorption of the protein by elution with an affinity ligand (43).

### 4.8.2.5 Ion Exchange Displacement Chromatography

The modes of chromatography were classified by Tiselius (111) as frontal elution and displacement. In displacement chromatography the components are resolved into consecutive “rectangular” zones of highly concentrated and purified substances rather than to peaks. The molecules are forced to migrate by an advancing wave of a displacer that has a higher affinity for the stationary phase than the feed solute. The displacer competes for the binding sites and displaces all molecules with lower affinity for the adsorbent. The displaced components will in turn displace other components with lower affinity. The displaced molecules move with the same velocity through the column and become separated into consecutive zones, rather than peaks (101).

Several types of displacers have been investigated, and some examples of Polymer™ displacers available from SACHEM are as follows:

- Anion displacement: CM-dextrans, chondroitin sulfate, dextran sulfate, polyvinylsulfate and heparin
- Cation displacement: DEAE-dextrans, methacrylic polyampholytes

A and B β-lactoglobulin were separated on a $7.5 \times 75$ mm column of TSK DEAE 5 PW by displacement with chondroitin sulfate and eluted in two rectangular zones with B ahead of A, which has one negative charge more. The load was $100 \text{ mg}$. The matrix was a hydroxylated vinyl polymer and could withstand washing with the sodium hydroxide required to clean the column of remaining displacer and protein. Silica columns, because of their sensitivity to high pH values, were not suitable. The two proteins were found to be pure by nondenaturing PAGE and reversed-phase HPLC. Following isocratic elution on the same column, only $20 \text{ mg}$ could be fractionated. Thus, the capacity of displacement elution was five times higher than that of isocratic elution (112).

In amphotolyte displacement chromatography, ampholytes for isoelectric focusing have been used as displacers. Two forms of a β-N-acetyl-d-hexose aminidase were separated by elution with a $4\%$ solution of ampholytes at pH 8–10 from a column of CM-cellulose equilibrated in $0.02 \text{ M Tris–HCl}$, pH 7.6. These forms of the enzymes were not resolved either by isoelectric focusing or IEC. As a pH gradient was obtained at the beginning of the chromatogram, the separation probably depended both on chromatofocusing and displacement chromatography (113).

Positively charged displacers based on pentaerythritol have been developed. These are dendritic polymers; that is, the molecules branch out from a central atom with quaternary ammonium groups at the ends of the branches. The other displacers mentioned here are linear polymers. Two of the dendritic polymers have molecular masses of about $500$ and $1600$ Da, while linear polymers have molecular masses that are $>2000$ Da. Examples of the dendritic polymers used as displacers in a cation exchanger and a discussion of displacement chromatography are to be found in Reference 114.

Narahari and colleagues (115) used two adsorbed buffering species as displacers. A strong anion exchanger TSK-Gel Q-4 PW ($7.5 \times 75$ mm) was equilibrated with $0.05 \text{ M MOPS}$ (pH 7.5). β-lactoglobulins (100 mg) were applied to the column, which was eluted with $0.05 \text{ M Naacetate}$ (pH 4.3); at lower pH the two variants A and B were displaced as discrete rectangular zones. At a load of only $1 \text{ mg}$, the two lactoglobulins co-eluted. With $100 \text{ mg}$ load, B was displaced ahead of A.

A recent review on displacement chromatography is found in Reference 116.
4.9 HANDLING OF ISOLATED PROTEINS

4.9.1 Determination of Protein Content: Colorimetric Methods, Spectrophotometry

There are several methods for determining protein content. The most accurate is the method developed by Moore and Stein in the 1950s, in which proteins are hydrolysed by HCl, and the amino acids are separated by cation exchange chromatography and then allowed to react with ninhydrin. The amount of each amino acid is determined from the absorbance at 570 nm, or 440 nm for proline. There are several variants of the Moore–Stein method, and picomolar quantities of protein can now be determined.

Simpler and more common methods for protein determination are the colorimetric methods by Lowry and Bradford, in which proteins react with the Folin–Ciocalteu (Lowry) reagent or Coomassie Blue (Bradford), and the amount of protein is obtained from a standard curve of a well-known protein, such as albumin.

A protein solution of 1 mg/mL is often assumed to have absorbance at 280 nm (absorbance at 570 nm) = 1. However, as the absorbance in the vicinity of 280 nm depends on the content of tryptophan, tyrosine, and cystine, it is obvious that the absorbance at 1 mg/mL can vary substantially. The UV absorbance at 280 nm does not usually reflect the amount of protein present.

At the 280-band, absorption coefficients of proteins can vary considerably. Proteins containing tryptophan and/or tyrosine have an absorption maximum close to 280 nm and a minimum at 250 nm. If a nontypical protein spectrum is obtained, the sample is likely to contain nonproteinaceous substances. It is good practice to run an absorption spectrum in the region 400–200 nm. Sometimes, several minor peaks are detected in the region 250–265 nm, indicating the presence of phenylalanine.

The molar absorptivity of a protein close to 280 nm is the sum of the molar absorptivities of Trp, Tyr, and cystine, plus a contribution from the secondary and tertiary structures. This last factor constitutes about 10% of the total absorbance of various snake neurotoxins having 57–75 amino acids and three to five disulfides, but may be different for other types of proteins. After reduction of the disulfides and alkylation (e.g., with iodoacetate), the molar absorbance is the sum of the absorbances of tryptophan and tyrosine. Some proteins have a UV absorbing cofactor, such as the heme group in myoglobin. A discussion of other spectrophotometric methods is also included. Absorption of the protein to the cuvettes must also be considered. Scopes (43) recommends a 5 mM phosphate buffer (pH 7) in the presence of 50 mM sodium sulfate to prevent adsorption to the cuvette. If only a few microliters of sample are added to a 3-mL cuvette, the increase in volume can be neglected.

\[
A^{0.1\%} = 34.14 \times \frac{A_{280}}{A_{205}} - 0.2, \]

gives a good estimate of the protein content of a number of proteins including glycoproteins, and chromophoric proteins such as myoglobin. A discussion of other spectrophotometric methods is also included. Adsorption of the protein to the cuvettes must also be considered. Scopes (43) recommends a 5 mM phosphate buffer (pH 7) in the presence of 50 mM sodium sulfate to prevent adsorption to the cuvette. If only a few microliters of sample are added to a 3-mL cuvette, the increase in volume can be neglected.

4.9.2 Concentrating Protein Solution, Lyophilization

Protein fractions can be concentrated by many techniques, including precipitation, evaporation, ultrafiltration (dialfiltration), or freeze-drying (lyophilization). In ultrafiltration, the protein solution is forced under pressure past a membrane that allows passage of salts and low molecular weight compounds. Care should be taken to utilize a membrane that will fully retain the protein of interest, as the molecular mass retained by the filter (NMWCO) is an average of a range and likely only tested for globular proteins. An example of ultrafiltration is the separation of albumin (67 kDa) and IgG (156 kDa) from factor VIII (240 kDa) (Section 4.11.2).
At the laboratory scale, protein fractions are often concentrated by freeze-drying. This requires a volatile buffer to be used or a change to be made to such a buffer (Table 4.5) though dialysis, gel filtration, or reversed-phase HPLC. Dialysis against a dilute volatile buffer can be better than dialysis against water. Proteins with hydrophobic regions may aggregate in water, but are less likely to do so when dialyzed against a buffer.

Ammonium acetate is suitable for lyophilization, but dilution to about 0.2 M is recommended. Ammonia is more volatile than acetic acid, and during freeze-drying the pH will drop as the ratio of ammonium to acetic acid reduces. This drop in pH can lead to protein aggregation. To avoid this problem the pH can be adjusted to 7–8. A second freeze-drying can be suitable. The sample is dissolved in a small volume of 0.01–0.02 M AmOAc and freeze-dried. The final product does not adsorb much to the walls of the container or become electrostatically charged. However, it contains some acetic acid and ammonium ions as counterions. These should be removed, for instance by gel filtration, before using proteins for experimentation, such as radioactive acetic anhydride, which reacts with ammonia.

Freeze-drying frequently produces small amounts of aggregates detectable by gel filtration, and even by IEC. Lyophilization can cause reversible changes in protein structure (119) and should not be used for proteins to be crystallized. Lyophilized proteins are normally stored at –20°C or lower. Sonication is recommended to bring proteins into solution. Freeze-drying will sometimes inactivate proteins. For instance, muscarinic toxins of type 2 (120) can be inactivated.

Lyophilization can cause reversible changes in protein structure (119) and should not be used for proteins to be crystallized. Lyophilized proteins are normally stored at –20°C or lower. Sonication is recommended to bring proteins into solution. Freeze-drying will sometimes inactivate proteins. For instance, muscarinic toxins of type 2 (120) can be inactivated when freeze-dried from 0.1% trifluoroacetic acid (TFA) following reversed-phase HPLC (E.K., personal observation).

For many assays proteins are diluted from a stock solution to a concentration of 1 μg/mL or less. To prevent adsorption of a large part of the protein to test tubes, pipettes, and so on, 1–2 mg/mL of a protein that does not interfere with the assay, often albumin, can be added to the sample.

4.9.3 Rechromatography, Resolution, Degree of Cross-Contamination

At the laboratory scale it can often be worthwhile to rerun a protein that eluted close to another component. For industrial separations rechromatography is not recommended, and overlapping peaks are resolved either by subsequent chromatographic methods (HIC, RPC, etc.) or indicative of a poorly designed method needing more development at the small scale.

Figure 4.10 shows that the cross-contamination of two not fully resolved peaks of equal size (a) is surprisingly low. Rechromatography of one of the peaks will remove most of the contaminating protein. If a contaminant is present in smaller amounts (b and c) it is much easier to remove it from the main component. A rerun also confirms that a substance is homogeneous with regard to the separation parameters used.

Sometimes, material from an early peak trails into later eluting peaks. Moreover, an earlier peak can also be contaminated with material from later eluting peaks. When a protein starts eluting from an ion exchanger it will displace small amounts of proteins still adsorbed to the column, acting in a displacement-like manner. This type of cross-contamination has also been observed in gel filtration.

The degree of cross-contamination can be calculated from the resolution, expressed by the formula

\[ R_s = 2\Delta V/(W_1 + W_2) \]

where \( R_s \) = resolution, \( \Delta V = \) difference in the elution volume of the peaks, and \( W_1 \) and \( W_2 \) = widths at the base of peaks.

4.9.4 Dissociation of Protein Complexes, False Peaks

False peaks indicating heterogeneity are sometimes observed due to poorly designed step wise elution (see Section 4.8.2.1). However, they can also occur in well conducted gradient chromatography, for various reasons (e.g., when a
noncovalent protein complex dissociates on the column and components elute in different positions. Several presynaptic neurotoxins that inhibit the release of acetylcholine consist of subunits with very different charge properties held together by noncovalent forces. One of the subunits is very basic, and if the toxin is adsorbed to a cation exchanger this component will be bound strongly and will not elute together with the other subunits (106–109).

Experience also suggests that binding a protein to the column far below its desorption concentration might give rise to artifacts and several peaks arising from protein denaturation.

Multiple peaks of homogeneous proteins can be seen on an overloaded column because all protein molecules will not interact with the same number of ion exchange groups and do not bind with the same strength. In such situations, rechromatography of a smaller aliquot should be performed. A gel filtration fraction of black mamba (*Dendroaspis polylepis*) venom was chromatographed on Bio-Rex 70. The load was about twice that of earlier chromatographies. Dendrotoxin I, a blocker of some voltage-dependent potassium channels, is the dominating component, accounting for >10% of the venom protein eluted as a multiple peak, although multiple peaks were not seen for other components. Rechromatography of a smaller aliquot of the pooled multiple peaks gave only one peak. At lower loading conditions all molecules had enough space to bind to the same number of sites.

Some substances alternate between two structural forms and yield two peaks when subjected to chromatography. Running one of the peaks should then yield a similar double peak pattern.

### 4.9.5 Storage of Isolated Proteins

There are many methods for storing purified proteins, and the best method will be case-dependent. One must consider both physical and chemical instabilities and the intended use after storage. Shear forces during pumping, adsorption, denaturation, aggregation, and freeze/thaw stability are all important criteria. Reactive groups capable of oxidation (e.g., disulfide/thiol), photocatalysis, hydrolysis, deamidation, protonation, and so on must be identified and stabilized. The more well characterized the target protein, the more easily critical physical and chemical instabilities can be identified.

Buffers selected for protein storage should not act as substrates or inhibitors, nor should they interfere with biochemical assays or the intended use of the protein after storage. Buffers, due to the surprisingly low denaturation Gibbs energy for globular proteins, can induce conformational changes. Anjum and colleagues (114) discuss the role of osmolytes in protein stability and the compatibility of osmolytes with Gibbs energy of stabilization for proteins.

Conformational changes affecting protein function can be very obvious (e.g., denaturation) or subtle (e.g., changes in a single domain or reactive center). Salts, especially when present in concentrations significantly greater than the buffering species, can act as buffer components and affect protein conformation. For a review of this topic see Reference 121.

Some enzyme preparations are stored as precipitates in the presence of saturated ammonium sulfate. The use of different glycerol or albumin concentrations to stabilize enzymes is a common practice. Freeze-drying is often used in laboratory-scale experiments, after which the protein is stored at −20°C or lower.

Repeated freezing and thawing of a protein solution can lead to inactivation. This may be avoided if the sample is divided into small aliquots for use as needed. One possible cause of inactivation by freezing and thawing is pH shifts due to the difference in freezing point for the mono-ionized salt compared to its free acid or base form. Such differences can lead to severe changes in the local pH environment of the protein. Pikal-Cleland and colleagues (122) found that significant drops in pH (up to 3.2 units), leading to denaturation in freeze/thaw, could be eliminated by simply replacing sodium phosphate buffer with potassium phosphate. The use of a zwitterionic buffer should also help minimize the effects of such pH shifts.

Even when a protein is stored −70°C, not all chemical reactions have been halted. The ammonia content of plasma increases slowly at −70°C, and proteolytic enzymes present in low levels (not detectable on SDS-PAGE) may still be catalytically active. The result of such activity should be apparent on SDS-PAGE gels as the appearance of low molecular weight degradation products, not present before storage. Therefore, one must consider the addition of protease inhibitors in cases where the protein content is not well defined.

### 4.10 HYDROXYAPATITE CHROMATOGRAPHY

Hydroxyapatite chromatography is discussed thoroughly by Boschetti (123) and Kawasaki (124) and in booklets from various manufacturers, for instance from Bio-Rad laboratories, and the references therein. We summarize these discussions.

Calcium phosphate had been used for protein adsorption with varying success for many decades when Tiselius and co-workers (125) showed that a much more useful adsorbent was obtained when the brushite, Ca₅(PO₄)₂, was changed into hydroxyapatite by boiling in 1% NaOH for one hour. The hydroxyapatite crystals generated fines more easily, but these were removed by decantation before column packing.

Hydroxyapatite made this way often has excess phosphate, which leads to the formation of unstable rectangular plate-shaped crystals that impair the flow properties and stability of the adsorbent. Later, hydroxyapatite with hexagonal crystals and the theoretical Ca/P ratio was synthesized and sintered.
at high temperatures to fuse the particles into a stable ceramic mass. This gives an adsorbent with better flow properties and capacity suitable for large scale operations. It is stable at pH values above 6.5, but can also be used in the pH interval 5.5–6.5 if CaCl₂ up to 1 mM is added to the buffer. At pH below 5.5, hydroxyapatite starts dissolving.

The formula of hydroxyapatite is Ca₁₀(PO₄)₆(OH)₂. In the crystal structure an even number of functional groups are regularly spaced on the crystal surface: pairs of positively charged Ca²⁺ ions (C-sites), clusters of six negatively charged oxygen atoms associated with six phosphates (P-sites), and hydroxyl groups. The hydroxyapatite–protein interactions are complex. Positively charged groups (amino groups and arginines) are attracted to the P-sites and repelled by the C-sites. The situation is reversed for carboxyls. The initial attraction is electrostatic, but the final binding to C-sites involves formation of stronger coordination complexes with carboxyls on the proteins. Consequently, ions that bind to calcium ions can be used as displacers (e.g., phosphate, citrate, and fluoride). The content and distribution of carboxylates on the surface of proteins is a major factor that determines how strongly different proteins bind to hydroxyapatite. Phosphate groups on proteins and other molecules bind even more strongly than carboxyls. Phosphoproteins bind very strongly and DNA binds well, but less strongly, probably because the spacing of its phosphate groups prevents an ideal match with the distribution of the C-sites. Endotoxins bind by their many phosphate groups on their core polysaccharides and lipid moieties.

The functional sites have both negatively and positively charged groups as well as noncharged hydroxyls regularly distributed over the crystal surface. This is different from the functional sites of ion exchangers, with only anionic or cationic groups. Consequently, the selectivity of hydroxyapatite is different than that of conventional ion exchangers.

A drawback of classical hydroxyapatite is its mechanical and chemical instability. This has been partly overcome by the introduction of ceramic fluoroapatite, Ca₁₀(PO₄)₆F₂, which apparently has the same chromatographic properties as hydroxyapatite. This seems to indicate that OH-groups of the hydroxyapatite do not interact with the proteins. Fluoroapatite is available as 10-μm (average diameter) spherical particles in prepacked HPLC columns or as 20-, 40- or 80-μm-diameter media for large scale chromatography.

The column is normally equilibrated and the sample applied in a low concentrations (1–10 mM) of sodium or potassium phosphate buffer (pH 6.8, about an equimolar mixture of H₂PO₄⁻ and HPO₄²⁻), and the proteins eluted in a concentration gradient of phosphate buffer. Owing to the low solubility of Na₂HPO₄, potassium phosphate should be used in a cold room.

The binding of basic proteins becomes stronger by lowering the pH, due to the increased positive charge on the proteins. Usually, lowering the pH will lead to stronger binding and a more concentrated phosphate buffer is required for desorption. Weak binding of basic proteins can be strengthened by adding 1 mM phosphate to the buffer. Phosphate ions pair with the C-sites and suppress their ability to repel positively charged proteins. Basic proteins can be eluted with gradients of chloride or phosphate. Hydroxyapatite behaves as an ion exchanger for basic proteins and adsorption is facilitated by the addition of 1 mM phosphate to neutralize the charge of the C-sites by ion pairing. Desorption can be achieved with NaCl.

Acidic proteins cannot be eluted with NaCl, even at concentrations >3 M, but instead require displacers with stronger affinity for Ca²⁺, such as phosphate, citrate, or fluoride. The presence of other salts such as sodium chloride or ammonium sulfate seems to affect the elution of acidic proteins. This can be taken advantage of by applying a sample from an ion exchange column eluted by a NaCl gradient directly to the apatite column. This salt tolerance has also been used for the purification of halophilic enzymes that are active in saturated NaCl (3.4 M).

Antibodies, which behave as basic proteins, can be eluted with sodium chloride, while acidic proteins, DNA, and endotoxins remain bound to the column. Double gradients can be used to isolate basic proteins (KCl gradient) and acidic proteins (potassium phosphate gradient) from the same column.

If the starting buffer is 10 mM phosphate, immunoglobulins may not be soluble at this low salt concentration. However, after addition of NaCl up to 100 mM, immunoglobulins will be soluble. This will not affect the binding to the column, at least not of basic proteins.

Hydroxyapatite seems to be particularly useful for the purification of medium and high molecular weight proteins and nucleic acids. Low molecular weight solutes show in general very low affinity for hydroxyapatite. A special possibility is to use the technique for purification of phosphoproteins.

The selectivity of hydroxyapatite has been used, for instance, to isolate isoforms of human ceruloplasmin, which were not separated by anion exchange chromatography (see Section 4.11.4).

The resolving power of hydroxyapatite can be extremely good, as exemplified by the separation of four human tumor necrosis factor proteins (hTNF). The molecule consists of three subunits of two subtypes, one a polypeptide of 157 amino acids with an N-terminal valine (subunit A), the second of 158 amino acids with an N-terminal methionine residue added to valine (subunit B). The four possible hTNFs, A₃, A₂B, AB₂, and B₃ could be separated by hydroxyapatite. PAGE with and without SDS gave only one band of hTNF composed of the four isoforms, and the four isoforms were not separated by reversed-phase HPLC or IEC.

Hydroxyapatite can also be used with organic solvents. The separation of saponins by a descending acetonitrile gradient of 90% to 70% has been applied (124).
Hydroxyapatite is commercially available from many suppliers of chromatographic media.

4.11 APPLICATIONS

4.11.1 Isolation of Glycolytic Enzymes from Chicken Muscle by HPLC Ion Exchangers Mono Q and Mono S

In the isolation of proteins from a complex sample, different selectivities are often used. This is illustrated by the isolation of enzymes from chicken muscle at two different pH values using both a cation exchanger and an anion exchanger.

A low ionic strength extract of chicken muscle was transferred to the starting buffer for the first step, cation exchange, by buffer exchange on Sephadex G-25. The sample was filtered (0.22 μm) and 800 μL was applied to a Mono S HR 10/10 column. The column was first eluted (1 mL/min) with the starting buffer, 0.05 M MES, pH 6.0, and then with a gradient of 0–0.5 M NaCl in the starting buffer. The enzymes were detected by the zymogram technique using substrates. Creatine kinase (CK) and phosphoglucomutase (PGM) eluted in the breakthrough peak, and other enzymes were resolved in the gradient: TPI, triosephosphate isomerase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ENO, enolase; and LDH, lactate dehydrogenase.

The breakthrough peak was collected and transferred to the starting buffer for the anion exchange (0.02 M Tris HCl, pH 8.0) by gel filtration on Sephadex G-25. A volume of 1 mL of the sample was applied to a Mono Q HR 5/5 and eluted (1 mL/min) with the starting buffer at a gradient of 0–0.5 M NaCl (Fig. 4.11). Creatine kinase (CK) was well separated from other proteins.

As mentioned previously, the breakthrough peak was not submitted to chromatography, only filtered through the column. Consequently, the full potential of IEC was not exploited. To do so, the breakthrough peak was chromatographed at another pH on an anion exchanger. This application demonstrates the power of high performance IEC at a relatively small sample load.

4.11.2 Large Scale Purification of Factor VIII, Human Serum Albumin, and IgG by Stepwise Elution

Highly purified human blood proteins are needed for a variety of scientific and therapeutic purposes. The usual procedure for large scale fractionation is the Cohn cold ethanol procedure. An alternative method utilizes both anion and
Figure 4.12 Overview of a purification process for Factor VIII, albumin and IgG. The schematic picture shows the chromatographic steps and other procedures necessary before a product can be used in clinic. In a booklet from GE Healthcare there is a more detailed discussion about the removal of viruses, bacteria and endotoxins, storage and shelf-life of the products. (See color insert.)
Figure 4.13  Negative chromatography for the purification of influenza virus. The cell culture was passed through a microfilter followed by size exclusion chromatography on Sepharose 6 FF. The virus eluted in the flow through the fraction as indicated by the hemagglutinin activity. Further purification was achieved by anion exchange chromatography on Q-Sepharose FF. Here, also, the virus passed unretarded through the column while the charged contaminants were retained, an example of two consecutive negative chromatography steps. Reproduced with permission of GE Healthcare Life Sciences, Uppsala, Sweden.

Figure 4.14  Hydroxyapatite chromatography and separation of isoforms of human ceruloplasmin. Ceruloplasmin is a blue copper protein that occurs in human plasma in two isoforms differing in their carbohydrate content. They can be separated on hydroxyapatite but not by IEC. Defatted serum was diluted with 0.03 M potassium phosphate and 0.1 M NaCl (pH 6.8) and applied to a column of DEAE-cellulose equilibrated with the same buffer. Ceruloplasmin was eluted with a gradient of 0.45 M NaCl in 0.03 M potassium phosphate (pH 6.8). The ceruloplasmin fractions (blue) containing about 0.25 M NaCl were dialysed against 0.075 M potassium phosphate buffer (pH 6.8) and applied to a column (2 × 75 cm) of hydroxyapatite in the same buffer. Hydroxyapatite was prepared as described by Tiselius and colleagues (126). The column was eluted with a 1000-mL linear gradient up to 0.5 M phosphate. The two ceruloplasmin forms contain 11% (major form) and 7% (minor form) carbohydrate. All buffers contained 0.02 M L-amino caproic acid to prevent plasminogen activation.
cation exchange chromatography and starting material is cryoprecipitated and Factor IX-depleted plasma (Fig. 4.12). Purification of three proteins is given in more detail. Factor VIII purification begins and ends with gel filtration. Two ion exchange steps with different selectivities, Q Sepharose HP and SP Sepharose HP, are used to concentrate and purify the sample. The serum fraction, after the first gel filtration step, can (after ultrafiltration) enter into the albumin/IgG process. After centrifugation and buffer exchange to 25 mM sodium acetate buffer, the pH is adjusted to pH 5.2 with acetic acid to precipitate other proteins. The plasma is then centrifuged and filtered before application to a column of DEAE-Sepharose Fast Flow equilibrated in this buffer. The loading is ~35 g protein per liter of ion exchanger. After eluting IgG under starting conditions, albumin is displaced by stepwise elution with the same buffer at pH 4.5, and applied directly to a column of CM Sepharose equilibrated in this buffer. Highly purified albumin (>99% by cellulose acetate electrophoresis) is obtained by elution with 0.11 M sodium acetate buffer, pH 5.5.

The chromatographic parameters were optimized for high purity at a large scale. Such optimization is not usually required at the laboratory scale, as additional chromatographic steps such as gel filtration and reversed-phase chromatography are used to obtain sufficient purity.

4.11.3 Isolation of Virus by Negative Chromatography

A high degree of purification can be obtained if the molecules of interest pass unretarded through the column while contaminants are adsorbed. This method is sometimes referred to as negative chromatography (see Section 4.7.2.3). The example in Figure 4.13 shows two consecutive negative chromatographies for purification of influenza virus. The method was developed by GE Healthcare for purification of influenza virus. The chromatographic steps such as gel filtration and reverse-phase chromatographies for purification of influenza virus. The negative chromatography begins and ends with gel filtration. Two ion exchange steps with different selectivities, Q Sepharose HP and SP Sepharose HP, are used to concentrate and purify the sample. The serum fraction, after the first gel filtration step, can (after ultrafiltration) enter into the albumin/IgG process. After centrifugation and buffer exchange to 25 mM sodium acetate buffer, the pH is adjusted to pH 5.2 with acetic acid to precipitate other proteins. The plasma is then centrifuged and filtered before application to a column of DEAE-Sepharose Fast Flow equilibrated in this buffer. The loading is ~35 g protein per liter of ion exchanger. After eluting IgG under starting conditions, albumin is displaced by stepwise elution with the same buffer at pH 4.5, and applied directly to a column of CM Sepharose equilibrated in this buffer. Highly purified albumin (>99% by cellulose acetate electrophoresis) is obtained by elution with 0.11 M sodium acetate buffer, pH 5.5.

The proteins that co-eluted on DEAE-cellulose were separated on hydroxyapatite. The two ceruloplasmin forms contained about 11% (major form) and 7% (minor form) carbohydrate. All buffers contained 0.02 M epsilon-caproic acid to inhibit plasminogen activation.

It would have been possible to apply the sample from the ion exchange column without dialysis, as NaCl would not interfere with the binding of an acidic protein (see Section 4.10).

4.12 ACKNOWLEDGMENTS

We thank Prof. Stellan Hjertén and Dr Gunnar Johansson for discussions on many aspects of chromatography, Drs Lars Rydén and John Brewer for contributions to this chapter made in the previous editions.

4.13 REFERENCES


5

HIGH-RESOLUTION REVERSED-PHASE CHROMATOGRAPHY OF PROTEINS

SYLVIA WINKEL PETTERSSON
Chromatography and Applications, Eka Chemicals AB/Akzo Nobel, Bohus, Sweden

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5.1 INTRODUCTION

Reversed phase high-performance liquid chromatography (RP-HPLC, henceforth abbreviated as RPC) has been an important and often-used technique within protein research and development for the last 25 years. For a long time, this efficient purification technique was used predominantly for analytical purposes, but it is now a common tool for preparative applications, and also an essential part in the downstream processing of many biopharmaceuticals. New stable stationary phases and the use of modern column equipment have been a prerequisite for this development.

Most chromatographers gain their initial experience of RPC in the analytical field. Although the principles of analytical and preparative RPC are the same, the goals and targets differ. In analytical RPC, the ultimate goal is to gain information regarding identification, purity and composition, but in preparative RPC, the recovery of purified products is the aim. In this chapter, some important aspects of the theory of RPC are summarized in order to establish a common ground for understanding the special features of preparative, overloaded RPC. Some general guidelines for method development for preparative RPC separations are given, with special emphasis on proteins. Finally, some basic information is given regarding the features of the packing material, together with general recommendations for its use.

The purpose of this chapter is to provide some answers to the questions that you, as a new user of preparative RPC, might face, and help you to avoid some of the most common pitfalls.

5.2 FUNDAMENTALS OF RPC

A basic understanding of retention and interaction principles is a prerequisite for controlling and ultimately optimizing RPC separations. This chapter describes the commonly accepted mechanisms leading to separation. Equations are kept at a minimum and basic relationships of parameters encountered in HPLC in general are explained in Chapter 2, “Introduction to Chromatography.” The intention here is to provide a brief insight into the RPC separation mechanism at a molecular level. For a more detailed theoretical background of RPC, the chapter “Reversed-Phase and Hydrophobic Interaction Chromatography of Proteins and Peptides” by Milton T.W. Hearn in Reference 1 is recommended.

5.2.1 RPC: Partitioning and Adsorption

For a solute to be retained in a chromatographic system, it must transfer from the mobile phase into or onto the stationary phase. The association with the stationary phase can either occur by partitioning or adsorption (Fig. 5.1) (2). In the case of proteins, the prerequisites for partitioning are not available, primarily because of their macromolecular character and sensitivity to, and low solubility in, organic solvents.

The adsorption mechanism is based on the solvophobic theory proposed by Melander and Horvath (3). They state that RPC retention arises from solute binding onto the stationary phase and is mainly a result of hydrophobic interactions between the solute and the mobile phase.

Retention should be mainly dependent upon the free energy of creation of cavities of the size of the solute in the mobile phase. The impact of the stationary phase is considered to be weak and indeed negligible. Interactions of the solute with the second “solvent,” the stationary phase, and the formation of solute-sized cavities in the surface modification of the stationary phase are neglected. Elution occurs when the adsorbed solute is displaced from its adsorption site either by a mobile-phase molecule or a competing species from the sample mixture that exhibits stronger affinity to the stationary phase than the solute in question. In the case of analytical separation, the mobile phase is the sole displacer, because the available adsorption sites by far outnumber the number of solutes, so no competition takes place between different solutes.

5.2.2 Interaction Mechanisms

RPC describes a type of chromatography in which the stationary phase is less polar than the mobile phase. Most RPC stationary phases are based on surface-modified silica materials. n-Alkyl silanes are covalently bonded to the
silanol groups of the silica bead. Most commonly used for proteins are C8 and C4 silanes, but there is a variety of other packing materials commercially available, including phenyl-, cyanopropyl-, or aminopropyl modified materials. In addition, organopolymer materials such as polystyrene-divinylbenzene (PS-DVB) can be used as RPC materials; they have the advantage of higher alkali stability but often show less efficiency when compared with silica-based column packings (4). The characteristics of RPC packing materials are discussed in detail in Section 5.6.1.

Intermolecular interactions, which form the basis for specific retention and therefore separation, can be classified into the following groups:

- hydrophobic interactions
- hydrogen-bonding interactions
- ion-exchange interactions
- metal ion coordination interactions.

Depending on the type of solute, stationary, or mobile phase, one or several different interactions take place and are responsible for the observed retention.

5.2.2.1 Hydrophobic Interactions Hydrophobic interactions are mediated by polarized electron donor or electron acceptor processes (5). In an attempt to simplify the terminology, van der Waals, \( \pi \rightarrow \pi \) and \( \pi \rightarrow \text{dipole} \) interactions also belong to this group. All types of solutes will undergo hydrophobic interactions with an RPC stationary phase, and the densely bonded packing materials have plenty of interaction sites. The larger the solute, the more it is retained due to the larger hydrophobic interaction surface. As discussed earlier, retention can either be based on partitioning or adsorption, or in some cases, by a combination of the two. Retention is controlled by the elution strength of the mobile phase, which in the case of RPC comprises a mixture of water (or buffer) and a water-miscible organic solvent. Commonly used solvents include [in elutropic order (increasing strength)]

- methanol
- ethanol
- propanol
- acetonitrile
- tetrahydrofuran (THF).

From a strictly chromatographic point of view, acetonitrile is to be preferred because it has several favorable properties, such as low viscosity and low UV cut-off. The choice of organic modifier will be discussed in more detail in conjunction with optimization and scale-up in Sections 5.4.2.4 and 5.5.

Retention \( (k') \) becomes shorter with increasing elution strength of the mobile phase, in other words, with increasing content of organic modifier. In most cases, a linear relation between \( \log(k') \) and the percentage of organic modifier can be found (Fig. 5.2). The slope of the curve depends on the size of the solute. Although the retention for a small molecule will only depend marginally upon the percentage of organic modifier present in the mobile phase, an increase or decrease of only a few percent has a dramatic effect on the retention of larger molecules, such as peptides and proteins. As a consequence of this behavior, the separation of larger molecules often requires gradient elution, where the content of organic modifier is increased continuously during the separation. Gradient features will be discussed in detail in conjunction with method optimization (see Section 5.4.2.5).

5.2.2.2 Hydrogen-Bonding Interaction Hydrogen bonding occurs between residual silanol groups of the silica backbone and the solute (Fig. 5.3). Although most manufacturers...
of silica-based RPC packing materials attempt to minimize the existence of unreacted silanol groups by performing a so-called end-capping step with, for example, trimethylchlorosilane (C1), a certain number of silanol groups will always remain available for hydrogen-bonding interactions.

The hydrogen-bonding interaction sites are few in number compared to hydrophobic interaction sites, but the bonding energy is much higher. Just a few strong interaction sites can lead to tailed peaks. A few solute molecules can form hydrogen bonds with the silanol groups, whereas solutes like proteins primarily undergo hydrophobic interactions with the stationary phase. The two different interaction mechanisms contribute with different partial retentions to the overall retention (Fig. 5.4). Such behavior is commonly seen for low molecular weight, basic solutes such as amines and some peptides. The extent of tailing depends on ligand density, end-capping, acidity of the silica matrix, pH, buffer type, and concentration (6). Generally, tailing is minimal for materials based on high purity silica, or materials where the silica backbone is modified by introducing organic functional groups (7). However, it is important to mention that hydrogen-bonding interactions are also beneficial. In many cases, small differences in the ability to undergo hydrogen-bonding interactions allow first separation of complex mixtures of peptides, for example.

5.2.2.3 Ion-Exchange Interactions Ion-exchange (IEX) interactions occur between deprotonated residual silanol groups and protonated solutes and can be classified as very strong interactions (IEX > H-bond > hydrophobic interaction). Silanol groups undergo deprotonation between pH 3 and pH 8 (8), depending on their vicinity. Isolated silanol groups are most acidic, and vicinal or geminal groups are less acidic. Furthermore, the acidity also depends on the purity of the silica backbone. Metal impurities will enhance the acidity of the silanol groups and cause them to deprotonate at lower pH than in the case of highly pure silica material (9). IEX interactions can be avoided in a similar manner as hydrogen-bonding interactions. Simply by choosing an acidic mobile-phase pH, IEX interactions can be circumvented. An example of IEX interaction between lysine and a deprotonated silanol group is shown in Figure 5.5.

5.2.2.4 Influence of pH The pH of the mobile phase is probably the most important tool when dealing with the separation of ionic or ionizable compounds. Depending on pH, they are either positively or negatively charged, or exist in an uncharged form. Pronounced changes in retention and band spacing, and therefore loading capacity, can be expected by varying the pH in the region pH = pK_a ± 1 where protonation/deprotonation takes place. Consequently, the buffer used in the mobile phase should have a pH separated by at least one unit from the pK_a of the compound of interest. Depending on the charge, they are more or less prone to undergo any of the above-described interactions with the RP stationary phase. Furthermore, the residual silanol

![Figure 5.4](image-url) Peak tailing (solid line) caused by unwanted ion-exchange interactions. Individual contributions are shown as dotted lines.
groups from the silica backbone also change their degree in protonation with pH. The importance of protonation/deprotonation is also maintained for amphotheric compounds, such as amino acids and consequently peptides and proteins. Under strong acid conditions (below $pK_a$), the amino acid is positively charged. Between $pK_1$ and $pK_2$, it exists as zwitterions. Above $pK_2$ it exists as an anion ($pK_1 < 6$), whereas basic amino acids (Lys, Arg) react weakly alkaline ($pK_2 = 7.5–10.7$) and acidic ones (Asp, Glu) weakly acidic ($pK_3 = 3–3.5$) (10).

Figure 5.7 shows the effect of a pH shift by one unit on the preparative separation of a 39-amino-acid peptide. Although the peptide with an isoelectric point $pI = 4.3$ exhibits the same degree of protonation at both pH 6 and 7, the residual silanol groups of the stationary phase are significantly more deprotonated at pH 7, thus enabling IEX interactions with the peptide. The addition of the IEX interactions to the separation leads to a different retention pattern for the target peptide and its impurities. By increasing the pH of the buffer from 6 to 7, recovery could be increased from 77% to 91%.

The implication of this behavior is that peptides behave very differently when separated at acid or basic pH values. The pH region around the isoelectric point is commonly avoided, as peptides there show poor solubility in aqueous systems, and pH values above $pK_2$ are usually such that the stability of the peptide is at risk. Consequently, peptides are most commonly separated at acidic pH $pH < pK_1$.

### 5.2.2.5 Influence of Buffer Concentration

Because pH plays a decisive role in the RPC of peptides and other charged species, buffers or other additives (acids) are commonly used in the mobile phase. The buffer acts in several ways, as described in the following.

#### 1. Buffering a Certain pH Value

The purpose of a buffer is to counteract pH fluctuations that will occur if an acid or base is added to an aqueous system (injection of an acid or base into the mobile-phase stream). Buffer capacity $\beta$ is a function of pH, concentration, and $pK_a$ of the weak acid (11). The buffer capacity reaches its maximum at $pH = pK_a$ and is individual for different buffer systems. Although sodium acetate is an appropriate buffer at $pH 3.6–4.9$ ($pK_a$ of acetic acid) = 4.75, it will exhibit poor buffer capacity at, for example, pH 6, but the opposite is valid for sodium bicarbonate ($pK_a = 6.37$). The higher the concentration of buffer, the higher the buffer capacity. As the buffer has to be miscible with the organic modifier, the concentration level is often limited by solubility aspects. Double peaks in preparative RPC are often a sign of insufficient buffer capacity. The solute elutes in two different forms due to nonuniform protonation.

#### 2. Blocking of Silanol Groups

The cations of the buffer salt undergo ion-pairing interactions with deprotonated silanol groups, and consequently suppress
such interactions between cationic solutes and the stationary phase due to numeric excess.

3. Ion-Pairing Agents

Highly polar ionic solutes, such as oligonucleotides or small peptides, are often difficult to retain under reversed-phase conditions, because they are unable to undergo sufficient hydrophobic interactions with the stationary phase. In this case, hydrophobic ion-pairing agents such as tetrabutylammonium acetate (TBA-Ac) can be used. The ionic solute forms a hydrophobic ion pair with the TBA-Ac (Fig. 5.8), which is much more retentive than the native solute.

5.2.3 RPC of Polypeptides

RPC is widely used for the separation and purification of peptides and proteins. The technique shows a much higher resolution power than IEX or size-exclusion chromatography (SEC). By means of RPC, polypeptides differing in only one CH₂ group of a side chain of one amino acid can be separated (e.g., human and rabbit insulin, serine for human insulin threonine for rabbit insulin) (12). RPC shows this resolving power both for small peptides of a few amino acids and for large proteins. The separation is based on subtle differences in the so-called hydrophobic foot of the peptide or protein molecule. Differences in the hydrophobic foot not only result from differences in the primary amino acid sequences, but also from differences in the conformation (secondary and tertiary structure) (12). The retention of polypeptides is controlled by the sensitivity these macromolecules show towards the concentration of organic modifier in the mobile phase. The concentration window within which the polypeptide desorbs from the stationary phase is extremely narrow (the larger the polypeptide, the narrower the window). This characteristic can be exploited by applying gradient elution, where each of the different polypeptides elutes within its specific concentration window. The sudden desorption produces sharp peaks that enhance the high resolution character of RPC. Although isocratic separations are known to produce a higher resolution than gradient elution in the case of small- to medium-sized analytes, isocratic elution of larger polypeptides will produce very broad peaks due to the slow diffusion process.

The elution mechanism is greatly affected, not only by the organic modifier concentration, but also by the pH and ion-pairing agent present in the mobile phase. The use of ion-pairing agents such as TFA (trifluoro-acetic acid) or different buffers, enable RP separations of very polar peptides due to their ion-pairing character.

5.3 DEVELOPMENT AND OPTIMIZATION OF ANALYTICAL SEPARATIONS

5.3.1 Theoretical Approach

The goal of analytical HPLC is to separate components of a mixture under such conditions that a satisfactory quantitative analysis can be obtained at a reasonable time and cost (13). The parameter to be maximized is accordingly the amount of information gained per unit of time. The costs are dominated by labor, so analysis time is an important parameter to consider when optimizing analytical HPLC separations.

The performance of a separation is commonly judged by the resolution \( R \) between the eluting peaks. Resolution correlates to the amount of information obtained by a separation, so the optimizing parameter can be defined as follows:

\[
\text{Conditions}_{\text{opt}} = \left( \frac{R}{t} \right)_{\text{max}}. \quad (5.1)
\]

It must be emphasized that an optimization can only be carried out seriously with known mixtures, where all components are identified. In the case of unknown sample mixtures, the analyst can only roughly estimate the resolving power of an HPLC separation.

Resolution is a function of three different factors: (a) efficiency \( N \), which expresses the width of the eluting bands, (b) selectivity \( \alpha \), which is a measure of how far apart the two considered compounds are eluting from each other, and (c) the relative retention \( k' \) of the later eluting peak, which describes the magnitude of retention:

\[
k' = \frac{t_R - t_0}{t_0}, \quad (5.2)
\]

\[
\alpha = \frac{k'_2}{k'_1}, \quad (5.3)
\]

\[
N = 5.54 \cdot \left( \frac{t_R}{w_{1/2}} \right)^2, \quad (5.4)
\]

\[
R = \frac{\sqrt{N}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k'_2}{1 + k'_2}, \quad (5.5)
\]

Maximizing resolution is often the first goal in the development of an analytical separation method (14). The efficiency is determined by the column parameters (particle
size) and operating conditions, such as flow rate and mobile-phase viscosity. However, some substances might show hindered diffusion into the porous system of the stationary phase (mass transport problem, see Section 5.3.1.1) or undergo interactions that lead to tailed peaks (see Sections 5.2.2.2 and 5.2.2.3). The relative retention $k'$ can be controlled by adjusting the elution strength of the mobile phase. Selectivity $\alpha$, however, is not as easy to control as the other two parameters, as it is defined by the mobile-phase and stationary-phase conditions. Nevertheless, maximizing $\alpha$ is a prerequisite for any chromatographic optimization. Although a poor $\alpha$ can to some extent be compensated by an increased $N$ or $k'_2$, the practical range of $N$ and $k'_2$ are limited (column length, particle size, analysis time) and the requirement for an acceptable $\alpha$ thus remains. It is subsequently of the utmost importance to find a combination of mobile and stationary phase that provides a high selectivity $\alpha$ for the concerned components.

### 5.3.1.1 Column Efficiency, Flow Velocity, and Pressure

Assuming that a chromatographic system (mobile and stationary phase) is chosen, $\alpha$ and $k'$ are fixed parameters. To optimize the separation with respect to resolution, $N$ has to be maximized for optimal resolution, and the flow velocity has to be increased for minimum analysis time according to Equation 5.1 (this applies to isocratic separations only). On the other hand, if one has a required value for resolution, the required theoretical plates can be calculated as follows:

$$ N_{eq} = 16 R^2 \left( \frac{\alpha}{\alpha - 1} \right)^2 \left( \frac{1 + k'_2}{k'_2} \right)^2. \quad (5.6) $$

The number of theoretical plates can be obtained in several ways, and is affected by changing column parameters such as length ($L$), particle diameter ($d_p$), and flow velocity ($F$). For columns of standard length (100 mm $< L < 500$ mm), the measured plate number of the column is proportional to its length. For very short columns ($L < 100$ mm), extra-column volumes can have a negative effect on column efficiency. The relation between plate number and column lengths is defined by HETP (height equivalent to a theoretical plate) which is abbreviated as $H$:

$$ H = \frac{L}{N}. \quad (5.7) $$

Furthermore, $N$ is a function of the flow velocity $F$ (mL/min) or the linear velocity $u$ (cm/sec). The van Deemter equation (Eq. 5.9) is widely used to characterize band broadening in a chromatographic column as a function of the linear velocity (15). The linear velocity $u$ is a function of the column cross-section and the total porosity of the packed bed:

$$ u = \frac{4 \cdot F}{d_p^2 \cdot \Pi \cdot \epsilon_{total}}. \quad (5.8) $$

If $A$, $B$, and $C$ are constants, $H$ describes the efficiency of the column according to the HETP equation:

$$ H = A + \frac{B}{u} + C \cdot u. \quad (5.9) $$

Constant $A$ describes the so-called eddy diffusion and reflects the quality of the packing. For a homogeneously packed bed, $A$ has a value of $\sim 1$, whereas for poorly packed beds, $A$ can be as high as 3 (16). Eddy diffusion describes the band broadening caused by the different path lengths the mobile phase has to pass on its way through the packed bed. Eddy diffusion is independent of the flow velocity.

The longitudinal diffusion is described by constant $B$. Band broadening due to longitudinal diffusion decreases with increasing flow velocity and only has significance at very low flow rates (below those applied in HPLC). It can be concluded that under normal circumstances, neither parameter $A$ nor $B$ are of importance in HPLC. The only constant to be aware of is $C$, which includes contributions from the binding kinetics as well as mass transfer (15). Thus, the van Deemter equation can be simplified to

$$ H = C \cdot u. \quad (5.10) $$

Peptides and proteins are more sensitive to changes in the flow rate than small molecules due to their significantly lower molecular diffusivity (5). Thus, the $C$ term for a protein is much higher than when the same packing material is tested with a small molecule. Figure 5.9 illustrates the contributions of parameters $A$, $B$, and $C$. As can be seen, $H$ reaches a minimum at a certain optimal linear velocity $u$. Working with $u_{opt}$ as the flow rate will give the highest possible efficiency for a certain column.

Mass transfer from the mobile to the stationary phase can only occur by diffusion. Subsequently, the diffusivity $D_m$ is of importance for the mass transfer, which is equivalent to

![Figure 5.9](image-url)  
*Figure 5.9*  Contributions of parameters $A$ (dashed line), $B$ (dash-dotted line), and $C$ (long-dashed line) to the van Deemter curve. Minimal plate height $H$ is obtained at the optima linear flow rate $u_{opt}$. 
a low value for \( C \). Diffusivity is a function of the viscosity of the mobile phase, temperature, the molecular volume of the solute, and is approximated by the Wilke–Chang equation (17). Furthermore, the particle diameter \( d_p \) is decisive for mass transfer. The smaller the particle, the shorter the longest possible diffusion path a solute has to pass in order to reach the farthest interaction site. The average time necessary for a molecule to diffuse across the distance \( d_p \) is \( d_p^2/2D_m \). Values for \( C \) should be \(<0.1\), and packing materials where \( C \) exceeds 0.1 should be avoided (18).

In order to compare results obtained from otherwise similar chromatographic systems, in which only the particle size and the diffusivity of the solute vary, Knox introduced a dimensionless form of the van Deemter equation:

\[
h = \left( \frac{B'}{v} \right) + A \cdot v^{1/3} + C \cdot v,
\]

where \( h = H/d_p \) and \( v = u \cdot d_p/D_m \). Plots of reduced plate height \( h \) versus reduced velocity \( v \) should result in identical curves for data obtained from identical packing material of different size fractions.

When pumping mobile phase through a particular bed, a pressure drop \( \Delta P \) will occur over the column. The relationship between particle diameter \( d_p \) and the flow velocity \( u \) is given by Darcy’s law for noncompressible solvents:

\[
\Delta P = \frac{u \cdot L \cdot \eta \cdot K_p}{k_0 \cdot d_p^2} \quad (5.12)
\]

Accordingly, \( \Delta P \) is proportional to the linear velocity \( u \), column length \( L \), and the viscosity of the mobile phase \( \eta \), and inversely proportional to constant \( k_0 \) and the square of the particle diameter \( d_p \). Constant \( k_0 \) is a function of the interstitial porosity \( e_i \), according to the Karman–Cozeny equation:

\[
k_0 = \frac{e_i^3}{180(1 - e_i)^2} \quad (5.13)
\]

Equation 5.13 shows that the interstitial porosity \( e_i \) has a tremendous influence on the permeability of the bed and therefore also on the pressure drop. For well-packed RPC columns, the interstitial porosity can be assumed to be 0.33, which leads to \( k_0 = 4.45 \times 10^{-4} \). In practice, the column permeability \( (d_p^2/k_0) \) depends entirely upon the particle diameter, the particle size distribution, and the nature of the surface modification. The latter is a very important parameter, because the particles of an HPLC packing material are never monodisperse. The particle size distribution is discussed in detail in Section 5.6.1.1. However, it can be mentioned that a broad distribution has two negative effects: (a) the largest particles control the efficiency, and (b) the smallest particles control the pressure drop. Consequently, broad particle size distributions should be avoided.

In conclusion, four important features from the van Deemter equation and from Darcy’s law should be emphasized:

1. There is an optimum flow velocity at which the plate height is minimal and the efficiency maximum.
2. Column efficiency is inversely proportional to the particle diameter.
3. The flow velocity at which column efficiency is maximal is also inversely proportional to the particle diameter.
4. The obtained pressure drop over the column is proportional to the flow velocity and inversely proportional to the square of the particle diameter.

5.3.2 Practical Approach

This chapter describes how to proceed with an analytical method development task. Method validation and other issues that are of major concern for quality control (QC) or quality assurance (QA) related problems are not covered.

Method development/optimization of an analytical HPLC method requires detailed knowledge of the sample mixtures. The scope of the separation is either to identify and/or quantify one or more components.

Identification of a compound takes place by comparing the retention time of a standard with that of the peak in question of an unknown sample. Correct peak identification is a prerequisite for the quantification of a compound in an unknown mixture. The compounds of interest have to be properly resolved from other peaks in order to quantify the peak area correctly. Quantification is then conducted by virtue of a calibration curve based on standards. The relative comparison of the quantity of several compounds by means of UV adsorption is only an option if the compounds have identical or at least similar extinction coefficients \( \varepsilon \), as this parameter correlates absorbance (Abs) with concentration \( c \) according to the Beer–Lambert Law (\( d \), optical path length):

\[
c = \frac{\text{Abs}}{\varepsilon \cdot d} \quad (5.14)
\]

Subsequently, calibration with either injections of known amounts of standard compound(s) or calibration with another analytical method is required if correct quantification is the objective.

5.3.2.1 Scope of the Separation Before starting any experimental work, one should have a clear picture of the type of sample and analysis one is dealing with. The flow chart in Figure 5.10 shows how the correct method of peak identification is chosen. If pure compounds are available and can be used as reference standards, this is to be
5.3 DEVELOPMENT AND OPTIMIZATION OF ANALYTICAL SEPARATIONS

Figure 5.10 Flow chart for choosing the right method of peak identification.

Figure 5.11 Flow chart for development/optimization of analytical separations of smaller peptides.
preferred, but mass spectrometry (MS) detection otherwise offers a solution.

5.3.2.2 Method Development: Small Peptides A general approach to method development is shown in the flow chart in Figure 5.11. A 3.5- or 5-μm C18 column is the backbone of the method optimization. Most analytical RPC separation problems can be solved by means of a C18 column. Nevertheless, testing other stationary phases, such as C8 or C4 modified phases, at the end of the method development might lead to a further improvement of the method. For highly polar analytes alone, which require a mobile phase with less than 5% organic modifier for decent retention, C18 phases are not recommended, as they are at risk of undergoing so-called phase collapse or de-wetting (see Section 5.2.4). In these cases, the use of less hydrophobic C4 or phenyl-modified phases is recommended. The key parameter in method development is, however, the mobile phase.

Buffered mobile phases should be used whenever charged analytes are to be separated. The correct elution strength (1 < k’ < 5) is determined by means of a steep screening gradient. For molecules with a molecular weight below 1000 g/mol, isocratic elution mode is recommended for best resolution. For large analytes, gradients will often prove to be the best alternative. Either a step gradient or a continuous gradient can be applied (Fig. 5.12). The steeper the gradient slope, the sharper the peaks will elute, but at the cost of resolution. Often a gradient of 0.05–0.3% organic modifier per minute gives the best results.

In the case of isocratic separations, the flow rate should be a compromise of short analysis time and high efficiency. By using packing material of small particle size (3 μm, 5 μm), relatively high flow rates (1.5–2.0 mL/min; column i.d., 4.6 mm) can be applied without losing efficiency. When gradients are applied, the flow rate is not controlling the analysis time in the same manner as for isocratic separations, as it is mainly the content of organic modifier that is responsible for the retention of the compounds. It is therefore advisable to use optimal flow rates for isocratic separations (see Section 5.5.1.1). For larger peptides and proteins, the optimal flow rates often become very low (e.g., 0.2 mL/min; column i.d., 4.6 mm) due to low diffusivity caused by molecule size.

![Figure 5.12](image)

Figure 5.12 (a) Step gradient (dashed line) and (b) linear gradient combined with an initial concentration and a final washing step (solid line).

5.3.2.3 Method Development: Proteins Minor polypeptides (<12 amino acids) can be separated according to the guidelines given previously for small peptides. By increasing the size of the polypeptide, small differences in the organic modifier concentration will cause quite significant differences in retention times. Consequently, to assure reproducible retention times, it is advisable to use a very shallow gradient (0.02% organic modifier per minute) in these cases.

For many pharmaceutical polypeptides and proteins, RPC methods are described in the US or European pharmacopoeia. Larger polypeptides are best separated using a 300 Å pore stationary phase, although polypeptides with a molecular weight below 5 kDa often show good mass transport properties with smaller (100 Å) pore packing material. Traditionally, polypeptides are often analyzed using a 300 Å C4 packing material in combination with a mobile phase based on water/acetonitrile +0.1% TFA. TFA has several advantages: it does not interfere with UV detection, it masks residual silanol groups, and it is an excellent ion-pairing agent for basic polypeptides. For other polypeptides, there is a wide range of other buffers and additives. Perchlorate, ammonium acetate, phosphate, formic acid, acetic acid, or triethylamine often lead to better peak shapes and selectivity values than TFA-based separations (19, 20). More hydrophobic packing materials are often a better choice than C4 materials for smaller polypeptides. On the other hand, larger polypeptides can suffer from contact with a highly hydrophobic surface such as a C18 modified packing material. With increasing contact time between the protein and the hydrophobic surface and/or organic modifier, there is a risk that the protein can undergo defolding, which can be reversible or irreversible. Irreversible defolding can result in loss of biological activity, which is mainly a problem in preparative liquid chromatography (LC).

Figure 5.13 describes a simple guideline for method development of analytical polypeptide separations. By means of four mobile phase systems and two to three columns, an acceptable separation should be achieved. For small polypeptides (<5 kDa), a C18 modified column is the best choice to start with; for larger polypeptides, a C4 modified packing material is to be preferred instead. Furthermore, packing materials based on monomeric silanation differ from those based on polymeric modification (see Section 5.6). Thus,
when screening columns from different manufacturers, it is advisable to look at both monomeric and polymeric modified products. Fine-tuning of the mobile-phase pH can further improve a separation, as well as testing buffers other than phosphates. The effect of temperature, however, is minute for polypeptides. What can often be observed is a slight increase in retention upon an increase in temperature due to unfolding (5).

5.4 DEVELOPMENT AND OPTIMIZATION OF PREPARATIVE SEPARATIONS

Preparative RPC is a process predominantly used for the purification of costly pharmaceutical products. Both the isolation of microgram quantities of a certain enzyme and the purification of ton quantities of, for example, insulin, are preparative applications. Subsequently, it is the objective of the separation and not the size of the column that divides preparative from analytical RPC.

The scope of any method development activity is a process that is able to fulfill the specifications set for the separation in question. Commonly, the specifications cover purity, recovery, and to some extent productivity. The scaffold of a method is the choice of stationary phase and mobile phase. Operational parameters, such as flow rate, column length, and gradient regime, are often chosen based on experience or technical limitations. Economic aspects play a subordinate role in the early part of method development. The goal is often defined by being able to purify a certain amount of material until a certain date. Time is precious and therefore the time spent on method development is minimized. This scenario is typical for small-scale preparative applications, for example, in the early stage of a development project.
Every method optimization is preceded by a method development. Optimization of the separation process is generally required when the purification step is taken to the industrial scale. However, even at a smaller scale, it might be worthwhile to extend method development work in order to improve, for example, yield or productivity. Parameters to be optimized are column length, flow rate, relative load, and particle size.

When industrial chromatographic processes are optimized, the ultimate goal is to minimize the purification costs ($/g_{purified}$). In this case, cost factors such as labor, solvent, equipment, lost crude (if yield is <100%), and packing material are taken into consideration. The optimization process is generally a combination of experimental work and computation.

Method development and method optimization are complementary activities. Although method development is usually required, further optimization of a method is time-consuming and not justifiable in many cases. For large industrial applications, elaborate method optimization is always required in order to reduce the operational costs to the largest possible extent. For small-scale preparative applications, there is often only limited time (and crude material) available, and method development has to be kept at a minimum, accepting suboptimal conditions. Figure 5.14 shows how the operational costs of an arbitrary separation are reduced with increasing time spent on method development. However, there is a time optimum, and any excessive time spent for optimization will not yield any further reduction in operational costs.

5.4.1 Basics in Preparative RPC

The general goal in preparative separations is achieving maximum throughput (g/h) within purity and yield constraints. Consequently, optimization in preparative RPC means looking for conditions that provide maximum relative loading (crude protein/kg or L packing) that renders products within the specified purity. Highest throughput will always be obtained under overloaded conditions, which are defined by operation in the nonlinear part of the adsorption isotherm (Fig. 5.15). Understanding of the features and consequences of overloaded conditions are therefore a prerequisite for successful development of preparative separation methods.

5.4.1.1 Volume Overload Versus Concentration Overload

A column can be overloaded in two ways: by volume or by concentration. Figure 5.16 shows examples of peak migration for analytical, volume–overloaded, and concentration–overloaded injections.

In the case of volume overloading, the volume $V_o$ of the injected sample of the mass $m_i$ is so large that the eluted peaks are significantly broader than from an analytical injection of the same amount $m_i$ injected in a smaller volume $V_A$. Purely volume-overloaded peaks appear flat-topped but symmetrical (not to be confused with a flat-topped peak due to saturation of the detector signal). As the solute concentration is low, one operates within the linear part of the adsorption isotherm, despite the broad peak profile.

In the case of concentration-overloaded injections, one operates outside the linear range of the adsorption isotherm, although the injection volume might be very small. Concentration-overloaded peaks typically elute in a triangular shape.

Pure volume overload is seldom encountered within preparative HPLC, but quite often coexists together with concentration overload. Knox and colleagues (21) show that the
final eluted peak profile under concentration-overloaded conditions for a given quantity of solute is independent of the sample volume up to a critical volume \( V_{C3} \), whereas \( V_{C3} \) is approximately half of the volume of the peak arising from a small volume injection. It is advisable to exploit the critical injection volume \( V_{C3} \) to obtain an injection solution that does not also excessively differ from the mobile phase with respect to viscosity.

In the case of a highly concentrated injection sample, the viscous plug of the injection solution will only slowly adsorb onto the stationary phase, which can lead to a severely tailed elution peak.

5.4.1.2 Isotherms and Peak Shape  Depending on the isotherm, the overloaded peak appears in different shapes (Fig. 5.17). Compounds can adsorb according to several types of isotherms, and in the case of a separation, the different compounds can compete for a certain interaction site, leading to competitive adsorption isotherms. The most commonly observed isotherms are the Langmuirian, anti-Langmuirian, and S-shaped isotherms.

In the case of a Langmuirian isotherm, the end of the overloaded peak coincides with the end of the analytical
peak, and the front propagates towards shorter retention times and becomes steeper with increasing load. For an anti-Langmuirian isotherm, the opposite is the case: the front of the overloaded peak coincides with the analytical peak, and the end of the overloaded peak propagates towards longer retention times and becomes steeper with increasing load. For S-shaped isotherms, the peak shape initially changes according to an anti-Langmuirian isotherm, and after passing the inflection point of the isotherm, the overloaded peak propagates according to a Langmuirian isotherm.

5.4.1.3 Touching Bands and Displacement Effects In preparative RPC, conditions are sought that provide the highest possible throughput, if purification costs are ignored. Often, lowest costs are obtained at the highest possible throughput. The throughput is defined by the injected amount of sample, the cycle time, and the yield of separation:

\[ P = \frac{m_{\text{inj}}}{t_{\text{cycle}}} \cdot \text{yield} \]  

(5.15)

As Equation 5.15 shows, a given throughput \( P \) can be achieved with different values for the injected amount of sample \( m_{\text{inj}} \), cycle time \( t_{\text{cycle}} \), and yield. The examples below illustrate how a throughput of 2.5 g/min can be achieved either by increasing the injected amount and sacrificing yield (Example 2) or by decreasing the cycle time and sacrificing yield (Example 3).

**Example 1**

\[
\begin{align*}
50 \text{ g} & \quad 20 \text{ min} & 100\% & = 2.5 \text{ g/min} \\
\end{align*}
\]

**Example 2**

\[
\begin{align*}
58.8 \text{ g} & \quad 20 \text{ min} & 85\% & = 2.5 \text{ g/min} \\
\end{align*}
\]

**Example 3**

\[
\begin{align*}
50 \text{ g} & \quad 17 \text{ min} & 85\% & = 2.5 \text{ g/min} \\
\end{align*}
\]

Preparative separations that produce yields of 100% (Example 1) are commonly called touching-band separations (Fig. 5.18). The column is loaded with feed until the product peak and the closest eluting impurity peak touch each other at the baseline.

Displacement effects can produce better separations than expected because they move the first peak to shorter retention (Fig. 5.19). Owing to the displacement effect, the overlap between the two blue peaks becomes smaller in chromatogram (b).

At high loads, the thermodynamics of the chromatographic system are modified by the sample. The retention behavior of a particular compound is modified by the presence of other compounds in the injection mixture. These mutual interactions between compounds can generate displacement effects. These effects are not always possible to achieve, and depend upon the relative adsorption isotherms.

The downside of a displacement effect is a so-called tag-along effect (Fig. 5.20). In this situation, the less concentrated compound elutes after the main compound. Instead of being compressed, the less concentrated compound is tagged along the first peak, contaminating the main compound.

5.4.1.4 Purity, Yield, Throughput, and Cost Preparative separations are commonly governed by constraints that are related to the factors that are seldom considered to be of importance within analytical HPLC. Sample stability and
solubility, cost and handling of large volumes of mobile phase, and the availability of stationary phase in multi-kilogram quantities are just some of the issues that can be named. The goal of any preparative run is to produce a maximum amount of product with a certain purity in the shortest possible time and/or at the lowest cost $/\text{g} \text{ purified product}.$

Purity, yield, and throughput are interlinked factors. At very low loading (analytical injection), both high purity and high yield can be obtained at the cost of low throughput (Fig. 5.21a). At high loading, either high purity or high recovery can be obtained (Fig. 5.21b and c). The core aim of preparative HPLC optimization is to balance these factors in such a way that the required purity is obtained at the highest possible throughput.

How these factors are weighted against each other depends on the type of preparative separation. While at the early stage of development work, high yield might be mandatory due to shortage of material, lowest possible costs will certainly be the dominating aspect in the implementation of an industrial preparative HPLC process.

The total cost of preparative RPC can be broken down into the following elements:

- equipment costs, including column hardware, control systems, solvent pumps, detection, fraction collection, electricity, maintenance
- the mobile phase, including buffers, organic solvents, solvent recovery
- the stationary phase, including packing material (cost will be highly dependent on the lifetime of the packing material)
- lost crude (unless the yield is 100%, a cost has to be allocated for the lost crude or the reprocessing of it as side fractions)
- labor, in other words, the personnel required to operate the purification process
- mobile phase removal (evaporation, lyophilization, or precipitation as required to isolate the purified product)
- quality control (purity assay).

On an industrial scale, the total costs are generally dominated by the mobile-phase costs (Fig. 5.22a). Thus, with a reduction of the mobile-phase costs by, for example, reduced consumption (L/\text{g} \text{ purified product}), and/or the exchange of an expensive solvent (acetonitrile) with a cheaper solvent (methanol), the total purification costs can be reduced drastically, whereas a
reduction of the personnel or stationary phase costs will hardly affect the overall costs.

The cost breakdown for a laboratory-scale preparative RPC separation, however, looks different (Fig. 5.22b). There, the relative personnel costs exceed the mobile-phase costs, and reducing the operational time or increasing the degree of atomization will reduce the total purification costs in an effective way.

Optimization of the purification costs can be accomplished by means of computerized optimization programs (22). If all cost elements and boundaries are known, optimization programs can optimize operational parameters such as relative load, column length, and flow rate against lowest total costs (23).

Even if computerized optimization of a preparative RPC separation is not always necessary, the costs can be reduced by a few simple choices:

- Selection of Mobile Phase  Alcohols such as methanol, ethanol and propanol are considerably cheaper than acetonitrile, but they will cause up to three times higher backpressure, which can affect the maximum flow rate and consequently throughput. The ease of removal of the mobile phase from the purified product should also be considered. Can the mobile phase including buffer salts simply be evaporated at a moderate temperature or is a desalting step necessary?

- Lost Crude  Before method development, decide how much loss of material can be accepted and set a minimum recovery.

- Stationary Phase  A long-lasting packing material is not only beneficial from a purchasing point of view; column unpacking/packing is time-consuming. Moreover, the packing material should withstand moderate regeneration procedures in order to remove strongly adsorbed species and therewith prolong the service life of the packing material. See Sections 5.6.3 to 5.6.5.

5.4.2 Method Development for Preparative Separations

The scope of the method development process is to provide a method, consisting of packing material, mobile phase, flow rate, and relative loading, that provides separation results (purity, yield) fulfilling the set specifications. Although, in principle, all preparative separations start as analytical separations, it is important to be aware of the different requirements for these two types of separations. As can be seen in the comparison in Table 5.1, the requirements for analytical and preparative separations have little in common. It is thus clear that a simple scale-up of a method that was developed for analytical purpose will hardly render an appropriate preparative RPC method. This applies for all preparative separations, independent of scale.

5.4.2.1 Considerations Concerning the Packing Material  The importance of the packing material is discussed in more detail in Section 5.6. Porous packing materials are defined by pore size and specific surface area. The

| TABLE 5.1 Comparison of the Focuses of Analytical and Preparative HPLC Separations |
|-----------------------------------------------|--------------------------|--------------------------|
| Goal                                         | Identification/          | Isolation/               |
|                                              | quantification           | purification             |
|                                              | of compounds             | of compounds             |
| Separation time                              | Very important           | Important                |
| Resolution of all components                 | Very important           | Not important            |
| Good detector response                       | Very important           | Not important            |
| Mobile phase costs                           | Not important            | (Very) important         |
| Environmental/safety aspects                 | Moderately important     | Very important           |
| Ease of further work-up                      | Not important            | Important                |
specific surface \((m^2/g)\) is reciprocal to the pore size. Average numbers are:

- \(80–120 \text{ Å} : 280–380 \text{ m}^2/\text{g}\)
- \(300 \text{ Å} : 80–110 \text{ m}^2/\text{g}\)

The higher the specific surface, the higher is the potential loading capacity. However, if the pore size is too small to allow unobstructed diffusion of the protein in and out of the porous system, then the accessible surface area, and therefore the loading capacity, will be less than expected.

For peptides with a molecular weight below 8000 Da, 100 Å pores usually result in the highest accessible surface area (see Section 5.6.1.2). For larger peptides and proteins, 300 Å pores are preferred.

The particle size controls the efficiency of the column, but also the backpressure. Increased efficiency with smaller particles results in higher resolution, while the selectivity obtained for a certain separation does not depend upon the particle size of the packing material. Consequently, if a method is developed with, for example, 5-µm packing material, the result of a large scale separation performed with 10-µm material can easily be predicted. However, this is only the case if both the 5- and 10-µm materials originate from the same manufacturing process, thus exhibiting identical surface properties.

The pressure drop is proportional to \(1/d_p^2\). Therefore, by reducing the particle size by a factor of 2, the pressure drop over the column will increase by a factor of 4. It is advisable to perform all method development with 10–15-µm material, as this particle size is commonly used in prep mode.

RPC packing materials are most often porous silica beads modified by either C4, C8, C18, or phenyl-silanes. The longer the chain length, the higher the observed hydrophobicity or retentivity. This is at least the case for small molecules, where the interaction mechanism is based on partitioning. As the molecular size of the analyte increases, the retention mechanism shifts from partitioning to adsorption/desorption, as briefly discussed in Section 5.2.1. The change is gradual; polypeptides > 1 kDa, however, are presumably only retained by adsorption.

In the case of an adsorption/desorption mechanism (where the analyte is adsorbed onto the packing material until the elution strength of the mobile phase reaches a critical value, whereupon the peptide desorbs and elutes), the chain length of the surface modification generally has no influence on the separation of polypeptides.

However, the chain length influences the actual pore diameter. Although C18 will reduce the actual pore diameter by \(2 \times 25 \text{ Å}\) (all-trans conformation), C4 will only reduce the diameter by \(2 \times 7 \text{ Å}\) (24). This might play an important role when using C4 modified material to start with; only for small peptides (<50 amino acids) should C18 be screened as well.

Some common organopolymer-based packings (e.g., polystyrene/divinylbenzene) exhibit an intrinsic hydrophobicity, and are therefore not modified with alkylsilanes (25).

### 5.4.2.2 Considerations Concerning the Mobile Phase

Mobile phase composition is the single most important parameter of an HPLC separation. Important factors include buffer type, buffer pH, buffer concentration, and to some extent, the organic modifier might also influence the outcome of the separation.

#### 5.4.2.3 Buffer

The buffer has to fulfill two tasks: it has to ensure that the peptide exists in a uniform protonation state, or it can act as a counterion or ion-pairing agent for both the peptide and the residual silanol groups for silica-based materials. Depending on the pH, and therefore the degree of protonation of the peptide and silanol groups, the retention of the peptide might vary significantly (see Section 5.2.2.4).

As a rule of thumb, one should always operate at least one pH unit from the isoelectric point of the peptide in order to assure a stable regime. It has to be emphasized that the pH of the buffer changes upon the addition of an organic modifier. Some examples are given in Table 5.2 (26).

#### 5.4.2.4 Organic Modifier

Generally, the type of organic modifier plays a subordinate role. The viscosity of the mobile phase is lowest with acetonitrile (Fig. 5.23) (27, 28).

### TABLE 5.2 Effect on pH by Mixing 0.1 M Buffer with Methanol (50/50 by Volume)

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH Before Mixing</th>
<th>pH After Mixing</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>9.94</td>
<td>9.83</td>
<td>−0.11</td>
</tr>
<tr>
<td>Sodium borate</td>
<td>9.93</td>
<td>10.17</td>
<td>+0.24</td>
</tr>
<tr>
<td>Sodium phosphate</td>
<td>10.00</td>
<td>10.65</td>
<td>+0.65</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>10.00</td>
<td>11.40</td>
<td>+1.40</td>
</tr>
</tbody>
</table>

---

**Figure 5.23** Viscosity of different mixtures of water with organic modifiers. Ethanol, methanol, and 2-propanol at 20°C and acetonitrile at 25°C.
28), which is advantageous both with respect to the observed backpressure in the system, and also the diffusivity (mass transfer). Ecological and economic aspects often make alcohols such as methanol, ethanol, and propanol the preferred choice, especially for large-scale preparative applications.

5.4.2.5 Gradient and Flow Rate

Gradients have a tremendous impact on both retention and resolution. Gradient elution enables the following features in a preparative separation:

- concentration step prior to elution → mandatory for large injection volumes
- elution of substances that vary greatly in their hydrophobicity within a reasonable time span
- reconditioning of the column.

A concentration step at the beginning of the separation is always recommended when dealing with preparative separations. Mobile phase composition is kept at a low content of organic modifier (5–10%) for approximately 2–5 minutes in order to ensure that the entire peptide load is adsorbed at the column inlet as a narrow band. If the injection solvent differs from the mobile phase, the concentration step should be prolonged, allowing the complete exchange of counterions. Furthermore, a high salt concentration that might exist after a previous IEX purification step has to be rinsed out before elution is commenced.

After a concentration step, elution can be initiated either by gradient elution or by one or several isocratic elution steps. Generally, small peptides (<10 amino acids) are best separated isocratically. The steeper the gradient, the weaker the resolution power of the separation of closely related species. For peptides larger than 10 amino acids, a gradient slope of 0.1–0.3% organic modifier/minute often leads to the best preparative separation results.

The elution time for preparative separations is often found to be optimal between 40 and 70 minutes, not taking the concentration step into account. After the target compound has eluted, the column should be reconditioned by increasing the organic modifier content to 70–90% for 2–3 column volumes, before re-equilibration to the initial condition is commenced.

Although the flow rate has a direct impact on the cycle time and therefore the productivity for isocratic separations, by applying gradient elution, this influence is weakened. However, during equilibration and reconditioning, the maximum flow rate should be applied (defined by the pressure limitations of the equipment). During elution, the optimal flow rate (Table 5.3), depending on particle size, will lead to the best possible efficiency.

5.4.2.6 Method Development

Based on the aspects discussed above, a method (stationary phase, mobile phase, gradient) will now be described, leading to a satisfactory separation of an analytical injection:

1. Select pore size (100 or 300 Å) depending on the molecular size.
2. Select particle size (10 μm is a good choice).
3. Select surface modification (C18 for peptides <5 kDa, C4 for larger ones).
4. Run screening gradient (10–70% MeCN/45 minutes at pH 2) to identify the approximate elution range.
5. Adjust the gradient to a slope of 0.1–0.3% (minimum) and judge the resolution under different pH values. Retention time of the main product should be 40–70 minutes. (The larger the peptide, the longer the required retention time.) It is advisable to probe three different pHs initially, pH 2, pH 4, and pH 8 by means of a 20 mM KH₂PO₄ buffer.
6. For the best pH value, test alternative buffers and/or organic modifier if the resolution is not complete (gradient might need parallel adjustment).

Examples of buffers are as follows:

pH 2–3: phosphates, glycine-HCl, Na-citrate
pH 4–5: acetates, KH-phthalate
pH 7–8: Tris, MOPS, HEPES, carbonates

**TABLE 5.3 Optimal Flow Rate (mL/min) for Columns of Different Internal Diameters (i.d.) and Packed with Different Particle-Sized Material**

<table>
<thead>
<tr>
<th>Particle Size, μm</th>
<th>4.6 mm i.d.</th>
<th>10 mm i.d.</th>
<th>20 mm i.d.</th>
<th>50 mm i.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.0</td>
<td>4.7</td>
<td>19</td>
<td>118</td>
</tr>
<tr>
<td>10</td>
<td>0.7</td>
<td>3.3</td>
<td>13</td>
<td>82</td>
</tr>
<tr>
<td>13</td>
<td>0.5</td>
<td>2.3</td>
<td>9.5</td>
<td>60</td>
</tr>
<tr>
<td>16</td>
<td>0.4</td>
<td>1.9</td>
<td>7.5</td>
<td>47</td>
</tr>
</tbody>
</table>

**Figure 5.24** Reconstructed elution profile from a preparative peptide separation. Purity and recovery values are calculated for fraction pools 5–10.
Once a satisfactory separation is achieved with an analytical injection, overloaded injections should be conducted to see if the chosen buffer system also works under overloaded conditions. Loading on a 4.6 × 250 mm column is gradually increased from 1 mg, to 5 mg, 10 mg, and 20 mg. From 20 mg, fractions can be collected and analyzed in order to judge the separation performance by purity and recovery results (Fig. 5.24).

### 5.5 SCALE-UP

Scale-up is the process that takes a method from a smaller scale to a larger scale without changing the properties and the outcome of the method. Consequently, all method development and optimizations should be completed on a small scale due to the lesser requirements for materials. Scale-up in preparative chromatography is often conducted in two steps. The method is first transferred from the laboratory scale to the pilot scale (e.g., i.d. column, 50 mm) and in a second step, the method is finally scaled up to the industrial scale.

Chromatography is perfectly scalable as long as the kinetic and dynamic properties are the same for both scales. This can be accomplished by maintaining a constant linear flow rate, temperature, relative loading, feed concentration, gradient, and bed length, as well as using identical packing materials (particle size, pore size, surface chemistry) and mobile phase. The productivity is thus increased by increasing column diameter (Fig. 5.25).

In doing so, the residence time of the product is kept constant. To ensure constant residence time, it is extremely important to keep the bed length constant during the scale-up (29). Based on the fact that the column is only scaled up in one dimension, there is only one scale-up factor to be considered, namely the cross-sectional ratio $r_2^2/r_1^2$:

$$\frac{V_2}{V_1} = \frac{r_2^2}{r_1^2}$$

(5.16)

The cross-section ratio is also used when calculating the loading and flow rate. In order to maintain the same relative loading and the same linear flow rate, Equations 5.17 and 5.18 are used:

$$\text{Load}_2 = \frac{\text{Load}_1 \cdot r_2^2}{r_1^2}$$

(5.17)

$$\text{Flow}_2 = \frac{\text{Flow}_1 \cdot r_2^2}{r_1^2}$$

(5.18)

In practice, however, the situation is often such that the final load is known based on productivity requirements, and a suitable column diameter is sought. In this case, Equation 5.17 has to be rearranged to Equation 5.18 as

$$r_2 = r_1 \cdot \sqrt{\frac{L_2}{L_1}}$$

(5.19)

Table 5.4 shows the scale-up of a peptide separation from laboratory scale (4.6 × 250 mm) to pilot scale (50 × 250 mm).

#### Table 5.4 Summary of a Two-Step Scale-Up of a Preparative RPC Peptide Separation

<table>
<thead>
<tr>
<th></th>
<th>Column diameter, mm</th>
<th>Column length, mm</th>
<th>Packing material</th>
<th>Packing density, g/mL</th>
<th>M (packing material), g</th>
<th>Flow rate, mL/min</th>
<th>M (crude), g</th>
<th>Relative load, g/g</th>
<th>Initial purity, %</th>
<th>Product purity, %</th>
<th>Recovery, %</th>
<th>Cycle time, h</th>
<th>Productivity, g&lt;sub&gt;crude&lt;/sub&gt;/h</th>
<th>Productivity, g&lt;sub&gt;crude&lt;/sub&gt;/(h * kg)</th>
<th>Productivity, g&lt;sub&gt;product&lt;/sub&gt;/h</th>
<th>Productivity, g&lt;sub&gt;product&lt;/sub&gt;/(h * kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column 1</td>
<td>4.6</td>
<td>250</td>
<td>KR100-10-C18</td>
<td>0.60</td>
<td>2.49</td>
<td>1.0</td>
<td>0.02</td>
<td>0.008</td>
<td>0.70</td>
<td>0.95</td>
<td>0.90</td>
<td>0.25</td>
<td>0.08</td>
<td>32.11</td>
<td>0.05</td>
<td>20.23</td>
</tr>
<tr>
<td>Column 2</td>
<td>50</td>
<td>250</td>
<td>KR100-10-C18</td>
<td>0.60</td>
<td>294.38</td>
<td>118</td>
<td>2.36</td>
<td>0.008</td>
<td>0.70</td>
<td>0.95</td>
<td>0.90</td>
<td>0.25</td>
<td>9.45</td>
<td>32.11</td>
<td>5.95</td>
<td>20.23</td>
</tr>
<tr>
<td>Column 3</td>
<td>300</td>
<td>250</td>
<td>KR100-10-C18</td>
<td>0.60</td>
<td>10597.50</td>
<td></td>
<td>85.07</td>
<td>0.008</td>
<td>0.70</td>
<td>0.95</td>
<td>0.90</td>
<td>0.25</td>
<td>340.26</td>
<td>32.11</td>
<td>214.37</td>
<td>20.23</td>
</tr>
</tbody>
</table>
and finally to plant scale (300 × 250 mm). As can be seen, many parameters are constant despite the differences in scale.

5.6 COLUMN PACKING MATERIAL

The column packing material can be regarded as the backbone of every chromatographic separation. Without a robust, chemically, and mechanically stable stationary phase, no reproducible chromatography can be achieved. The importance of the key features of the packing material is described below. This section covers only spherical packing materials. Small and rigid particles are a prerequisite for high performance applications, where high eluent flow rates and pressures are commonly applied. A list of commercially available RPC packing materials is presented in Table 5.5. Monolithic columns are not discussed in this section.

5.6.1 Particle Characteristics

The vast majority of RPC packing materials are based on totally porous particles. The following sections therefore deal with this material type. However, superficially porous particles have recently been introduced (e.g., Poroshell 300 SB-C18) and offer an interesting improvement for analytical separations of macromolecules. These spheres consist of a solid core covered by a porous shell that enables rapid separations of macromolecules without sacrificing column efficiency (30). The application of superficially porous material in preparative RPC has to be considered minimal, as the specific surface area, and thus the potential loading capacity, is significantly lower than for totally porous materials.

5.6.1.1 Particle Size and Particle Size Distribution

A narrow, and above all, consistent particle size distribution leads to columns with maximum efficiency and good bed stability. By having a narrow particle size distribution, high pack pressure due to low bed porosity is avoided. The particle size distribution is commonly defined by the d_{50}/d_{10} ratio (Fig. 5.26). A broad particle size distribution has two negative aspects: efficiency is defined by the largest particles, and the pressure drop is controlled by the smallest particles.

Particle size and particle size distribution are usually measured with the electrical sensing zone method. To obtain a true particle size, mass calibration is used to compensate for the porosity of the particles.

5.6.1.2 Pore Size: Accessible Surface Area

The correct pore size and pore morphology is crucial for an optimized separation. Although an overly large pore will always work for analytical separations, it will have a detrimental effect on preparative separations. The pore has to be large enough to allow unobstructed mass transfer in and out of the pore. On the other hand, large pores mean low specific surface areas according to Equation 5.20 (assuming cylindrical pores, specific surface area (SSA) in m²/g, pore volume (PV) in cm³/g and pore diameter (PD) in Å):

\[
SSA = \frac{40,000 \cdot PV}{PD} 
\]

The pore size and pore size distribution are generally measured by nitrogen adsorption. This method produces reliable results up to a pore size of 200–300 Å. For larger pores, mercury intrusion should be chosen to analyze the pore structure of a packing material. For high performance RPC, a narrow pore size distribution is a prerequisite. Small pores, so-called micropores (<50 Å), contribute to the total pore volume measured by nitrogen; however, they are hardly accessible for both the surface modification agents (C18-silanes) and most of the analytes. The accessible surface area available for interactions with the analytes is thus significantly below the nominal specific surface areas stated on a datasheet (Fig. 5.27).

The absence of micropores is a requirement for high performance packing materials, regardless of whether it is intended for analytical or preparative separations. Furthermore, it is also of the utmost importance that the pores are cylindrically shaped. So-called bottleneck pores (large pore with a small opening) will significantly impair the accessible surface area.

RPC packing materials with pore sizes of 85–120 Å are most common. They usually have surface areas between 280 m²/g (120 Å) and 400 m²/g (85 Å). Compounds that can be separated with such materials have molecular weights up to 6 kDa; however, depending on the conformation of, for example, a polypeptide, even larger molecules may undergo unobstructed mass transfer with a 100 Å material if the geometry of the pore is cylindrical. A packing material of 300 Å is generally used for polypeptides with a molecular weight exceeding 6 kDa (i.e., proteins). For analytical purposes, 300 Å pore-sized material might already be the best choice for peptides and proteins with lower molecular weights.

5.6.2 Surface Properties

The surface properties of a packing material are responsible for the interaction between the stationary phase and the analytes to be separated. Because the surface is responsible for the function of the packing material, it is probably the most important characteristic of a packing material. However, the surface properties not only control retention and selectivity, but are also a key factor regarding the chemical stability (see Section 5.6.3) of the packing material. The properties of a RP packing material are dependent both on the nature
<table>
<thead>
<tr>
<th>Packing</th>
<th>Manufacturer</th>
<th>Material Type</th>
<th>Pore Size</th>
<th>Particle Size</th>
<th>Bulk Availability</th>
<th>Website</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amberchrom™ XT</td>
<td>Rohm and Haas</td>
<td>Polystyrene/divinylbenzene</td>
<td>300 Å</td>
<td>20 μm 30 μm</td>
<td>Yes</td>
<td><a href="http://www.amberchrome.com">www.amberchrome.com</a></td>
</tr>
<tr>
<td>DAISOGEL–SP-BIO</td>
<td>Daiso Co., Ltd</td>
<td>Silica, C4, C8, C18 modified</td>
<td>120 Å 200 Å</td>
<td>5 μm 10 μm</td>
<td>Yes</td>
<td><a href="http://www.daiso-co.com">www.daiso-co.com</a></td>
</tr>
<tr>
<td>DAISOGEL ODS-AP</td>
<td>Daiso Co., Ltd</td>
<td>Silica, C18 modified</td>
<td>120 Å 200 Å</td>
<td>10 μm 15 μm</td>
<td>Yes</td>
<td><a href="http://www.daiso-co.com">www.daiso-co.com</a></td>
</tr>
<tr>
<td>Luna™</td>
<td>Phenomenex</td>
<td>Silica, C8, C18, Phenyl modified</td>
<td>100 Å</td>
<td>3 μm 5 μm</td>
<td>Yes</td>
<td><a href="http://www.phenomenex.com">www.phenomenex.com</a></td>
</tr>
<tr>
<td>Jupiter™</td>
<td>Phenomenex</td>
<td>Silica, C4, C5, C18</td>
<td>300 Å</td>
<td>3 μm 5 μm</td>
<td>Yes</td>
<td><a href="http://www.phenomenex.com">www.phenomenex.com</a></td>
</tr>
<tr>
<td>Kromasil®</td>
<td>Akzo Nobel</td>
<td>Silica, C4, C8, C18, Phenyl modified</td>
<td>100 Å 300 Å</td>
<td>3.5 μ 5 μm</td>
<td>Yes</td>
<td><a href="http://www.kromasil.com">www.kromasil.com</a></td>
</tr>
<tr>
<td>Inertsil® WP-300</td>
<td>GL-Science</td>
<td>Silica, C4, C8, C18 modified</td>
<td>300 Å</td>
<td>5 μm</td>
<td>No</td>
<td><a href="http://www.inertsil.com">www.inertsil.com</a></td>
</tr>
<tr>
<td>PLRP-S</td>
<td>Polymer Labs/Varian</td>
<td>Polystyrene/divinylbenzene</td>
<td>100 Å 300 Å</td>
<td>10 μm</td>
<td>Yes</td>
<td><a href="http://www.polymerlabs.com">www.polymerlabs.com</a></td>
</tr>
<tr>
<td>Source™ 15RPC</td>
<td>GE Healthcare</td>
<td>Polystyrene/divinylbenzene</td>
<td>Proprietary</td>
<td>15 μm</td>
<td>Yes</td>
<td><a href="http://www.gehealthcare.com">www.gehealthcare.com</a></td>
</tr>
<tr>
<td>Sunfire™</td>
<td>Waters</td>
<td>Silica, C18 modified</td>
<td>100 Å</td>
<td>3.5 μ 5 μm</td>
<td>No</td>
<td><a href="http://www.waters.com">www.waters.com</a></td>
</tr>
<tr>
<td>Symmetry300™</td>
<td>Waters</td>
<td>Silica, C4, C18 modified</td>
<td>300 Å</td>
<td>3.5 μ 5 μm</td>
<td>No</td>
<td><a href="http://www.waters.com">www.waters.com</a></td>
</tr>
<tr>
<td>TSK-Gel TMS-250</td>
<td>Tosoh Corporation</td>
<td>Silica, C1 modified</td>
<td>250 Å</td>
<td>10 μm</td>
<td>No</td>
<td><a href="http://www.tosoh.com">www.tosoh.com</a></td>
</tr>
<tr>
<td>TSK-Gel Octadecyl-4PW</td>
<td>Tosoh Corporation</td>
<td>Proprietary polymer, C18 modified</td>
<td>500 Å</td>
<td>7 μm</td>
<td>No</td>
<td><a href="http://www.tosoh.com">www.tosoh.com</a></td>
</tr>
<tr>
<td>Vydac TP</td>
<td>Grace</td>
<td>Spheroidal silica, C4, C8, C18 modified</td>
<td>300 Å</td>
<td>10–15 μm 15–20 μm 20–30 μm</td>
<td>Yes</td>
<td><a href="http://www.discoverysciences.com">www.discoverysciences.com</a></td>
</tr>
<tr>
<td>YMC-ODS-AQ</td>
<td>YMC</td>
<td>Silica, C18 modified, hydrophilic end-capping</td>
<td>120 Å 200 Å</td>
<td>10 μm 15 μm</td>
<td>Yes</td>
<td><a href="http://www.ymc.de">www.ymc.de</a></td>
</tr>
<tr>
<td>YMC –Butyl</td>
<td>YMC</td>
<td>Silica, C4 modified</td>
<td>120 Å 200 Å</td>
<td>10 μm 15 μm</td>
<td>Yes</td>
<td><a href="http://www.ymc.de">www.ymc.de</a></td>
</tr>
<tr>
<td>Zorbax 300StableBond</td>
<td>Agilent</td>
<td>Silica, C3, C4 and C18 modified</td>
<td>300 Å</td>
<td></td>
<td>No</td>
<td><a href="http://www.chem.agilent.com">www.chem.agilent.com</a></td>
</tr>
</tbody>
</table>
of the silica matrix and the surface modification, or in the case of polymer-based material, the type of monomers. The best chromatographic results are obtained with perfectly spherical particles that are free from microporosities, and are therefore topographically smooth. The surface silanol groups should be evenly distributed and of a relatively neutral nature. The acidity of the silanol groups is dependent on the purity of the silica (e.g., metal impurities) and on the manufacturing process in general.

Metal impurities can either be present in the silica matrix, where especially strongly electronegative ions (Fe$^{2+}$, Al$^{3+}$) enhance the acidity of the silanol groups in their proximity, or they can be adsorbed to the surface (Fig. 5.28).

The surface modification of silica-based packings is conducted by reacting bare silica with silanes (31). The use of monochlorosilanes leads to a so-called monomeric surface modification, while using di- or trichlorosilanes results in so-called polymeric surface modification. Polymeric functionalization by di- or trifunctional reactants leads to additional silanol groups when unreacted groups are hydrolyzed when treated with water (Fig. 5.29). These organo-silanol groups have a significant influence on the retention behaviors of peptides and proteins. One can thus expect different separation patterns for RPC packings based on monofunctional or polyfunctional surface modification. Monomeric surface modifications are easier to control and subsequently often lead to more reproducible results (31).

### 5.6.3 Chemical Stability

Chemical stability is a key parameter that not only determines the service life of a column, but is also essential with respect to potential contamination of the eluent. In the case of analytical HPLC, the leaking phase might interfere with detection, or in the case of preparative separations, the purified product would be contaminated. As well as the contamination risk, the surface properties change in the case of poor chemical stability, and reproducibility is impaired.

The extractable species from bonded-phase silica can be divided into two categories: bonded-phase hydrolysis products (organic silicon compounds) and soluble silicates (or silicic acid, inorganic silicon compounds). Bonded-phase hydrolysis products are formed as a result of the hydrolytic cleavage of the siloxane bonds (Si–O–Si) through which the organic groups (silanes) are attached to the inorganic particles (silica gel).
The initial hydrolysis product (I) is a silylalcohol (a silanol) that may dimerize to form a silylether (a siloxane) \((31)\).

In bonded phases that are end-capped (reacted in a second step with trimethylsilyl groups), the corresponding trimethyl species (III) and (IV) may be formed. In addition, species (V) can be formed through the reaction of (I) and (III).

All these hydrolysis products, I–V, are quite hydrophobic and are only eluted from a bonded phase by mobile phases containing a high amount of organic solvent \((>50\% \, \text{v/v})\). The rates of hydrolysis and dimerization are strongly dependent on the pH \((32)\). At low pH, the degradation is mainly the result of a cleavage of the covalently attaching siloxane-bond \((\text{Si}–\text{O}–\text{Si})\), and at high pH, the degradation is mainly due to the dissolution of the silica support matrix.

The second category of extractables comprises soluble silicates or silicic acid. These are formed by hydrolysis of the silica support at high pH:

\[
\text{SiO}_2 + 2\text{H}_2\text{O} \rightarrow \text{Si(OH)}_4
\]

\(\text{Si(OH)}_4\) may undergo further reaction to form a wide variety of oligomers. The extent of the hydrolysis is strongly dependent on pH, type of buffer and organic modifier, and on the composition of the mobile phase \((\% \, \text{organic modifier})\) in contact with the silica \((33)\). Chemical stability is largely influenced by the surface silanols and the coating density of the silane. The surface of Kromasil consists of uniformly distributed, neutral silanol groups. The number of undesirable acidic silanols is minimized. High coating density promotes high hydrolytic stability. Kromasil-bonded phases are produced with monofunctional silanes.

Chemical stability is measured by exposing the packing material for several hundreds of thousands of column volumes of a mobile phase of interest. The chromatographic behavior (retention times, asymmetry) is tested regularly and the eluent is analyzed for silicon content by, for example, atomic absorption spectroscopy and gas chromatography-mass spectrometry (GC-MS).

With respect to chemical stability, polymer-based packings demonstrate the intrinsic advantage of silica-based packings. Generally, polymer-based materials will not undergo any dissolution under common chromatographic conditions. With respect to pH stability, polymers can be subjected to mobile phases from pH 1 to pH 14, without risking any adverse effects on the stationary phase.

### 5.6.4 Mechanical Stability

The mechanical stability of the packing material is crucial for successful preparative applications. Columns have to be packed at high piston pressures in order to achieve the highest possible efficiency. Furthermore, high mobile phase velocity is used for high throughput. As a result, high backpressure is
developed in the system. In large diameter dynamic axial compression (DAC) columns, the mechanical stress on the particles can be significant. In addition, one column is often used for several products. If the packing is to be dedicated to one product, the column has to be unpacked and repacked for each new project, putting high demands on the mechanical strength of the packing. If the packing shows insufficient mechanical stability, it will partially collapse under these conditions and will form fines, which can clog not only the frits but will also reduce the interstitial porosity of the packed bed. Consequently, the pressure drop increases and eventually reaches the pressure limit. The deteriorated packing has to be replaced and the shortened service life increases the costs of the packing material, and therefore the entire separation costs. Furthermore, the formation of fines will not only lead to an increase in pressure drop, but will also change the surface properties of the packing due to bare silica surfaces becoming available for interactions.

The mechanical strength of silica packing materials is mainly influenced by the following factors:

- **Particle Shape**: spherical particles are much stronger than irregular ones, due to there being more contact points between the particles in the bed.
- **Composition of the Matrix**: rigid inorganic oxides or polymer-based beads.
- **Pore Volume, Pore Diameter**: large pore volume and pore diameter weaken the silica.
- **Manufacturing Process**: proprietary treatment of the silica matrix can combine high surface area with mechanical strength.

The mechanical stability of the packing material can be tested by two different methods. The simplest test is to increase the flow rate of the mobile phase step by step and then measure the backpressure. As long as the packing pressure increases linearly with the flow rate, the particles are assumed to be intact. Once the pressure drop deviates from the linear slope, the particles are either crushed (weak silica material) or deformed (polymer materials) (25).

Another possibly more relevant way to assess the mechanical stability of packing materials is to pack a short bed in a 50-mm i.d. DAC column with a stepwise increased piston pressure (40–120 bar). The pressure drop is noted for each packing pressure. When increasing the packing pressure, the pressure drop first increases linearly due to denser packing. Once attrition is such that the silica begins to be crushed, the pressure drop increases drastically (Fig. 5.30).

### 5.6.5 Regeneration

RPC packing material is best reconditioned with a high content (80%) of organic modifier. Moderately adsorbed impurities can often be eluted with MeOH/water or MeCN/water, but for tougher cases, THF/water may be necessary.

If the adsorbed impurities are even more hydrophobic, the column can also be purged with DMF or DMSO, or with 100% CH₂Cl₂, after preconditioning with 100% THF or IPA. Before the column can be used again in RP mode, the CH₂Cl₂ must be replaced by 100% IPA or THF as an intermediate solvent.

An alternative washing procedure comprises using 0.2 M acetic acid/organic modifier 80/20. The column can be purged thoroughly with this mobile phase; however, it is not advisable to store the column with this eluent.

If the mentioned treatments fail (e.g., denaturation of proteins or peptides), an alkaline wash can be performed. This treatment is very harsh and will reduce the service life of silica-based packings, so it should only be chosen as a last resort. The column can be purged with 0.1 M NaOH/organic modifier 40/60 for a maximum of 30 minutes. Thereafter, the bed must be neutralized immediately with 0.2 M acetic acid/organic modifier 50/50, until the eluent at the column outlet is acidic. Polymer-based packing materials can be treated with 1 M NaOH CIP without any risk of deteriorating the stationary phase.

### 5.7 COLUMN DESIGN

A chromatographic column is a cylindrical tube with a defined diameter and length. The packing material is kept inside the column with porous discs, so-called frits, at the beginning and end of the tube. The porosity of the frit is such that the particles of the packing material are retained inside the column, while the liquid mobile phase can pass through. Chromatographic columns can be made of stainless steel, glass, or plastic. The column has to be mechanically stable with high flow rates and pressure, and chemically stable under the eluent conditions.
During operation, the hydrodynamic force of the eluent that passes through the particulate bed in the column can force the packing material to rearrange into a more densely packed bed. Any kind of reorganization of the packed bed during chromatography has a deteriorating effect on efficiency, as a void is formed at the column inlet (Fig. 5.31). The scope of any column design thus includes minimizing the possibility of the bed rearranging and forming a void at the column inlet.

For a column with a small inner diameter, that is, an analytical column, the bed is generally stabilized by the column walls. For an analytical column of 4 mm i.d. packed with 5-μm material, there are no more than 800 particles across the column diameter. For preparative-scale columns, the situation is different. Even for a moderately sized column of 50 mm i.d. packed with 15-μm material, there are 3000 particles across the column diameter. Consequently, the particle arrays in a preparative-sized column do not experience the same wall support as an analytical-sized column, and are therefore more prone to undergo rearrangement (34). Another parameter that makes columns with larger diameters more susceptible to forming voids is packing at lower pressure. Analytical-sized columns are typically packed at pressures up to 600 bar, but the pressure rating for larger columns is generally in the 70–100 bar range. The lower packing pressure leads to higher bed porosity, and bed reorganization is thus more likely to occur when the mobile phase is pumped through the column.

5.7 COLUMN DESIGN

5.7.1 Optimal Column Dimensions

As previously mentioned, the column dimensions are defined by the inner diameter and length. The column length is dependent upon the efficiency that is required for a certain separation, as the separation power increases linearly with column length. Consistent efficiency can be obtained with a short column packed with small particles, or a long column packed with larger particles. The trend in analytical RPC is to exploit the high efficiency of <2-μm particles in combination with very short columns, for example, 30–50 mm long (35). However, the optimal column length for preparative separations is still in the 20–30 cm range, as larger particles need to be used to be in agreement with the pressure limitation of the hardware. As discussed in context with scale-up in Section 5.5, an increase in loading should be compensated by an increase in column diameter and not by the length.

5.7.2 Fixed-Bed Columns

In analytical RPC, the columns are without exception of fixed length, so the length of the packed bed is defined by the length of the tube. The bed is stabilized by the walls up to an internal diameter of 30–50 mm, as discussed in Section 5.7. Fixed-bed columns are generally packed by a column supplier and are ready to use. If the bed shrinks due to rearrangement or in extreme cases, chemical dissolution of the packing material under incompatible conditions, there is no possibility to

Figure 5.31 Formation of a void due to rearrangement of the bed by hydrodynamic forces.
5.7.3 Axial-Compression Columns

Columns having a feature that allows compression of the bed in an axial direction are used to offset any void formation as previously discussed. The compression can either be carried out as a distinct action or can take place continuously (Table 5.6). The latter case is called dynamic axial compression. Columns that allow distinct, nondynamic compression are commonly found in low pressure chromatography. The bed can be compressed by screwing the top-frit into the bed. The pressure that is applied on such a chromatographic bed is typically in the range 3–5 bar (AxiChrom™, Quickscale®). A similar technique is also used in high pressure LC, where the bed can be adjusted with a hydraulic pump (Self-Packer®, Load & Lock™) or by adjusting the packing with a central screw (Pack-n-Sep™).

Dynamic axial compression is achieved when a movable column piston is permanently pushed into the bed by a hydraulic pump at a set pressure, typically 50–100 bar. The piston pressure that can be applied must be in agreement with the mechanical stability of the packing material (e.g., polymer-based packings will undergo deformation in the abovementioned pressure range).

The operation backpressure must always be lower than the piston pressure. Dynamic axial compression columns are a prerequisite for large scale, high performance RPC.

5.7.4 Column Performance Tests

In order to obtain a high plate count, it is of the utmost importance to reduce the extra-column dead volumes to a minimum by, for example, choosing small i.d. capillaries and shortening them as much as possible.

Depending on the packing solvent, the column may need to be purged with a miscible solvent before being equilibrated with the mobile phase (e.g., C18 silica packed with toluene/IPA 50/50 → purge with 100% IPA → equilibrate with mobile phase):

Mobile phase: acetonitrile/water 70/30 (v/v)
Test solution: 1 μL acetophenone + 1 μL toluene/mL mobile phase
Detection: 254 nm

Flow rates and injection volumes are dependent upon the column and particle diameters. Optimized conditions are displayed in Table 5.7. Plate numbers are calculated based on the assumptions made in the calculation of the plate height and the determination of the plate number (above). The operation backpressure must always be lower than the piston pressure.

### Table 5.6 Selection of Commercially Available Chromatographic Columns with Axial Compression

<table>
<thead>
<tr>
<th>Name</th>
<th>Supplier</th>
<th>Compression Principle</th>
<th>Available Diameters</th>
<th>Website</th>
</tr>
</thead>
<tbody>
<tr>
<td>AxiChrom™ column</td>
<td>GE Lifescience</td>
<td>Central screw mechanism—</td>
<td>50, 70, 140, and 200 mm</td>
<td><a href="http://www.gelifescience.com">www.gelifescience.com</a></td>
</tr>
<tr>
<td>Hipersep</td>
<td>Novasep</td>
<td>Hydraulics—dynamic</td>
<td>50–110 mm</td>
<td><a href="http://www.novasep.com">www.novasep.com</a></td>
</tr>
<tr>
<td>InPlace™</td>
<td>Bio-Rad</td>
<td>Central screw mechanism—</td>
<td>100–1000 mm</td>
<td><a href="http://www.bio-rad.com">www.bio-rad.com</a></td>
</tr>
<tr>
<td>Load &amp; Lock™</td>
<td>Varian</td>
<td>Central screw mechanism—</td>
<td>1”, 2”, 3”</td>
<td><a href="http://www.varianinc.com">www.varianinc.com</a></td>
</tr>
<tr>
<td>Pack-n-Sep™</td>
<td>Novasep</td>
<td>Hydraulics—dynamic</td>
<td>50 mm</td>
<td><a href="http://www.novasep.com">www.novasep.com</a></td>
</tr>
<tr>
<td>Peak LP/MP/HP DAC</td>
<td>Peak Biotech</td>
<td>Wrench—nondynamic</td>
<td>100–1600 mm</td>
<td><a href="http://www.peakbiotech.com">www.peakbiotech.com</a></td>
</tr>
<tr>
<td>Quickscale® column</td>
<td>Millipore</td>
<td>Central screw mechanism—</td>
<td>70–630 mm</td>
<td><a href="http://www.millipore.com">www.millipore.com</a></td>
</tr>
<tr>
<td>Self Packer®</td>
<td>Merck</td>
<td>Hydraulics—nondynamic</td>
<td>25, 50, 100 mm</td>
<td><a href="http://www.merck.de">www.merck.de</a></td>
</tr>
<tr>
<td>Spring column™</td>
<td>Grace</td>
<td>Mechanical spring—dynamic</td>
<td>50, 100 mm</td>
<td><a href="http://www.grace.com">www.grace.com</a></td>
</tr>
</tbody>
</table>

### Table 5.7 Optimized Flow Rates and Injection Volumes for Column Performance test of RPC Columns

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<tr>
<th>Column i.d.</th>
<th>Particle Size</th>
<th>Injection Volume for 25-cm Bed Length</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>10 μm</td>
<td>13 μm</td>
</tr>
<tr>
<td>4.6 mm</td>
<td>0.7 mL/min</td>
<td>0.5 mL/min</td>
</tr>
<tr>
<td>10 cm</td>
<td>330 mL/min</td>
<td>235 mL/min</td>
</tr>
<tr>
<td>20 cm</td>
<td>1.3 L/min</td>
<td>930 mL/min</td>
</tr>
<tr>
<td>30 cm</td>
<td>3.0 L/min</td>
<td>2.1 L/min</td>
</tr>
<tr>
<td>45 cm</td>
<td>6.7 L/min</td>
<td>4.8 L/min</td>
</tr>
</tbody>
</table>
5.8 PRACTICAL RECOMMENDATIONS: HOW TO AVOID AND SOLVE PROBLEMS

5.8.1 Extra-Column Volumes

All volumes through which the solute passes between the injector and the detector, and which are outside the column, are called extra-column volumes, and they affect the separation negatively because they cause band broadening. As a result, the detected peaks are broader or are more tailed than expected from band broadening within the column (van Deemter theory, Section 5.3.1.1). The extra-column contribution to the overall band broadening is additive to the broadening caused by the column (16):

\[ w = 4 \sqrt{\sigma_{\text{inj}}^2 + \sigma_{\text{cap}}^2 + \sigma_{\text{det}}^2 + \sigma_{\text{col}}^2} \]  

(5.21)

In order to minimize overall band broadening, it is important to reduce the extra-column volumes to the largest possible extent. Whereas it is difficult to modify the instrument with regard to the injector and detector, the right choice of tubing and connectors will contribute significantly in keeping the extra-column volume at a minimum.

5.8.1.1 Capillary Tubing

When working with analytical HPLC instruments, capillaries with external diameters of 1/16 inch (1.6 mm) are generally used to connect the different parts of the instrument. Those parts that do not affect the extra-column volume should have internal diameters (i.d.) of 1 mm. They entail little risk for clogging and demonstrate minimum flow resistance, which contributes to the extra-column pressure drop. For those connections critical to the extra-column volume, an i.d. of 0.17 mm is recommended. Such capillaries add only 23 µL additional volume per meter of tubing (1.0 mm i.d. tubing: 800 µL/m). To avoid clogging of such fine capillaries, it is of the utmost importance to filter both mobile phases and sample solutions.

For preparative columns, the tubing must be scaled up in order to reduce the flow resistance caused by the higher flow rates (\( \Delta P_{\text{cap}} \approx \frac{d_{\text{cap}}^4}{16} \); see Equation 5.12, Section 5.3.1.1):

- 20 mm i.d. columns → 0.25 mm i.d. tubing
- 50 mm i.d. columns → 0.50 mm i.d. tubing
- 100 mm i.d. columns → 1.0 mm i.d. tubing

5.8.1.2 Fittings

Fittings connect the distinct parts of the HPLC instrument. Steel fittings usually have a separate ferrule, though some manufacturers use two ferrules – a ring and a conical component. Upon first tightening, the ferrule is permanently pressed and the enclosed capillary is to some extent compressed. The ferrule can no longer be removed from the capillary. Plastic (PEEK) fittings are made of a single component (nut, ferrule) and are installed by finger-tightening only. Over-tightening (common with steel fittings) must be avoided, because disproportionate force can damage the threading and the capillary. It is advisable to only tighten the screw slightly with a wrench and then test if the connection is tight, meaning that no leakage occurs when the mobile phase is pumped through the system (36).

5.8.2 Mobile Phase Preparation

Mobile phases can be prepared in many ways, and the described procedure is certainly not the only one that leads to accurate and reproducible separation results. However, we find that when considering the following recommendations, one is generally dealing with robust chromatography.

5.8.2.1 Mixing of the Mobile Phase

Mobile-phase compositions are usually described as the volumetric ratio between the aqueous and the organic solvent, for example, water/methanol 40/60 (v/v). It is a well known phenomenon that the mobile-phase components will undergo volume contraction upon mixing, which means that the volume of the mixture is smaller than the sums of the volumes of the components. The effect is most pronounced for water and alcohols; in water and acetonitrile or THF the effects are less critical. Four different methods for the preparation of 1 L of the previously mentioned mobile phase, water/methanol 40/60, are possible:

- To 400 mL of water, methanol is added to produce 1 L.
- To 400 mL of water and 600 mL of methanol are measured individually and mixed thereafter.
- The HPLC instrument mixes the mobile phase from channel A (water) and channel B (methanol).
- To 600 mL of methanol, water is added to produce 1 L.

Separations obtained with the differently prepared mobile phases lead to chromatograms where the retention times vary significantly, and the importance of a clear description of the mobile-phase preparation method becomes apparent (37).

It is advisable to prepare the mobile phases according to the second method, where the mobile phase components are weighed to eliminate fluctuation due to temperature effects:

Example: water/methanol 40/60 (v/v)

\[ \rho(\text{H}_2\text{O}): 1.0 \text{ g/mL}; \rho(\text{MeOH}): 0.78 \text{ g/mL} \]

\[ m = \rho \cdot V \rightarrow m(\text{H}_2\text{O}) = 1.0 \text{ g/mL} \cdot 400 \text{ mL} = 400 \text{ g} \]

\[ m(\text{MeOH}) = 0.78 \text{ g/mL} \cdot 600 \text{ mL} = 467.0 \text{ g} \]
5.8.2.2 pH Adjustment of Buffered Mobile Phases
Buffers are commonly used to control the mobile-phase pH whenever ionic or ionizable compounds are to be separated. Buffered mobile phases can be prepared in two ways:

- preparing the buffer, including pH adjustment, prior to mixing with organic modifier
- mixing buffer and organic modifier prior to pH adjustment.

Although pH is a well defined parameter in aqueous systems, it is not as straightforward to define or measure proton concentration in partially organic solvent systems (26). Mobile phases containing buffers should always be filtered through a 0.45-μm filter prior to use.

5.8.2.3 Premixing
Most manufactures of HPLC instruments claim that the quaternary pumps are fully capable of mixing pure aqueous and pure solvents to a sufficiently accurate degree. While the results of online mixing have certainly improved significantly during the last 20 years, more reproducible results will still be obtained if the mobile phases are premixed. On-line mixing takes place by alternately pumping liquids from channels A and B. For mixing 50% water (channel A) with 50% acetonitrile (channel B), the HPLC instrument will pump, for example, 0.2 sec from channel A, followed by 0.2 sec from channel B, and so on. Although a certain blending between the two different pulsed liquids will take place due to diffusion and convection, it cannot be assured that a homogeneous mixture is obtained, especially if the two different liquids are very different with respect to their viscosity.

It is therefore advisable to use premixed mobile phases; for example, channel A, water/organic modifier 95/5; channel B, water/organic modifier 10/90. By doing so, one still has the significant advantage of letting the instrument mix the mobile phase with the required elution strength (or gradient slope), rather than having to prepare many different mobile phases during method development. Moreover, a mobile-phase container containing at least a few percent of organic modifier is much less susceptible to bacterial growth than a solution of pure water or buffer.

5.8.3 Column Storage
Correct column storage is a prerequisite for problem-free chromatography and long column service life. Reversed-phase stationary phases should be kept in a mixture of water/organic modifier, free from any buffers or additives that might precipitate during storage. It is thus advisable to wash the column whenever chromatography will be stopped for more than 15 minutes with water/organic modifier 50/50, regardless of whether the column will be detached from the HPLC instrument or not. If chromatography is only to be interrupted for a couple of hours, it might be less laborious to keep the buffer containing mobile phase running at a very low flow rate. If the column is detached, close the inlet and outlet with a screw to avoid drying of the stationary phase.

5.8.4 Wettability
The use of mobile phases with a low content of organic modifier (<5%) generally leads to what is known as phase collapse (38, 39). The latter is best described as a dewetting phenomenon, in which the highly aqueous mobile phase is excluded from the hydrophobic pore system due to surface tension. The effect is predominantly observed with reversed-phase packing materials with high ligand density (>3.2 μmol/m²). The loss of wetted surface results in a decrease in accessible interaction sites for the solute, and leads to a loss in retention (40) and/or reduced loading capacity (Fig. 5.32). The process can easily be reversed by purging the packed bed with a high content of organic modifier (>50%). The phenomenon is most dominant for C18 and C8 packing materials, but even occurs with C4 modified packing materials. Aqueous mobile phases of 100% should be avoided to ensure robust chromatography. Packing materials with a pore size of 300 Å are less prone to dewetting than stationary phases with smaller pores.

If the peptides are highly polar and will not be retained with an organic modifier present in the mobile phase, the use of wide-pore packing material (pore diameter, 300 Å) will in most cases enable the use of 100% aqueous mobile phases. Furthermore, applying post-column pressure (25 bar) to the column will also result in a fully wetted RP column (100-Å pore diameter) as the mobile phase is forced into the pores (41).

Figure 5.32 Breakthrough curve of insulin under (a) dewetted (0% acetonitrile) and (b) wetted (5% acetonitrile) conditions.
5.8.5 Clogging

Columns that are subjected to preparative loadings often suffer from increased backpressure. In most cases, the increase in backpressure is caused by clogging of the inlet frit. The following precautions will reduce the risk of clogged frits:

1. Whenever possible, use the mobile phase as an injection solvent.
2. Filter (0.25 µm) the injection solution prior to injection.

If a frit nevertheless seems to be clogged, which is observed by increased pressure drop over the column and/or loss in resolution, the following washing procedure should be run in the order listed:

1. Back-flush the column with a high content (80–90%) of organic modifier.
2. Use a stronger solvent, such as THF, DMF, DMSO, or CH₂Cl₂ (the latter requires an intermediate step of 100% IPA or THF).
3. Use 0.5 M acetic acid/ethanol 50/50.
4. Use 0.1 M NaOH/ethanol 50/50 or even plain 0.1 M NaOH (pure NaOH should only be used for treating the frit itself, unless the column is packed with organo-polymer packing). The column must be unpacked prior to treatment. Neutralize after treatment with 0.5 M acetic acid.

5.9 REFERENCES

## HYDROPHOBIC INTERACTION CHROMATOGRAPHY

**Kiell-Ove Eriksson and Makonnen Belew**  
*GE Healthcare Bio-Sciences AB, R&D Department, SE-751 84 Uppsala, Sweden*

### 6.1 Introduction

According to Jencke (1), the hydrophobic interaction (HI) is “an interaction of molecules with each other which is stronger than the interaction of the separate molecules with water and which cannot be accounted for by covalent, electrostatic, hydrogen bond or charge-transfer forces.” This is thus a negative definition of HI and falls far short of a more comprehensive account of the hydrophobic effect itself. Another concept is based on the principle that “like attracts like” and has served as an explanation for the strong attraction between two oil drops in water leading to their fusion into one. However, according to Tanford (2) “…the attraction of non-polar groups (such as hydrocarbon chains) for each other plays only a minor role in the hydrophobic effect. The effect actually arises primarily from the strong attractive forces between water molecules …”. It is therefore apparent that “…HI was applied by different authors to describe and interpret quite different phenomena occurring in aqueous solutions” (3).

This chapter is not an academic exercise that attempts to discuss the divergent theories on HI and HIC [hydrophobic interaction chromatography (4)], but is rather intended to demonstrate the great potential that this biochemical separation method affords for the separation and purification of a wide variety of biomacromolecules. We have also tried to maintain a balance between theory and practical application examples in order to highlight the versatility of this growing area of separation science. The chapter is divided into six sections:

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main sections: “History,” “RPC vs HIC,” “Theory,” “Adsorbents for HIC,” “Factors that Impact HIC,” and “Applications.” This is done primarily to organize the presentation knowing too well that a chromatographic event is a unit operation comprising several steps, that is, column packing, equilibration, sample preparation, and so on. We have also consistently used the terms solute, solvent, and adsorbent in order to obtain a unified approach when describing the components of a chromatographic system.

### 6.2 HISTORY

In a classical paper published in 1948 entitled “Adsorption Separation by Salting Out,” Tiselius (5) laid down the foundation for a biochemical separation method that we now know as HIC. He noted that “…proteins and other substances which are precipitated at high concentrations of neutral salts (salting out), often are adsorbed quite strongly already in salt solutions of lower concentration than is required for their precipitation, and that some adsorbents which in salt-free solutions show no or only slight affinity for proteins, at moderately high salt concentrations become excellent adsorbents.” Fourteen years later, Porath (6) repeated Tiselius’ experiments using Sephadex™ G-100 as the adsorbent, a partially purified human serum fraction as solute, and a negative salt gradient of ammonium sulfate (85–35% saturation, i.e., 3.7 M–1.5 M) as the eluting solvent. By this simple arrangement, he obtained a remarkable separation of the sample components into at least three fractions, which, upon paper electrophoresis, were shown to be distinct groups of proteins.

As far as we know, only sporadic reports have been published using “salting out” or “zone precipitation” as separation methods (7–12); these are summarized in Table 6.1. More references are found within the references cited below. This work is of older date and the technique seems to have gained little attention in recent decades.

Introduction of the cyanogen bromide method for chemical activation of gel matrices (e.g., dextran, agarose) and the subsequent coupling of a wide variety of ligands (e.g., peptides, proteins, and a wide variety of low molecular organic compounds) to such activated polymers (13–15) opened a new chapter in the field of protein separations. Shortly following this technological advance, two important chromatographic separation methods were developed: affinity chromatography (16) and HIC (17). Immobilized metal ion affinity chromatography (IMAC) followed in 1975 (18). It should be pointed out that the cyanogen bromide activation and coupling method introduces positively charged groups on the gel matrix (19), which could be undesirable in specific applications. Other types of activation and coupling chemistries (which did not introduce charged groups) were soon developed and characterized (20).

**TABLE 6.1 Some Examples where the Technique of “Salting Out” Adsorption Chromatography has been used as the Major Separation Principle**

<table>
<thead>
<tr>
<th>Adsorbent</th>
<th>Mobile Phase</th>
<th>Solute</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>Sephadex G-50</td>
<td>2.7 M (NH₄)₂SO₄, pH 6.0</td>
<td>Serum proteins</td>
<td>7</td>
</tr>
<tr>
<td>Sepharose 4B</td>
<td>2 M (NH₄)₂SO₄ in 0.03 M phosphate pH 6.0</td>
<td>Enzymes and tRNA from yeast</td>
<td>8</td>
</tr>
<tr>
<td>Sepharose 4B</td>
<td>2.5 M (NH₄)₂SO₄ in 0.05 M phosphate pH 6.6</td>
<td>Extract from halophilic bacteria</td>
<td>9</td>
</tr>
<tr>
<td>Cellulose</td>
<td>2.4–3 M (NH₄)₂SO₄ in 0.1 M phosphate pH 6.5/8.0</td>
<td>Yeast enzymes</td>
<td>10</td>
</tr>
<tr>
<td>Sepharose 4B &amp; Ultrogel AcA 44</td>
<td>2 M (NH₄)₂SO₄ in 10 mM acetate, pH 4.3</td>
<td>4 tRNA species</td>
<td>11</td>
</tr>
<tr>
<td>Sepharose CL-6B</td>
<td>70–35% satd. (NH₄)₂SO₄ in 0.2 M Tris-HCl, pH 7.8</td>
<td>Mouse serum transferrin</td>
<td>12</td>
</tr>
</tbody>
</table>

*Mobile phase = column equilibration buffer.

Note: More references are found within the references cited.

The first type of hydrophobic adsorbents were synthesized by Yon (17). He activated Sepharose 6B with cyanogen bromide and then coupled 1,10-diaminodecane to the gel. The concentration of the immobilized ligands was very high compared to what is now the practice. Not surprisingly, the resulting adsorbent showed both ionic and hydrophobic character, and the elution of some proteins required harsh conditions that could lead to their denaturation. Almost simultaneously, Er-el, Zaidenzaig and Shaltiel (21) reported the synthesis of a homologous series of alkylamino-Sepharoses that contained positively charged groups. During the same period, Hofstee (22, 23) also reported the synthesis and characteristics of aromatic amines immobilized on CNBr-activated Sepharose 4B. In each instance, the adsorption behavior of the “hydrocarbon-coated Sepharoses” was established using a series of purified proteins and some crude biological extracts. The results obtained indicated that at equal ligand concentrations on similar base matrices (i.e., similar ligand surface grafting densities), there was good correlation between the length of the immobilized alkyl chains and the strength of the “hydrophobic” binding of the proteins (see Section 6.6.1.1) (17, 21, 24, 25). It is also worth noting that the adsorption of proteins on the α-alkane-substituted Sepharoses [which we shall refer to as Seph—NH(CH₂)n.H, following the nomenclature introduced by Shaltiel et al. (21, 24)] was achieved at low ionic strength of the equilibration buffer. Elution of bound proteins was accomplished by a stepwise increase of the salt concentration in the solvent. It is thus
apparent that the chromatographic mode of operation is similar to that of ion-exchange chromatography, but the underlying mechanism may be due to both hydrophobic and ionic interactions (21–25).

The synthesis and preliminary characterization of the second type of hydrophobic adsorbents (i.e., charge-free amphiphilic gels) was first reported by Porath and colleagues in 1973 (26). The adsorbent was prepared by a simple procedure involving the reaction of crosslinked Sepharose 6B with benzyl chloride for 5 h at 80°C in the presence of 5 M NaOH and sodium borohydride. The gel was washed thoroughly, packed in a 15-mL column and equilibrated with either 0.05 M sodium phosphate buffer, pH 7.0 (containing 0, 1, or 3 M NaCl) or 0.1 M sodium formate buffer, pH 3.0 (containing 0 or 1 M NaCl). As solutes, they used a solution of cytochrome c and a crude extract of kidney beans.

The results showed that the adsorption of proteins increased strongly with increasing concentration of salt in the equilibration buffer and was further enhanced by lowering the pH. Elution of bound proteins was achieved by lowering the salt concentration, increasing the pH, and/or lowering the polarity of the elution buffer. The conditions for adsorption and elution of proteins on this new adsorbent are thus different from those used in adsorbents prepared by the CNBr method. This prompted Porath and colleagues (26) to call this new chromatographic principle “a method for protein fractionation based on hydrophobic salting-out adsorption in non-ionic amphiphilic gels.”

In a series of articles published between 1973 and 1975, Hjerten and colleagues (27–30) reported the synthesis and some general characteristics of charge-free hydrophobic adsorbents based on alkyl or aryl derivatives of Sepharose. The adsorbents were synthesized by an essentially two-step procedure (27) involving (1) preparation of the glycidyl ether derivatives of the alcohols, using boron trifluoride ethyl ether as a catalyst and (2) coupling the glycidyl ethers to a Sepharose matrix in an essentially organic environment in which was dissolved boron trifluoride ethyl ether as a catalyst. The replacement of the water in the Sepharose matrix was a tedious procedure involving several washing steps. The adsorbent so prepared is charge-free and has a short spacer arm of three carbon atoms.

The hydrophobic adsorbents prepared by the above two research groups share the same general structure [Seph–O–(CH2)n–H or Seph–O–aryl] and chromatographic mode of operation that will be presented later on in this chapter. It is also fair to say that the work of these two Uppsala groups laid the foundation for HIC as it is practised today.

The third variant of hydrophobic adsorbents was synthesized by Maisano and colleagues (31) with the general structure Seph–S–(CH2)n–H. They activated Sepharose 6B with bis-epoxides and then coupled n-alkane thiols of varying alkyl chain lengths and concentrations. The coupling of the alkane thiols was reproducible and almost quantitative. The gel media so produced are charge-free and have a long, hydrophilic spacer arm of 10 carbon atoms. Its adsorption characteristics towards serum proteins were also qualitatively different from the Seph–O–(CH2)n–H type adsorbents, which may be due to the superimposed charge-transfer effect exerted by the thio-ether moiety and/or some contributions from the long spacer arm. Other types of adsorbents based on immobilized thioaromatic ligands have been described (32), but their adsorption characteristics were found to be quite distinct from the Seph–S–(CH2)n–H or Seph–O–(CH2)n–H type adsorbents. Demiroglou and Jennissen (33, 34) have also reported the synthesis of

\[
\begin{align*}
A. & \quad \text{NH}_2^+ \quad \text{O} \quad CH_2 \quad \text{(CH}_2)_n \quad \text{H}_3 \\
B. & \quad \text{OH} \quad \text{O} \quad \text{CH}_2 \quad \text{CH}_2 \quad \text{H} \\
C. & \quad \text{OH} \quad \text{O} \quad \text{CH}_2 \quad \text{CH}_2 \quad \text{(CH}_2)_n \quad \text{H}_3 \\
D. & \quad \text{Si} \quad \text{NH}_2^+ \quad \text{O} \quad \text{CH}_2 \quad \text{CH}_2 \quad \text{H} \\
E. & \quad \text{OH} \quad \text{O} \quad \text{CH}_2 \quad \text{CH}_2 \quad \text{(CH}_2)_n \quad \text{H}_3
\end{align*}
\]

Figure 6.1 Structures of different hydrophobic ligands coupled to a gel matrix, M.
uncharged, spacer-free alkyl–S–S–gels and their general adsorption characteristics. The synthetic route they adopted apparently led to the introduction of an extra sulfur atom in the final adsorbent. The structures of various HIC media are shown in Figure 6.1.

6.3 NOMENCLATURE

HIC has been known by several names, including hydrophobic bonding (17), hydrophobic chromatography (9, 21), hydrophobic affinity chromatography (17, 22), non-ionic adsorption chromatography (35), salting out adsorption in amphiphilic adsorbents (26), hydrophobic interaction chromatography (27, 28), and salt-promoted adsorption chromatography (36). The assignment of so many names to a single chromatographic separation method might reflect a lack of agreement among the various researchers as to the mechanism underlying the adsorption of proteins to charged or uncharged types of these adsorbents. This issue does not seem to be totally resolved (35), although the term coined by Hjertén (27, 28), “hydrophobic interaction chromatography” (HIC) seems to have gained general acceptance.

6.4 RPC vs HIC

The development of reversed-phase chromatography (RPC) has its roots in partition chromatography, which earned a Nobel Prize for Martin and Synge in the early 1940s (37). They packed silica gel in a column and percolated an aqueo-organic solvent mixture through it. The aqueous components of the solvent were partitioned preferentially towards the silica gel, and the organic components were partitioned towards the mobile phase. Selective retardation of the solute (e.g., a biological extract) applied to such a column was considered to be due to the varying distribution between the mobile organic phase and the stationary aqueous phase. This method can be considered as “normal phase partition chromatography,” of which paper chromatography has been one of the best success stories.

In 1950, Howard and Martin (38) reacted silica powder with dialkyl-dichlorosilane vapour to form a hydrophobic coating of alkyl groups. When used in a column format, this product retained the less polar components of an aqueo-organic solvent mixture and could thus be used as a stationary phase in partition chromatography with a polar mobile phase. This method was named “reversed-phase partition chromatography” (RPC) to distinguish it from the related method described above. Present-day RPC media are highly substituted with alkyl or aryl groups and are capable of retaining the organic components of an aqueo-organic solvent. As a consequence, many proteins are denatured upon exposure to such a hydrophobic environment, making RPC more suitable for the high-resolution analysis of peptides and proteins in their denatured state. However, some stable proteins such as insulin can be readily processed using RPC.

With the above discussions as a background, one can outline the major similarities and differences between these two closely related separation methods. To begin with, HIC media have lower ligand substitution levels (alkyl or aryl ligands in the range of 10–40 μmol/mL adsorbent) compared with RPC media, thereby reducing the risk of denaturation of proteins on HIC media. The adsorption of solutes to HIC media occurs in an essentially hydrophilic environment and is most often mediated by moderate to high concentrations of so-called water structuring salts of the Hoffmeister series (39). In RPC, the solute–adsorbent interaction occurs in an essentially hydrophobic micro-environment, and does not require the presence of mobile-phase additives such as salts. Instead, RPC relies on the use of solvent phase modifiers such as acetonitrile to vary selectivities. The recovery of proteins from HIC media is, in most instances, relatively high, thus making such adsorbents suitable for purifying a variety of biomolecules in their native state. For further discussions on this topic, see References 40 and 41.

Despite some differences at the operational level, RPC and HIC share a common mechanism of interaction at the molecular level, that is, hydrophobic interactions (see Section 6.1). Consequently, RPC should be re-named RP-HIC (i.e., reversed-phase HIC) and HIC as SP-HIC (salt-promoted HIC; see References 36 and 42) to emphasize the identity between these two closely related separation methods. It is also reasonable to propose that “where SP-HIC ends, RP-HIC begins.” These two nomenclatures will be adopted throughout this chapter, even though there is as yet no general consensus on this matter.

6.5 THEORY

In general, it is well appreciated that charge-based interactions involve much longer range forces than hydrophobic, hydrogen-bond or other van der Waals interactions. So, if you have an amphipathic surface the charge forces will tend to dominate initial interactions with amphipathic colloids such as proteins. Once the colloid is localized at the surface, the other forces will come into play and can even dominate. HIC media that have charged and hydrophobic groups are classic examples of this. Of course, many other interactions may also play a role, such as asymmetric partition of salt and solvent between the bulk and media localized liquid-phase regions.

Since the introduction of the charged type of HIC adsorbents (17, 21, 22) there have been controversies about the mechanism underlying the binding of proteins to such media. Yon (17) proposed that both ionic and hydrophobic interactions could contribute to the adsorption of proteins.
Er-el and colleagues (21, 24), Halperin and Shaltiel (25) and Halperin and colleagues (43) suggested that the hydrophobic component is the driving force for the observed interactions. This conclusion was based on the observation that the length of the n-alkyl chain of Seph–NH–Cn adsorbents “... has a marked effect on the capacity of the column to bind phosphorylase b, passing from no retention, through retardation, to reversible binding up to a very tight binding, as the hydrocarbon chain is gradually lengthened” from C-1 to C-6 (21). It should be mentioned here that each of these adsorbents carries one positive charge per mole of adsorbent, and their varying adsorption characteristics cannot be accounted for by electrostatic interactions. Hofstee (22) attributes the observed phenomena to “… the combined (and possibly mutually reinforcing) effects of hydrophobic and electrostatic forces.” This view was shared by Wilchek and Miron (44), who proposed that the primary event in the binding of proteins to the charged-type hydrophobic columns is ion exchange, which is subsequently strengthened by a secondary, hydrophobic event. An interesting result obtained by Hofstee and Otitlio (35) further suggests that the interaction observed is dependent on the concentration of salt in the equilibration buffer. Thus, as the salt concentration is increased, ionic binding is gradually abolished, and at further increase of the salt concentration, binding increases, presumably due to hydrophobic interactions. This is corroborated by one of Hjerten’s earliest results (28), where he separated dialyzed human serum into at least four distinct fractions after chromatography on a packed column of pentyl amine—Sepharose equilibrated with buffer containing 4 M NaCl. The bound fractions were eluted with buffer containing no salt and by raising the pH to 9.8. This result seems to suggest that charged and uncharged types of hydrophobic adsorbents operate in the same manner if adsorption is carried out in the presence of a sufficiently high concentration of salts.

For the uncharged type of hydrophobic adsorbents, a number of theories have been proposed over the years, one of which is based on the thermodynamics of the hydrophobic bond (2, 45–49) and seems to have gained popular acceptance. This theory has its roots in the classical investigations of Frank and Evans (47), who used solutions of hydrocarbons in water or aqueous solutions of small organic molecules (containing nonpolar groups) as model systems. They found that the thermodynamic changes arising from the transfer of hydrocarbons from a nonpolar solvent to water is invariably accompanied by a very large decrease in the entropy and a small negative change in the enthalpy of the system. A plausible explanation, provided by Frank and Evans (47), suggests that the water molecules in the immediate vicinity of the hydrocarbon molecule are well arranged with less randomness and better hydrogen bonding than in the bulk water at the same temperature. When two or more hydrocarbon molecules in water are near to each other, they interact almost instantaneously because their affinity towards one another is much higher than their affinity to water. This results in the displacement of the ordered structure of water molecules in the immediate vicinity of the hydrocarbon molecules, with a subsequent increase in the entropy of the system. It is this gain in entropy that is the underlying principle that accounts for hydrophobic interactions.

Hjerten (45, 46) formalized this hypothesis with reference to the thermodynamics of the whole system; that is, \( \Delta G = \Delta H - T \Delta S \). He proposed that the adsorption of proteins to a HIC medium leads to an increase in entropy \( (\Delta S) \), resulting in a negative value for the change in the total free energy \( (\Delta G) \) of the system. Consequently, the ligand–protein interaction (adsorption) is thermodynamically favorable. One should realize, however, that the discussions presented here assume that there is a parallelism between the interaction of small organic molecules in an aqueous environment and the adsorption of proteins to hydrophobic ligands attached to an insoluble gel matrix. The available evidence seems to suggest that this is most likely the case when one considers that a substantial number of apolar amino-acid residues are exposed on the surface of proteins and are therefore accessible to solvent molecules (50–53). Although the hypothesis outlined here seems to be generally accepted, other explanations for the hydrophobic effect have also been proposed (for reviews, see References 41, 54, and 55).

Hydrogen bonding has been implicated as one of the driving forces in the binding of proteins to HIC media (4, 10, 35). Direct evidence for this effect was obtained by Fujita and colleagues (10), who showed that yeast enzymes that were adsorbed on a column of cellulose (equilibrated with a buffer containing 2.4 M ammonium sulfate) can be eluted with the same equilibration buffer containing, in addition, 2.7–4.4 M urea. The bound enzymes could also be eluted by decreasing the salt concentration just like in a “normal,” salt-promoted, HIC mode of operation. The general applicability of this approach was tested in our laboratory using Phenyl or Butyl Sepharose™ 6 Fast Flow under the same experimental conditions used by Fujita and co-workers. The bound proteins could not be eluted with 6 M urea in the adsorption buffer, but were readily eluted with buffer containing no salt or urea. This apparent difference might be due to the fact that Fujita and colleagues used unsubstituted cellulose, in contrast to the commercially available HIC media that are widely used today.

According to Srinivasan and Ruckenstein (54), van der Waals attraction forces (comprising dispersion, orientation, and induction) constitute the most predominant driving forces involved in the binding or adsorption of solutes to HIC media. The opposite effect, that is, desorption or elution of the bound solutes, is ascribed to repulsive van der Waals forces (56). The authors also claim that the elution of bound proteins begins when the surface tension of the elution buffer decreases to a point lower than that evoked by the protein in question.
The effects of various neutral salts on the adsorption of biomolecules to hydrocarbon-coated insoluble matrices (e.g., Sepharose<sup>TM</sup>, cellulose) is a central theme in HIC and has its roots in the well-known Hofmeister (or lyotropic) series (57) for the precipitation of proteins from aqueous solutions (see Table 6.2). Melander and Horvath (58, 59) extended this concept, and showed that the molality and the molal surface tension increment (brought about by a series of neutral salts dissolved in water) are the most important parameters that determine the extent of retention of biomolecules on HIC media. Thus, an increase in the molality of the salt in the mobile phase, or a change to a salt that confers higher molal surface tension increment, will result in increased binding of solutes to HIC media. They also found a close parallelism between the lyotropic series of neutral salts and the measured molal surface tension increments for the same salts (see Tables 6.2 and 6.3). The results they obtained thus laid down the basis for a quantitative appraisal of the salting-out effects of various neutral salts in terms of their intrinsic molal surface tension increments.

Although the theoretical model proposed by Melander and Horvath (58, 59) has been shown to be valid for the majority of salts investigated so far, some exceptions have been noted, particularly for divalent cationic salts such as MgCl<sub>2</sub> (60–64). These and some other divalent cations exhibit one of the highest surface tension increments, but do not promote protein binding to HIC media. Contrary to expectations, these salts act as salting-in agents and enhance protein solubility. This phenomenon has been explained with reference to specific binding of MgCl<sub>2</sub>, and other divalent cations, to proteins, thereby overcoming salt exclusion and leading to a preferential hydration of the proteins (60).

At sufficiently high concentrations of antichaotropic salts, the molar concentration of water may be reduced to a point where the solubility of proteins may be affected (65). This can lead to aggregation of the proteins on the hydrophobic adsorbent and their virtual precipitation on the column. Such situations can arise in instances where biological extracts are dissolved in buffer containing a relatively low concentration of salt and then applied to a column equilibrated with buffer containing a high concentration of the same salt. Furthermore, charge–charge attraction/repulsion forces on the protein surface may be virtually eliminated to the extent that the protein molecule behaves as a neutral dipole. It has thus been postulated that high concentrations of salt evoke slow conformational changes on the surface of proteins (66, 67), thereby affecting their re-orientation towards, and adsorption onto, the immobilized hydrophobic ligands. On the basis of the results he obtained, Oscarsson (67) postulated that there is no simple correlation between the extent of hydrophobic interactions and the effects of various salts on surface tension increments, as suggested by Melander and Horvath (58, 59). He therefore proposed that conformational changes of the proteins may be more

**TABLE 6.2 The Hofmeister Series of some Anions and Cations Arranged in Order of their Effects on the Solubility of P in Aqueous Solutions**

| Increasing precipitation ("salting-out") effect |
|-----------------|-----------------|
| Anions: citrate<sup>3-</sup> > phosphate<sup>2-</sup> > sulfate<sup>2-</sup> > F<sup>-</sup> > Cl<sup>-</sup> > Br<sup>-</sup> > I<sup>-</sup> > NO<sub>3</sub><sup>-</sup> > ClO<sub>4</sub><sup>-</sup> |
| Cations: N(CH<sub>3</sub>)<sub>4</sub> <sup>+</sup> > NH<sub>4</sub> <sup>+</sup> > Cs<sup>+</sup> > Rb<sup>+</sup> > K<sup>+</sup> > Na<sup>+</sup> > H<sup>+</sup> > Ca<sup>2+</sup> > Mg<sup>2+</sup> > Al<sup>3+</sup> |

**TABLE 6.3 Molal Surface Tension Increment (σ) of Various Salts**

<table>
<thead>
<tr>
<th>Salt</th>
<th>×10&lt;sup&gt;3&lt;/sup&gt; (dyn g cm mol&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Salt</th>
<th>×10&lt;sup&gt;3&lt;/sup&gt; (dyn g cm mol&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.45</td>
<td>KSCN</td>
<td>1.96</td>
<td>K&lt;sub&gt;2&lt;/sub&gt; tartarate</td>
</tr>
<tr>
<td>0.55</td>
<td>NaClO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>2.00</td>
<td>Ba(NO&lt;sub&gt;3&lt;/sub&gt;)</td>
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<tr>
<td>0.74</td>
<td>NH&lt;sub&gt;4&lt;/sub&gt;I</td>
<td>2.00</td>
<td>LiF</td>
</tr>
<tr>
<td>0.79</td>
<td>LiI</td>
<td>2.02</td>
<td>Na&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
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<td>KI</td>
<td>2.10</td>
<td>NiSO&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>0.85</td>
<td>NH&lt;sub&gt;4&lt;/sub&gt;NO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>2.10</td>
<td>MgSO&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>0.86</td>
<td>KClO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>2.10</td>
<td>MnSO&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>1.02</td>
<td>NaI</td>
<td>2.15</td>
<td>CuSO&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
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<td>2.16</td>
<td>(NH&lt;sub&gt;4&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
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<td>2.27</td>
<td>ZnSO&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>1.16</td>
<td>LiNO&lt;sub&gt;3&lt;/sub&gt;</td>
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</tr>
<tr>
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<td>Na&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
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<td>2.73</td>
<td>Na&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>1.39</td>
<td>CsI</td>
<td>2.78</td>
<td>Li&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>1.39</td>
<td>NH&lt;sub&gt;4&lt;/sub&gt;Cl</td>
<td>2.78</td>
<td>FeCl&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
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</tr>
<tr>
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<td>FeSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>3.12</td>
<td>K&lt;sub&gt;3&lt;/sub&gt;Fe(CN)&lt;sub&gt;6&lt;/sub&gt;</td>
</tr>
<tr>
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<td>LiCl</td>
<td>3.16</td>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
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<td>NaCl</td>
<td>3.66</td>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
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<td>3.9</td>
<td>K&lt;sub&gt;3&lt;/sub&gt;Fe(CN)&lt;sub&gt;6&lt;/sub&gt;</td>
</tr>
<tr>
<td>1.82</td>
<td>CuSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>4.34</td>
<td>K&lt;sub&gt;3&lt;/sub&gt;Fe(CN)&lt;sub&gt;6&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

Source: Reprinted from Reference 58, with permission.
important in determining whether or not a particular protein in a mixture of other proteins will bind to the HIC adsorbent or not. This hypothesis is contested by a recent publication (68), in which the authors claim that differences in conformation are not responsible for the selectivity changes observed for some stable proteins they had analyzed. It all goes to show that the theory of HIC is a complex phenomenon indeed.

6.6 FACTORS THAT IMPACT HIC

Separation of biomolecules by HIC is based on an interplay between the hydrophobicity of the medium, the nature and composition of the sample, the prevalence and distribution of surface-exposed hydrophobic amino-acid residues, and the type and concentration of salt in the binding buffer. Unlike RPC, the adsorption of solutes to mildly hydrophobic adsorbents is promoted, or otherwise modulated, by the presence of relatively high concentrations of antichaotropic salts—kosmotropes (58)—such as ammonium sulfate, sodium sulfate, and so on (Table 6.2). Consequently, HIC is sometimes correctly referred to as salt-promoted adsorption chromatography (SPAC; see Reference 36). Desorption of bound solutes is achieved simply by stepwise or gradient elution with buffers of low salt content. It is thus one of the simplest chromatographic separation methods in its modus operandi. It is also one of the earliest separation methods described in the literature.

The main chromatographic components that affect HIC are as follows:

- nature of the adsorbent (type of base matrix, structure, concentration of immobilized ligands, etc.)
- composition of the solvent (type and concentration of salts in the equilibration buffer, pH, and temperature)
- characteristics of the solute and other components of the sample.

Of these parameters, the type and concentration of ligand as well as the type and concentration of salt in the adsorption buffer (Table 6.2) are of paramount importance in determining the outcome of an HIC event. In general, the type of immobilized ligand determines its adsorption selectivity towards the proteins in a sample, and its concentration determines its adsorption capacity. The contributions of the three parameters outlined here will now be discussed briefly.

6.6.1 The Adsorbent (Stationary Phase)

Base matrices and spacers all contribute to hydrophobicity, which is why some more hydrophobic base matrices have lower ligand densities than more hydrophilic ones. The ideal polymer matrices useful for immobilizing ligands for HIC should, by themselves, not contribute significantly to the hydrophobicity of the HIC adsorbent. To this group belong hydrophilic, non-crosslinked, carbohydrate-based polymers (such as cellulose, dextran, and agarose). In order to increase the rigidity and flow characteristics of such polymers, a high degree of crosslinking has been necessary, with a subsequent increase in background hydrophobicity. Despite this, however, these natural polymers are still more hydrophilic than their synthetic counterparts (e.g., styrene-divinyl benzene copolymers) that have been “hydrophilized” by grafting low molecular weight hydroxyl compounds on these matrices.

6.6.1.1 Type of Ligand and Coupling Chemistries

The most widely used ligands for synthesizing HIC media are alkyl or aryl compounds linked covalently to an insoluble base matrix (17, 21, 22, 26, 27). The coupling chemistries used for immobilizing the ligands have profound effects on the adsorption characteristics of the HIC media. Thus, activation of the basic matrix with cyanogen bromide and coupling of alkyl amines (17, 21, 22) invariably leads to the introduction of positive charges on the medium. This in turn affects the chromatographic mode of operation; that is, adsorption is performed at low ionic strength and elution of bound proteins is achieved by increasing the salt concentration. Superficially, this mode of operation is similar to that in ion-exchange chromatography. In a few instances, the charged HIC media have also been operated at high salt concentration during the adsorption step, and elution is performed by decreasing the salt concentration (28, 35, 69). It thus appears that the charges introduced during the activation of the gel matrix with CNBr are sufficiently quenched by salt at a concentration of 0.3 M or higher (28, 35) such that the charged HIC media, and their uncharged counterparts, behave similarly in many of their hydrophobic properties and adsorption characteristics.

The second alternative for preparing HIC media is by direct coupling of glycidyl ether derivatives of aliphatic and aromatic alcohols (27) on the agarose gel. A third alternative is the coupling of n-alkyl thiols to epoxy-activated agarose, as described earlier in this chapter (31, 33, 34). In both instances, the resulting media are essentially charge-free and exhibit “pure” hydrophobic characteristics. In the case of the n-alkyl thiol-substituted gels, additional interactions of a charge-transfer character appear to exist (70). For structures of common HIC media, see Figure 6.1.

6.6.1.2 Alkyl Chain Length

At a constant degree of ligand substitution, a homologous series of n-alkyl derivatives of Sepharose™ exhibit increasing “hydrophobicity” with increasing chain length of the n-alkyl substituents (21, 24, 27). In a classical paper, Er-el and colleagues (21) showed that the adsorption characteristics of two purified muscle enzymes they chose to study (i.e., glyceraldehydes 3-phosphate dehydrogenase and phosphorylase b) were quite different.
upon chromatography on a series of hydrocarbon-coated Sepharose™ gels (i.e., Seph–C₈ series). They found that the glyceraldehyde 3-phosphate dehydrogenase was not bound on columns packed with Seph–C₁–Seph–C₆ media, but phosphorylase b was retarded on Seph–C₃ and strongly bound on Seph–C₄. This is a clear demonstration of the differences in affinity of the two enzymes for the same HIC adsorbents, varying only in their chain lengths. Their results also demonstrated that an increase in the chain length increases the strength of binding of proteins on such media. The most useful range of n-alkyl chain lengths lies between C₁ and C₈ for the majority of the proteins investigated to date. This is reflected in the small range of commercially available HIC media, which are dominated by C₁–C₄ and phenyl derivatives of various gel polymers. It can also be generalized that the those in the lower series (i.e., C₁–C₄) are most useful for strongly hydrophobic solutes and those in the range C₅–C₈, and possibly higher series, are most useful for weakly hydrophobic proteins. In the latter instance, one should be aware of the fact that the higher the n-alkyl ligand, the stronger becomes the solute–adsorbent interaction, to the extent that some proteins are bound irreversibly and require aqueous organic solvents for their elution. In most instances, this leads to the denaturation of the proteins upon elution from the column. Such a problem can possibly be minimized by using a lower series of n-alkyl substituted gels and varying the concentration of the immobilized ligand or the concentration of salt in the adsorption buffer. Another possible approach is to prepare step-graded hydrophobic adsorbents based on a homologous series of n-alkane residues (24, 36, 71) and serially connect the packed columns. The sample is applied to the connected columns, starting with the least hydrophobic one. The unbound fraction is then washed out from all the columns and the individual columns disconnected. The bound proteins on each column are then eluted with a suitable buffer. Such an experiment has indicated that each protein tends to be bound to the column that provides the minimum degree of hydrophobicity required for binding and thereby an increased recovery and purity of the protein of interest.

### 6.6.1.3 Concentration of Ligands

This is one of the most important parameters in HIC that affects the overall chromatographic performance of the adsorbent with respect to its adsorption capacity, strength of binding, recovery of bound solutes, and selectivity. An example is provided in Figure 6.2, where an increase in ligand concentration of a butyl Sepharose 4 Fast Flow gel results in an increase in both the selectivity and retardation of a mixture of four proteins run under standardized conditions. At a constant chain length of a hydrophobic medium, its adsorption capacity for proteins increases exponentially with an increase in the concentration of the ligands immobilized on its surface (27, 31, 35, 72–74) until it eventually reaches a constant plateau. This implies that any further increase in the ligand concentration does not lead to a noticeable increase in the adsorption capacity of the medium but rather to an increase in multipoint, or “multivalent,” attachment (27, 35, 74, 75) of the proteins on the surface of the adsorbent. This phenomenon has been ascribed to cooperative interaction between multiple ligands and a protein molecule (69).

In many instances, the tight binding of solutes on the adsorbent may lead to denaturation of the proteins upon elution. Although this is not desirable when purifying proteins, the concept has been exploited for the reversible immobilization of some enzymes to highly substituted agarose gels to form a so-called “enzyme reactor bed” (76, 77). A proof of this principle was provided by immobilizing β-amylase on a highly substituted hexyl-agarose gel. It was demonstrated that such an “enzyme reactor bed” could be used for month-long continuous hydrolysis of starch at room temperature.

An interesting method for “tuning-in” the hydrophobicity of the adsorbent with that of a protein of interest has been advocated by Jennissen (73, 75) since about 1975. The method, which he named “critical hydrophobicity,” involves the determination of the adsorption capacity of a series of alkyl-substituted Sepharose gels that differ only in the concentration of ligands immobilized on their surfaces. Each experiment was performed following a standardized procedure using a protein of interest as the sample. A plot of the amount of protein bound...
versus the ligand concentration invariably results in a sigmoidal curve. This curve is then used to establish the "critical hydrophobicity" of the adsorbent, that is, the degree of ligand substitution where the adsorption of a protein on the adsorbent just begins (73, 75). It has been suggested that the half-maximal saturation point on the sigmoidal curve also corresponds to the optimal ligand concentration for a given HIC medium (75). Despite the attractive features of the critical hydrophobicity concept, its application is limited (71) and has not attracted the attention of biotechnology companies that produce chromatographic media for general use. However, the concept is generally useful in terms of understanding the stochastic and thermodynamic nature of HIC and may, in the future, find application in the design of custom HIC media for specific applications.

### 6.6.2 The Mobile Phase

This section will deal with the components of the mobile phase and their effects on the outcome of an HIC event. The topics that shall be discussed are: type and concentration of salts, pH, temperature and additives.

#### 6.6.2.1 Type and Concentration of Salts

The effects of neutral salts on the adsorption of proteins to insoluble polymer matrices have been either one of a general character, affecting primarily the solubility of proteins in various salts (5–10), or of a specific nature where the salts act as modulators (or mediators) of the interaction between immobilized hydrophobic ligands on the surface of insoluble polymer matrices and hydrophobic patches on the surface of protein molecules (26, 27, 32, 33). The former takes place on non-derivatized polymer matrices in the presence of very high salt concentrations (e.g., 2 M ammonium sulfate or higher) and results in "zone precipitation" (6) or "group separation" of the constituents in the sample. The latter functions in a relatively low salt concentration (e.g., 0.5–1.7 M ammonium sulfate) on adsorbents carrying hydrophobic ligands and can give rise to good resolution of the bound proteins upon elution by a linear decrease in the salt concentration.

The first demonstration of the role of high concentrations of salts in HIC was provided by Porath and colleagues (26) in 1973. They showed that, at pH 7.0, the capacity of the packed medium (benzylated Sepharose 6B) for cytochrome c increased by a factor of eight as the concentration of NaCl in the equilibration buffer was increased from 1 M to 3 M. When the experiment was repeated at pH 3.0, the capacity of the column for cytochrome c was increased by a factor of 15 as the NaCl concentration in the equilibration buffer was increased from 0 to 1 M. The basic concepts that Porath and colleagues (6) introduced in this one publication can be considered as the foundation for HIC as it is practised today. Following this, Hjertén and colleagues (27–30), and many other research groups, published several papers and firmly established HIC as a new method in the protein separations field.

The effects exerted by salts in HIC parallel those of their established effects on the precipitation of proteins (see Reference 57 and Table 6.2). Thus, both anions and cations that are on the left side of Table 6.2 stabilize protein structures and have a positive effect on HIC. Those that are on the right side of Table 6.2 have a destabilizing effect on protein structure ("chaotropes") and a negative effect ("salting in") on HIC. Thus, the type of salt used for the adsorption of proteins to HIC media is of paramount importance. So is the concentration of salt used in the binding buffer, as this can affect the selectivity and capacity of the adsorbent for the target protein.

The relationship between the concentration of salt in the equilibration buffer and the adsorption capacities of a number of HIC media for human serum albumin (HSA) is of a convex nature (Fig. 6.3). At low concentrations of ammonium sulfate in the equilibration buffer (~0.4–0.8 M), the relationship is apparently linear. As the salt concentration is increased further, the relationship becomes nonlinear and is of a markedly convex form. The results seem to indicate that, as the ammonium sulfate concentration in the equilibration buffer is increased, the applied protein begins to

![Figure 6.3](Image 347x289 to 585x438) Adsorption capacities of five HIC media for purified HSA as a function of the ammonium sulfate concentration in the binding buffer. The media (see inset for details) are products of GE Healthcare Life Sciences, Uppsala, Sweden. Method: frontal analysis to full saturation. Column: XK16/10 packed with 20 mL of each media. Protein: 4 mg/mL of purified HSA dissolved in binding buffer. Binding buffer: 20 mM sodium phosphate containing the indicated concentrations of ammonium sulfate, pH 7.0. Elution buffer: 20 mM sodium phosphate, pH 7.0. Flow rate: 3 mL/min (90 cm/h). Butyl Sepharose 4 FF has a higher capacity than Butyl-S Sepharose 6 FF, mainly due to its higher ligand concentration (50 μmol/mL) compared with the latter (10 μmol/mL). The commercially available HIC media shown here cover a wide range of hydrophobicities in order to accommodate the large differences in hydrophobicity of proteins in biological extracts. (Work from GE Healthcare Life Sciences, Uppsala, Sweden.)
aggregate or “precipitate” on the column. The results also indicate the following:

1. At \(\sim 0.3\) M ammonium sulfate, practically no protein is bound on any of the adsorbents.
2. The amount of HSA bound increases with increasing ligand concentration (see above), regardless of the nature of the ligand (phenyl or alkyl) immobilized on the gel.
3. Phenyl Sepharose 6 FF (high sub) has a relatively high capacity even at low salt concentrations (<1 M ammonium sulfate). It can thus be used in situations where the solutes of interest are weakly hydrophobic or to bind proteins at relatively low salt concentrations. The latter point is getting more attention today (71) due to environmental considerations.

The effect of different salts (at constant ionic strength) on the binding of proteins to HIC adsorbents approximately follows the order (30)

\[
\text{Na}_2\text{SO}_4 > (\text{NH}_4)_2\text{SO}_4 > \text{NaCl} > \text{NH}_4\text{Cl} > \text{NaBr} > \text{NaSCN}
\]

Of these salts, \(\text{Na}_2\text{SO}_4\), \(\text{NaCl}\), and particularly \((\text{NH}_4)_2\text{SO}_4\) are most often used in practical HIC experiments. Apart from differences in strength of interaction, the different salts also show some additional selectivity (78).

### 6.6.2.2 pH and Temperature

The effect of pH in HIC is dubious and not straightforward. In general, an increase in pH weakens hydrophobic interactions (26, 28), probably as a result of increased titration of charged groups, thereby leading to an increase in the hydrophilicity of the proteins. On the other hand, a decrease in pH results in an apparent increase in hydrophobic interactions (26). Therefore, proteins that do not bind to a HIC adsorbent at neutral pH bind at acidic pH (43). Hjerten and colleagues (78) found that the retention of proteins changed more drastically at pH values above 8.5 and/or below 5 than in the range pH 5–8.5 (Fig. 6.4). There are also indications that some proteins with high pI values bind strongly to HIC media at elevated pH values, although no general trend has been observed linking the strength of binding to the pI of proteins. These findings thus suggest that pH is an important separation parameter in the optimization of HIC experiments and it is therefore advisable to check the applicability of these observations to the particular separation problem at hand.

As discussed earlier (see Section 6.5), the binding of proteins to HIC adsorbents is entropy-driven [i.e., \(\Delta G = (\Delta H - T\Delta S)\)], which implies that the interaction increases with an increase in temperature (74, 79–82). It is interesting to note that the van der Waals attraction forces, which operate in hydrophobic interactions (54), also increase with an increase in temperature (83). However, an opposite effect was reported by Visser and Strating (84), indicating that the role of temperature in HIC is of a complex nature. This apparent

![Figure 6.4](image)

**Figure 6.4** The pH dependence of the interaction between proteins and an octyl agarose gel expressed as \(V_e/V_T\) (where \(V_e\) is the elution volume of the different proteins and \(V_T\) is the elution volume of a nonretarded solute). Elution by a linear decrease in salt concentration. Model proteins: STI (soy trypsin inhibitor), A (human serum albumin), L (lysozyme), T (transferring), E (enolase), O (ovalbumin), R (ribonuclease), ETI (egg trypsin inhibitor), C (cytochrome c). (Adapted from Reference 78.)
discrepancy is probably due to the differential effects exerted by temperature on the conformational state of different proteins and their solubility in an aqueous environment.

In actual laboratory work, one should thus be aware that a downstream purification process developed at room temperature might not be reproduced in the cold room, or vice versa.

6.6.2.3 Additives

Low concentrations of additives (e.g., water-miscible alcohols, detergents) compete effectively with proteins for the binding sites on HIC ligands. This results in the weakening or abolition of protein--ligand interactions, leading to the displacement or elution of the bound proteins. Solutions of chaotropic salts also have a similar effect by virtue of their negative effects on the ordered structure of water close to the proteins and immobilized HIC ligand. Both types of additives also decrease the surface tension of water (Table 6.3), which also has a negative effect on hydrophobic interactions. In some instances, additives have been used to improve selectivity during desorption of bound proteins, but one must be aware of the risk that proteins could be denatured upon exposure to such additives. On the other hand, some additives can be very useful for cleaning in place (CIP) of HIC columns exposed to very hydrophobic solutes.

6.6.3 The Solute (Sample)

Any biological extract or fluid contains a wide variety of macromolecular solutes that differ in size, composition, charge, solubility, intrinsic hydrophobicity, and so on. The purification of a single protein from such a mixture relies upon a cascade of biochemical separation methods that exploit differences, however small, in the surface characteristics of the impurities and the protein of interest. HIC is one such method, but the mechanism underlying the adsorption of proteins on HIC surfaces, in the presence of high concentrations of salt [so called salt-promoted adsorption (36)], is a matter of conjecture. Existing theories that attempt to address this issue have already been discussed in Section 6.5 of this chapter. In the following, an attempt will be made to present the various viewpoints regarding the intrinsic and salt-induced hydrophobicity of proteins.

It is estimated that as much as 40–50% of the accessible surface area of proteins is nonpolar (50, 51) and would theoretically be available for interaction with hydrophobic ligands immobilized on gel matrices. For this to materialize, one assumes that the hydrophobicity of protein surfaces is the sum of the hydrophobicities of the exposed amino acids.

To estimate the hydrophobicity of the individual amino acids, two approaches have been used. The first is based on direct measurements of the solubilities of individual amino acids in water and organic solvents (85–88) or the free energy of transfer of the amino acids from ethanol or dioxane to water. The second is based on empirical inspection of known protein structures (52, 89–92). Here, several hydrophobicity scales are based on, for example, the environment of the different amino acids, the fraction of amino acids that is buried in the protein, a side-chain interaction parameter, or a fractional accessibility to the surrounding solvent of the different residues. For a comparative study of published hydrophobicity scales, see the work by Cornette and colleagues (93).

According to Melander and Horvath (58), the above discussions do not give sufficient insight about the relative hydrophobicity of proteins, a parameter that is very useful in the context of HIC. They also concluded that neither the average hydrophobicity nor the frequency of the nonpolar side chains correlate well with the relative surface hydrophobicity of the proteins they examined. To overcome this situation, they proposed the use of a probe that measures the actual nonpolar surface area instead of the total surface area. To this end, they proposed using salting-out data where the protein molecules themselves act as hydrophobic probes in the salting-out process. However, because salting-out and retention data are not readily available for the large majority of proteins, they must be determined on a case to case basis. It is also worth mentioning that recent reports in this area (94, 95) do not seem to add much substance to what is already known. Most deal with such topics as prediction of retention times in HIC based on three-dimensional structural data, and identification of hydrophobic sites on protein surfaces using spectroscopic and fluorescence techniques. These topics are highly specialized and will not be dealt with in any detail in this chapter.

6.7 EXPERIMENTAL DESIGN

6.7.1 Strategic Considerations

Because the factors that impact HIC are numerous, the relevant chromatographic parameters that lead to the selective purification of the protein of interest should be optimized on a case to case basis. The important factors that impact HIC have already been outlined in Section 6.6 of this chapter. In the following, a summary of some practical hints that may be of assistance when planning, performing, and optimizing an HIC experiment will be presented.

The basic aim when designing a downstream purification protocol is to keep the number of unit operations (i.e., capture, intermediate purification, and polishing) to the bare minimum, so that an essentially “pure” product is obtained with the highest possible recovery and at a minimum cost. To attain this goal, a logical approach is to combine the separation techniques on the basis of different principles and thereby exploit differences in the surface properties of the substances to be separated. However, the sequence in which the chosen techniques are used must be carefully analyzed and executed. In many applications, HIC is particularly suitable to use after
techniques that leave the sample in a high salt concentration (e.g., initial precipitation with ammonium sulfate, affinity-based separations, ion-exchange chromatography) as the sample can often be applied directly. Used as the first step in a downstream purification process, HIC can also serve as an effective step for concentrating dilute samples.

The conductivity of most cell culture supernatants is in the range of 15–30 mS/cm, which makes HIC an attractive alternative to IEC as the first step in the downstream purification scheme of a target protein in such a biological sample. Other types of biological samples have even higher conductivities, and some type of conditioning (e.g., desalting, diafiltration, dilution) is therefore necessary before IEC is used as the initial capture step. In contrast, the only conditioning that is necessary when using HIC is to add sufficient salt to the sample to promote the binding of the solute to the adsorbent. In instances where the biological extract has been precipitated by high concentrations of ammonium sulfate, HIC fits in naturally as the first step of a downstream purification process.

6.7.2 General Considerations

The following is a list of some important “take home messages” that one should be aware of when using HIC in a downstream purification process:

1. The HIC medium should bind the target protein at a reasonably low concentration of salt. This is often dependent on the type of salt chosen (e.g., up to four times higher concentration of NaCl might be necessary to obtain a binding effect comparable to that obtained with ammonium sulfate).

2. The salt concentration used should be below the concentration that causes precipitation of different proteins in the feed stock. Ammonium sulfate (1 M) is a good starting concentration for screening experiments. If the target protein is not bound on the column, then a more hydrophobic medium should be chosen. The correct choice of HIC medium can often lead to lower consumption of salt in the process. This in turn has a bearing on the economical and environmental aspects of the purification process, especially for large scale applications.

3. The bound protein should be eluted with salt-free buffer and with high recovery (80% and higher). If nonpolar solvents are required for elution, a less hydrophobic medium should be tried.

4. The pH of the starting buffer and the type of salt used can be exploited to maximize selectivity during the adsorption process. This is achieved by checking the adsorption properties of the medium at different pH values and with different types of salts when screening different media.

5. Because the hydrophobic interaction is dependent on temperature, it is important that method development is performed at the intended final working temperature.

6.8 APPLICATIONS

HIC has found many applications in the purification of a wide variety of proteins, including

- monoclonal antibodies for diagnostic or clinical applications
- vaccines
- truncated forms of r-proteins
- interferons
- EGF (epidermal growth factors)
- human growth hormone.

In the following will be presented some further examples with some details of the experimental conditions used in order to illustrate the main elution protocols that are used for elution of proteins bound to HIC media.

6.8.1 Purification of Human Pituitary Prolactin (96): Elution by Decreasing Polarity

In the purification of human pituitary prolactin, HIC on Phenyl Sepharose was used (96). The entire preparation was performed at 5°C, and the starting material was frozen human pituitary glands. The glands were homogenized and extraction was carried out at elevated pH. The first chromatographic step was gel filtration on Sepharose CL-6B at pH 9.8. The fractions that contained prolactin were pooled and chromatographed on a column packed with Phenyl Sepharose (Fig. 6.5). To this column (3.2 × 25 cm) was applied a sample containing 200–400 mg of protein. The column was equilibrated with 0.2 M glycine/NaOH buffer (pH 9.8) and the elution was carried out at pH 9.8 by a stepwise decrease in the concentration of buffer (down to 0.02 M glycine/NaOH) and finally, by inclusion of ethylene glycol (50%, v/v). Fractions of about 15 mL were collected at a flow rate of 45 mL/h. The prolactin activity was eluted by the buffer containing ethylene glycol and recovery was 95%. The purification of the prolactin was completed by an additional gel filtration (Sephadex G-100 Superfine) and an ion-exchange step on DEAE-Sepharose CL-6B.

6.8.2 Purification of Recombinant Hepatitis B Surface Antigen (r-HBsAg): Elution by Stepwise Decrease in Salt Concentration (97)

Recombinant hepatitis B surface antigen is a vaccine against hepatitis B virus infection that is produced by genetically
modified Chinese hamster ovary (CHO) cells grown in Dulbecco’s modified Eagle’s medium containing 3–5% fetal calf serum. The cells were harvested every 48 h and the supernatant separated from the cells. In the supernatant was dissolved solid ammonium sulfate to a concentration of 0.6 M. The pH was adjusted to 7.0 and then centrifuged (or filtered over a membrane) to remove cell debris and other particles. The clarified supernatant was used as the starting material for purifying the r-HbsAg by an essentially three-step chromatographic process, that is, HIC, IEC, and gel filtration. A typical laboratory-scale purification process, at the capture step, is described briefly below.

All experiments were performed at room temperature (22°C). A column (XK 50/20), was packed with Butyl-S Sepharose(TM) 6 Fast Flow (where –S indicates sulfide) to a volume of 130 mL and equilibrated with Buffer A (20 mM sodium phosphate, 0.6 M in ammonium sulfate, pH 7.0). The flow rate was maintained at 2 L/h (100 cm/h). To this column was applied 300 mL of the clarified and concentrated cell culture supernatant (CCS) containing 12 mg of the r-HbsAg. This was followed by washing with Buffer A to elute the unbound fraction. The bound fraction (containing the r-HbsAg) was eluted with Buffer B (20 mM sodium phosphate, pH 7.0). Any strongly bound material was then eluted with buffer C (30% isopropanol dissolved in Buffer B). Finally, the column was washed with 0.5 M NaOH solution (2 CV) for sanitization purposes and to remove any material that might still be bound to the column.

A typical chromatographic elution profile is shown in Figure 6.6. The absorbance at 280 nm and distribution of
r-HBsAg activity (an ELISA-based assay) were determined for each of the three pooled fractions. The results showed the following:

1. At least 92% of the A<sub>280</sub> applied to the column is eluted in the flow-through fraction (Fig. 6.6a). The major components in this fraction are low molecular weight solutes (amino acids, pH indicators, etc.), as well as calf serum proteins (IgG, BSA, transferring, fetuin, and so on).

2. The bound fractions (Fig. 6.6b, c) account for \( \approx 6\% \) of the applied A<sub>280</sub>, but for \( \approx 85\% \) of the r-HBsAg activity. Figure 6.6 also shows the SDS-PAGE (non-reduced) patterns of the CCS, fraction B and the purified r-HBsAg obtained after the three-step purification process for r-HBsAg in the CCS of transformed CHO cells.

6.8.3 Purification of a Phospholipase C from *Trypanosoma brucei*: Elution by Detergent (98)

In the purification of phospholipase C from *T. brucei*, HIC was used at an early stage. The trypanosomes were lysed and the membranes solubilized and extracted with buffers containing \( n \)-octyl glucoside. A fraction containing the phospholipase activity was precipitated with an equal volume of saturated ammonium sulfate solution. After centrifugation, the supernatant (50 mL, containing 8.7 mg protein) was applied to a Phenyl Sepharose column (1.2 \( \times \) 14 cm) equilibrated with 50% saturated ammonium sulfate in 25 mM sodium succinate, pH 6.0 (Fig. 6.7). A linear gradient (100 mL) with decreasing salt concentration from 50% saturation to buffer without ammonium sulfate was applied, followed by further washing with 50 mL buffer. The phospholipase was eluted with buffer containing detergent, 1% CHAPS (3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate). The chromatogram (Fig. 6.7) shows that most of the protein was eluted in a sharp peak after introducing the detergent. On the other hand, the phospholipase was eluted in a broad peak. The trailing fractions of the activity peak, which contain \( \sim 70\% \) of the activity but relatively little protein, were pooled. The yield in the HIC step was 62% and the purification 22-fold. The HIC step was followed by an ion-exchange step (CM-Sephadex C-25) and gel filtration chromatography (Sephacryl S-200).

6.8.4 Exchange of Detergents Bound to Membrane Proteins (99)

Unfortunately, with most intrinsic membrane proteins, no single detergent is usually well suited to all the different steps in the purification scheme. Methods for detergent exchange are therefore needed. One method is phenyl-Sepharose mediated detergent-exchange chromatography. The alkyl detergents lauryl maltoside, octyl glucoside, and dodecyl sulfate were each successfully exchanged for Triton X-100, Triton N-101, or Nonidet P-40 present in solution of either cytochrome c oxidase, a mixture of inner mitochondrial membrane proteins, or a mixture of erythrocyte membrane proteins. Below follows a description of an exchange of lauryl maltoside for cytochrome c oxidase bound Nonidet P-40 (Fig. 6.8).

A 0.5 \( \times \) 10 cm bed of Phenyl Sepharose was presaturated with 13 mL of 10 mM lauryl maltoside in a pH 9.0 buffer at an ionic strength of 0.01 (Tris-HCl containing 0.1 mM EDTA), followed by 8 mL of 2 mM lauryl maltoside in the same buffer. A 0.6 mL protein sample was applied (containing 3 mg of protein/mL and 26 mM Nonidet P-40) to the above buffer, to which enough lauryl maltoside was added to achieve a concentration of 2 mM. Elution was effected by the above buffer containing 2 mM lauryl maltoside, and

![Figure 6.7](image1.png)

*Figure 6.7* HIC of a crude preparation of phospholipase C from *Trypanosoma brucei*. The vertical arrow shows the point at which elution with 1% CHAPS was initiated. Fraction numbers are shown on the abscissa; note the change in scale after fraction 44. (Reprinted from Reference 98 with permission.)

![Figure 6.8](image2.png)

*Figure 6.8* Exchange of lauryl maltoside for protein-bound Nonidet P-40. (Reprinted from Reference 99 with permission.)
fractions were collected and analysed. At pH 9 and an ionic strength of 0.01, only 5–15% of the Nonidet P-40 solubilized cytochrome c oxidase complex was bound to the column that had been saturated with lauryl maltoside. Less than 0.1% of the original amount of Nonidet P-40 remained in the complex after detergent exchange.

6.8.5 Purification of Alcohol Dehydrogenase by Expanded Bed Adsorption (EBA): Elution by Decreasing Salt Concentration (100)

Expanded bed adsorption (EBA) using a Streamline-Phenyl media was used for direct recovery of alcohol dehydrogenase (ADH) from yeast cell homogenate (1). Bakers’ yeast was disrupted by high pressure homogenization (five discrete passes at 500 bar). For the EBA a Streamline-50 column containing 250 mL Streamline-Phenyl matrix was equilibrated and expanded at 200 cm/h, with the upper adaptor fixed at 0.7 m. The equilibration buffer was 0.02 M potassium phosphate, pH 7, containing 0.78 M ammonium sulfate. The yeast homogenate was adjusted to 0.78 M ammonium sulfate, using solid salt, after the homogenate had been adjusted (diluted) to a protein concentration of 10 mg/mL. When the sample had been applied to the column, the media was allowed to settle, and the adaptor was adjusted down to the settled bed. Elution was thus performed with a settled bed and was done in reverse flow direction. The elution buffer used was 0.02 M potassium phosphate, pH 7. A typical chromatogram is shown in Figure 6.9. The purification factor for the EBA step was in the range 8–14, and the yield typically above 90%. The recovery was followed for five cycles. No reduction in recovery was seen using the Streamline-Phenyl media; this is in contrast to experiments where packed media were used.

The main benefit of using EBA is that it is possible to reduce the number of steps in a process by the elimination of the clarification steps needed if a packed bed is used in the capture step.

6.9 REFERENCES

IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY

LENNART KÅGEDAL
GE Healthcare Bio-Sciences AB, SE-751 84 Uppsala, Sweden

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7.1 INTRODUCTION

In 1975, Porath and colleagues published a paper entitled “Metal chelate affinity chromatography, a new approach to protein fractionation” (1). The principle of the suggested method was based on differences in the affinity of proteins for metal ions bound in a 1:1 complex of iminodiacetic acid (IDA) immobilized on a chromatographic support. The principle was not new; it had already been suggested in 1961 by Hellferich, who named it “ligand exchange chromatography” (LEC) (2), and the technique has been widely used since then. However, Porath’s paper showed for the first time that immobilized metal ions could be used to advantage to frac-
tionate and purify proteins. As will be demonstrated in this review, the technique has become an important tool for the isolation of many proteins.

In the past, the term metal chelate affinity chromatography (MCAC) was the accepted term for LEC when used in bio-
chemistry. Other names have also been used, such as metal chelate interaction chromatography (MCIC). Following a proposal by Porath and co-workers (4), the term immobilized metal ion affinity chromatography (IMAC) will be used in this chapter.

A comprehensive review of LEC was published in 1977 by Davankov and Semechkin (3). The theory they presented is also, to a large extent, relevant to IMAC, and we refer to them for a more complete coverage of the literature up to 1977. Some general aspects of the technique as applied to proteins...
were discussed in early reviews by Porath and Belew (4), Lönnerdal and Keen (6), Sulkowski (7), Porath and Olin (8), Porath (9), Rassi and Horvath (10), and Chaga (11). Mathematical modeling of IMAC was carried out by Vunnum and co-workers (12).

A number of reviews have been published in recent years. The articles by Ueda, Gout and Morganti (13), Gaberc-Porekar and Menart (14), and Chaga (15), paint a general picture of the development of IMAC, all with a certain emphasis on the use of IMAC for the purification of histidine-tagged proteins, which has become very popular and a very important tool in molecular biology in particular. It is the specific subject of reviews by Bornhorst and Falke (16) and by Fiedler and Skerra (17). The use of hard metal ions such as $\text{Fe}^{3+}$ for the purification of phosphorylated proteins and peptide has also been further developed in the search for useful preparative methods, as shown in reviews by Anderson (18) and by Holmes and Shiller (19). IMAC has also found widespread use as a purification tool in high throughput production of proteins for functional proteomics (20).

Most proteins can form complexes with metal ions. Many of these are multidentate complexes (chelates) that allow for the purification of the proteins by IMAC. The strength of the complexes formed varies from protein to protein, which, in many cases, gives rise to the high specificity of IMAC.

The chromatographic sorbent used in IMAC (see scheme in Fig. 7.1) consists of a suitable chromatographic support to which a metal-chelating substance (B) has been attached by a leash or linkage group (A). The structure of the complex formed when metal ions are added must be such that some coordination sites are left free for the binding of solvent or solute molecules (ligands). Alternatively, the complex should be able to rearrange itself to allow incoming ligands to participate in the formation of chelates or complexes with the metal ion. Solvent or buffer molecules will occupy “free” coordination sites of the metal in the absence of ligands with higher affinity for the metal ion.

Muzzarelli and colleagues have suggested the following definition of LEC (21): “Ligand exchange chromatography is based on the principle that a molecule or ion, which is part of a complex fixed on a support, can be released because a different molecule or ion enters to form a more stable complex, or because the complex collapses when the medium is altered.” The definition may be somewhat limited compared to that proposed by Davankov and Semechkin (Reference 3, p. 314) but it gives a sufficiently clear basis for the understanding of IMAC as reviewed in this chapter.

Some of the special features of IMAC of proteins can be summarized as follows:

- Exposure of certain amino-acid residues (histidine, cysteine, tryptophan) on the “surface” of the proteins is required for the adsorption of proteins.
- The steric arrangement of the protein chain plays an important role, which means that molecules with closely similar properties with respect to charge, molecular size, amino-acid composition, but with differences in their secondary and tertiary structure, can be separated.
- Simple ionic adsorption and other complicating factors can be suppressed or modified by buffers of high ionic strength.
- Binding is influenced by pH. Low pH causes elution of adsorbed substances. Exceptions to this are known (22).
- The technique can be made very efficient and simple in performance by the use of histidine tags.
- Several elution techniques are available (pH gradient, competitive ligands, organic solvents, chelating agents).

![Figure 7.1 Principle of immobilized metal ion affinity chromatography (IMAC). A = linkage (spacer) group, B = chelating group, Me = metal ion, L = solvent or buffer molecule.](image-url)
IMAC is a general technique for purifying proteins. Metalloproteins do not bind specifically at their metal coordination sites but rather through amino-acid residues exposed at the protein surface.

Clearance of viruses has been shown to be effective in a number of cases (23).

IMAC is an established technique for the purification of proteins on a laboratory scale and is beginning to find industrial applications. Its acceptance may have been rather slow initially, but its usefulness and versatility has been unequivocally demonstrated to a sufficient extent to convince biochemists that it is a valuable addendum to the arsenal of preparative methods.

The apparent complexity of the method because of the number of factors influencing the IMAC process may have added to the reluctance of biochemists to adopt the method. Although there are several question marks remaining concerning the exact mechanisms and chemical structures involved, there is sufficient information available for a systematic optimization of preparative experiments. For example, we know how to modify adsorption and desorption by the correct choice of chelating group, metal ion, pH, and buffer constituents.

The specificity of IMAC obviously depends on the exploitation of the combined effects of primary structure (occurrence and position of limited number of metal-binding amino-acid residues) and secondary and tertiary structure (exposure of certain amino acids on the protein surface). It may well be that the degree of proximity of the metal-binding amino acids are also important.

7.2 METAL CHELATE GELS

7.2.1 Choice of Chromatographic Support

Basically, the requirements of the support in affinity chromatography of biological molecules apply also to IMAC (Reference 24, p. 20). Ideally the support should have the following features:

- It should be easy to derivatize.
- It should exhibit no unspecific adsorption.
- It should have good physical, mechanical, and chemical stability.
- It should be of high porosity to provide easy ligand accessibility.
- It should permit high flow rates.
- It should be stable to eluents, including, for example, denaturing additives.
- It should allow regeneration of the column without deterioration of the gel bed.
- It should provide a stable gel bed with no shrinking/swelling during the chromatographic process.

Beaded agarose is the support predominantly used for IMAC of proteins, and published protocols in most cases describe the use of Sepharose™ 4B or Sepharose 6B to which iminodiacetic acid (IDA) had been coupled by the original bisoxirane method described by Porath and colleagues (1).

Silica-based IDA gels have been constructed for use in preparative work (25–28). Gels for use in high performance liquid chromatography (HPLC) and fast performance liquid chromatography (FPLC) have been prepared using hydrophilized resins (29, 30), crosslinked agarose (31; K.-A. Hansson, G. Moen, L. Kågedal, unpublished results) (10 μm), and silica (32) (5 μm) using IDA as the chelating ligand.

Membranes have also been used as supports for chelating groups (33).

7.2.2 Chelating Ligand and Strength of Chelates: Capacity for Metal Ions

IMAC in general can be carried out using various types of ion exchangers (3). In IMAC of proteins, however, only chelating groups have been used to fix the metal ion to the support, the reason being that metal ions bind more strongly to chelating groups. The binding energy of transition-metal cations, as calculated by Schmuckler, is 2–3 kcal/mol with ordinary cation exchangers and 15–25 kcal/mol with chelating groups (34). Table 7.1 lists the formation constants for 1:1 chelates of some chelating compounds. Compounds I and II have a structure similar to that of the chelating group in IDA Sepharose and compound III is a close analog of the so-called TED group, introduced by Porath and Olin (8). Studies of the formation constants of the IDA groups of Dowex A-1 indicate that formation constants of the same order of magnitude are obtained for analogous compounds free in solution or bound to a chromatographic support (3).

The relative stability of complexes formed with an IDA derivative of cellulose and divalent metal ions are in the order Cu(II) > Ni(II) > Zn(II) ≥ Co(II) ≥ Ca(II, Mg(II), as shown by Horvath and Nagydiosi (35). They also concluded that Ca(II) and Mg(II) ions do not form chelates with the IDA group because their presence did not affect the shape of the potentiometric acid–base titration curve.

The fixing of the IDA ligand to the support has in many cases been effected by Porath’s original method of coupling to agarose into which reactive epoxy groups have been introduced by reaction with 1,4-bis-(2,3-epoxypropoxy)-butane (1). The method has the dual advantage of providing a chemically stable ether linkage to the ligand and a 12-atom spacer. A three-atom spacer is obtained if activation of the agarose into which reactive epoxy groups have been introduced by reaction with 1,4-bis-(2,3-epoxypropoxy)-butane (1). The method has the dual advantage of providing a chemically stable ether linkage to the ligand and a 12-atom spacer. A three-atom spacer is obtained if activation of the agarose into which reactive epoxy groups have been introduced by reaction with 1,4-bis-(2,3-epoxypropoxy)-butane (1). The method has the dual advantage of providing a chemically stable ether linkage to the ligand and a 12-atom spacer. A three-atom spacer is obtained if activation of the agarose into which reactive epoxy groups have been introduced by reaction with 1,4-bis-(2,3-epoxypropoxy)-butane (1). The method has the dual advantage of providing a chemically stable ether linkage to the ligand and a 12-atom spacer. A three-atom spacer is obtained if activation of the agarose into which reactive epoxy groups have been introduced by reaction with 1,4-bis-(2,3-epoxypropoxy)-butane (1). The method has the dual advantage of providing a chemically stable ether linkage to the ligand and a 12-atom spacer.
Results obtained by Hansson and colleagues (unpublished results) show that the metal ion capacity of the IDA gel influences the retention of proteins (Fig. 7.2). IDA derivatives of SuperoseTM 12 carrying varying amounts of the chelating moiety were used. The resolution of the sample proteins was also affected, indicating that the extent of the influence on retention is dependent on the properties of the proteins. Commercially available column packings for IMAC of proteins are listed in Table 7.2.

### 7.3 FACTORS INFLUENCING ADSORPTION AND DESORPTION

#### 7.3.1 Chelate Structure and Metal Ions

The exact structure of the stationary complex depends upon the metal ion used and on the composition of the eluent buffer. According to Davankov and colleagues, Zn\(^{2+}\) and Cu\(^{2+}\) will leave one and Ni\(^{2+}\) three sites for solvent or buffer molecules when the three-dentate IDA is used (3). Others have published results showing octahedral coordination of the Zn\(^{2+}\) and Cu\(^{2+}\) ions (38, 39).

Figure 7.3 shows the postulated planar structure of the chelate formed between Cu\(^{2+}\) and Chelating Sepharose Fast Flow including the linkage group. One coordination site is occupied by a water molecule (or possibly by some buffer constituent) that will be substituted by a sample molecule during the course of the chromatographic experiment.

Porath and Olin have used N,N,N-tricarboxymethylethylene diamine coupled to Sepharose (TED Sepharose) (8). Because the TED group is a five-dentate chelating group, it can occupy five of the six coordination sites of the Ni\(^{2+}\) ion. Consequently, Ni\(^{2+}\)-TED Sepharose is a weaker adsorbent than the corresponding IDA column (8, 40, 41); this is demonstrated by, for example, the adsorption on Ni\(^{2+}\)-IDA Sepharose of proteins that pass through a Ni\(^{2+}\)-TED Sepharose column. Of great importance for IMAC technology were the publications by Hochuli and colleagues (42) about the use the Ni\(^{2+}\) chelate of a nitrilotriacetic acid derivative (NTA) for proteins containing adjacent histidine residues.

O-Phosphoserine (OPS), a tridentate chelating group, has been found to afford different selectivities compared to IDA (43). Carboxymethylated aspartic acid (CM-Asp) and NTA are tetradentate chelating groups. Some other chelating groups are mentioned in Table 7.2.

It is reasonable to assume that the sorption of ligands from the sample will cause rearrangement of the stationary complex (Reference 3, p. 326), which in turn may alter the stability of some of the stationary complexes. Such a supposition is supported by the observation that when a protein sample is applied to a column of chelating gel incompletely charged with copper ions, that is, the lower part of the column is still white, the blue zone of the copper complex will move down the column to some extent (L. Kågedal, unpublished data).

Cu\(^{2+}\), Ni\(^{2+}\), Zn\(^{2+}\), and Co\(^{2+}\) form a series of metal ions with decreasing binding strength to IDA as shown by Porath and others (4, 7, 44). Other ions such a Cd\(^{2+}\) may be used in a similar way (24).

Ca\(^{2+}\) and Fe\(^{3+}\) seem to represent special cases. Ca\(^{2+}\) has been used for the purification of *Dolichos biflorus* seed lectin (45) and factor VIII:C coagulant activity. The lectin has very high affinity for Ca\(^{2+}\) and was eluted from the column using EDTA. The special character of the interaction of Fe\(^{3+}\)-IDA gels with proteins is reflected in the requirement for special elution modes, see Section 7.3.3.

---

### TABLE 7.1 Formation Constants of Complexes of Chelating Compounds and Metal Ions

<table>
<thead>
<tr>
<th>No.</th>
<th>Formula</th>
<th>Ca(^{2+})</th>
<th>Fe(^{2+})</th>
<th>Fe(^{3+})</th>
<th>Co(^{2+})</th>
<th>Ni(^{2+})</th>
<th>Cu(^{2+})</th>
<th>Zn(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td><img src="image1" alt="Formula" /></td>
<td>3.8</td>
<td>6.3</td>
<td>7.6</td>
<td>8.7</td>
<td>11.1</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td><img src="image2" alt="Formula" /></td>
<td>4.8</td>
<td>6.8</td>
<td>8.1</td>
<td>9.4</td>
<td>11.7</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td><img src="image3" alt="Formula" /></td>
<td>8.2</td>
<td>12.2</td>
<td>19.5</td>
<td>14.5</td>
<td>17.1</td>
<td>17.5</td>
<td>14.6</td>
</tr>
</tbody>
</table>

*From Reference 36 with permission.

*b* In the chelate, the ligand is present in its ionized form.
<table>
<thead>
<tr>
<th>Designation</th>
<th>Manufacturer</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iminodiacetic acid Sepharose® CL-4B Matrix, Iminodiacetic acid Sepharose®, and Iminodiacetic acid StreamLine®</td>
<td>Affiland, Belgium</td>
<td>IDA bound to Sepharose and StreamLine matrices</td>
</tr>
<tr>
<td>PDC-StreamLine® Quartz Base Matrix, PDC-Sepharose® 4 Fast Flow Matrix, and PDC-Sepharose® CL-4B Matrix Polyvalent metal ion binding resin</td>
<td></td>
<td>Pentadentate chelator bound to Sepharose and StreamLine matrices</td>
</tr>
<tr>
<td>High Density IDA—Agarose 6 BCL Metal Free, Low Density IDA—Agarose 6 BCL Metal Free</td>
<td>GE Healthcare Life Sciences</td>
<td>No data available</td>
</tr>
<tr>
<td>Chelating Sepharose Fast Flow</td>
<td></td>
<td>Also available charged with Cu²⁺, Ni²⁺, Zn²⁺, or Co²⁺</td>
</tr>
<tr>
<td>Chelating Sepharose® 6B</td>
<td></td>
<td>Agarose, IDA</td>
</tr>
<tr>
<td>Chelating Superose®</td>
<td></td>
<td>Crosslinked agarose, IDA</td>
</tr>
<tr>
<td>Ni Sepharose High Performance</td>
<td></td>
<td>Crosslinked agarose, 34-µm beads, delivered charged with Ni²⁺</td>
</tr>
<tr>
<td>HiTrap™ Chelating</td>
<td></td>
<td>Crosslinked agarose, prepacked columns, 1 mL and 5 mL, IDA</td>
</tr>
<tr>
<td>POROSTM MC</td>
<td>Applied Biosystems</td>
<td>IDA bound to hydrophilized polystyrene-divinylbenzene particles; Contains magnetic agarose particles</td>
</tr>
<tr>
<td>QuickPick™ IMAC kits for proteins</td>
<td>Bio-Nobile Oy, Turku, Finland</td>
<td></td>
</tr>
<tr>
<td>ChromaCELL</td>
<td>Chemicell GmbH, Berlin, Germany</td>
<td>Cellulose with triphosphate metal complexing groups charged with Ni²⁺, Co²⁺, Cu²⁺, or Zn²⁺</td>
</tr>
<tr>
<td>TALONTM Superflow Metal Affinity Resin, TALONTM CellThru Resin, TALONTM Metal Affinity Resin</td>
<td>CLONTECH Laboratories</td>
<td>Tetradentate chelator precharged with Co²⁺ for his-tagged proteins on crosslinked agarose</td>
</tr>
<tr>
<td>Fractogel® EMD Chelate 650(M), Fractogel® EMD Chelate 650(S)</td>
<td>E. Merck, Darmstadt, Germany</td>
<td>Polymer support with polymers extending from the support and carrying the chelating groups</td>
</tr>
<tr>
<td>Novarose™ IDA High, Novarose™ IDA Low, Novarose™ TREN High, Novarose™ TREN Low, Novarose™ DPA High and Novarose™ DPA Low</td>
<td>Invitrogen Corporation, Carlsbad, CA, USA</td>
<td>NTA bound to highly crosslinked 6% agarose</td>
</tr>
<tr>
<td>Ni-NTA Agarose</td>
<td></td>
<td>IDA bound to highly crosslinked 6% agarose</td>
</tr>
<tr>
<td>ProBond™ Nickel-Chelating Resin</td>
<td>Iontosorb, Czech Republic</td>
<td>Macroporous beaded cellulose with diethylentriamine, diethylene-triamine-tetraacetic acid, 8-hydroxyquinoline, or salicylic acid as chelating ligands</td>
</tr>
<tr>
<td>Iontosorb DETA, Iontosorb DTTA, Iontosorb OXIN, Iontosorb SALICYL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EZview® RED HIS-Selecta® HC Nickel Affinity Gel</td>
<td>Sigma Chemical Comp. St Louis, USA</td>
<td>Red gel</td>
</tr>
<tr>
<td>HIS-Selecta® Nickel Affinity Gel</td>
<td></td>
<td>NTA on agarose</td>
</tr>
<tr>
<td>Iminodiacetic acid–agarose</td>
<td></td>
<td>Agarose, IDA</td>
</tr>
<tr>
<td>NTA-Agarose, Metal-charged loaded with manganese</td>
<td></td>
<td>6% agarose</td>
</tr>
<tr>
<td>Tris(carboxymethyl)ethylendiamine–Agarose</td>
<td>Tosoh, Japan</td>
<td>4% beaded agarose, lyophilized powder</td>
</tr>
<tr>
<td>Toyopearl AF-Chelate-650M</td>
<td></td>
<td>Polymer resin</td>
</tr>
</tbody>
</table>
The use of the purified protein may also influence the choice of metal ion. Kipriyanov has suggested standard protocols for the purification of histidine-tagged single-chain antibodies (46, 47). Although purification can be done with Ni\(^{2+}\)-loaded columns, Cu\(^{2+}\) is preferred because of the clinical use of the protein product.

### 7.3.2 Effect of Protein or Peptide Structure

The amino-acid residues responsible for the adsorption of proteins in IMAC have been discussed in a number of papers (5, 44, 48–55), yet there are several questions left open. According to Porath and colleagues, some studies with amino acids indicate that histidine and cysteine would be most likely to cause strong interactions with metals in IMAC (1). Tryptophan, which contains the indole structure, was also thought to contribute to the binding. Porath later demonstrated that Fe\(^{3+}\) bound to IDA Sepharose has a rather specific affinity for tyrosine-containing proteins (50).

The finding by Sulkowski (55) that there is an abrupt change in the adsorption of bovine serum albumin to a Ni\(^{2+}\)-IDA gel around pH 6.5 supports the notion that histidine residues are involved in the sorption process in this case. Above pH 8 the \(\alpha\)-amino group of the amino terminal has an affinity for Cu\(^{2+}\)-IDA (56, 57).

Desorption of ribonuclease with an imidazole gradient was found to be influenced by the pH of the eluent, a higher pH requiring a lower imidazole concentration.

Using the \(\alpha\)-chymotrypsinogen A and \(\alpha\)-chymotrypsin pair and glycosylated and non-glycosylated ribonuclease, Sulkowski (55) has shown that the microenvironment of the histidine residues influences the retention of proteins significantly.

Sulkowsky and colleagues have used IMAC as a tool for the study of the surface topography of interferons (44). The same group has also used some model proteins to establish the influence of various potential complex-forming amino-acid residues on the behavior of proteins in IMAC. Their results showed that for angiotensin I, angiotensin II, and pancreatic ribonuclease A, the strength of binding to different metals decreases in the following order: Cu\(^{2+}\) > Ni\(^{2+}\) > Co\(^{2+}\) > Zn\(^{2+}\). The difference between the strength of binding to Co\(^{2+}\) and Zn\(^{2+}\) was rather small. In the absence of tryptophan and cysteine on the protein surface, an increasing number of histidine residues cause stronger retention. Pancreatic trypsin inhibitor, which lacks histidine, tryptophan, and cysteine on the protein surface, was adsorbed

**Figure 7.2** Cu\(^{2+}\)-IMAC of proteins on IDA Superose\textsuperscript{TM} 12 gels with different metal ion capacity (K. A. Hansson, G. Moen, L. Kågedal, unpublished results). Buffers: A, 20 mM sodium phosphate, 1 M NaCl, pH 7.2; B, 20 mM sodium phosphate, 1 M NH\(_4\)Cl, pH 7.2. Column: 5 × 50 mm (HR 5/5 GE Healthcare Life Sciences, Uppsala, Sweden). Sample: 50 µL of a solution consisting of cytochrome c (peak 1), lysozyme (peak 2), \(\beta\)-lactoglobulin (peak 3), lens lectin (peak 4), and \(\alpha\)-lactalbumin (peak 5) dissolved in half-diluted buffer A. Flow rate: 0.6 mL/min. Conditioning of columns before sample application: elution with water, 5 mL; 0.25 M Cu\(_2\)SO\(_4\), 5 mL; water, 5 mL; buffer B, 20 mL. Elution of proteins: from 100% A to 100% B in 20 min. Cu\(^{2+}\)-capacity of packed gels (µmol/mL): (a) 18, (b) 26, (c) 32, (d) 43.

**Figure 7.3** Postulated planar Cu\(^{2+}\) chelate with iminodiacetic acid as chelating group.
only onto Cu$^{2+}$ at pH 7.4, “presumably due to coordination via amino groups.”

In a study by Hammacher and co-workers (31), IMAC was used in the structural characterization of platelet-derived growth factor (PDGF) purified from human platelets. PDGF is the general name for any one of several dimers that differ in the composition of the two homologous polypeptide chains denoted A and B. The chains are linked by disulfide bridges into homodimers of either the PDGF-AA or -BB type or a heterodimer of the PDGF-AB type. Depending on the cell type used as a source of PDGF, the purified product is either a single isoform (PDGF-AA, PDGF-AB, or PDGF-BB) or a mixture of different isoforms. The A and B chains differ in the content of histidine, B having one and A three residues. When applied to IMAC (Cu$^{2+}$-IDA), PDGF-BB was not adsorbed whereas PDGF-AA was retained on the column. The unknown sample, PDGF purified from human platelets, was partly retained. The retained material eluted before the A homodimer and was concluded to be a heterodimer (PDGF-AB).

Using carboxypeptidase A as a model protein, Muszynska and colleagues (40) studied the possibilities of using IMAC for the characterization of metalloproteins and their preparation in metal-free forms.

In general it seems that whether a protein is a metalloprotein or not has no bearing on the binding or nonbinding of the protein to the chromatographic support in IMAC. For example, Lönnnardal and co-workers showed that Fe-lactoferrin, Cu-lactoferrin, and apolactoferrin exhibited identical sorption and desorption properties; the Fe-lactoprotein was or not has bearing on the binding or nonbinding of the protein has in many cases been fused to a peptide consisting of six histidine residues. This modification of the protein ensures that the hybrid protein after expression in the appropriate microorganism can be efficiently adsorbed onto a chelating column and that the binding will be sufficiently strong to allow for a high degree of removal of other components before the protein is desorbed from the column. Adsorption can occur even if the sample consists of protein from inclusion bodies dissolved in a denaturing medium such as 8 M urea or 6 M guanidinium chloride. A number of papers have been published (59–80) that demonstrate that chelating groups such as IDA and NTA in combination with a suitable metal ion (Cu$^{2+}$, Ni$^{2+}$, Co$^{2+}$, Zn$^{2+}$) can be used. Further references are found in recent reviews (13–17). A high degree of purity after only one chromatographic step has been claimed in several cases. Efficient proteolytic cleavage and removal of the affinity tail and the protease has also been demonstrated (67).

Yilmaz and colleagues have shown that a cluster of His residues can be introduced in a chosen part of a protein by site-directed mutagenesis to serve as a complexing site when the N- or the C-terminus cannot be modified because they are part of the active site of an enzyme (81).

The use of various metal ions with IDA as the chelating group has been studied in a series of experiments with recombinant protein A tagged at the C terminus with (HisGly)$_n$His (unpublished work, GE Healthcare Life Sciences, Uppsala, Sweden) and expressed in Escherichia coli. The size of the peptide was varied with $n = 1, 2, 3, 4, \text{and } 6$. Isolation procedures were tested both under denaturing conditions with 6 M guanidinium chloride and under nondenaturing conditions. Under nondenaturing conditions, the most suitable metal ion was found to be Zn$^{2+}$ because of its relatively low affinity for native E. coli proteins. With Zn$^{2+}$, $n = 4$ or larger was required for binding of the fusion protein. In the presence of GuHCl, a metal ion with strong affinity for the protein of interest was required (e.g., Cu$^{2+}$ or Ni$^{2+}$).

In critical separations it has been found to be beneficial to extend the histidine tail to 10 residues (82).

Belew and colleagues (83) have made quantitative studies of the interaction between proteins and Cu$^{2+}$-IDA on Chelating Sepharose Fast Flow using both frontal analysis and equilibrium binding analysis. Closely similar results were obtained with the two methods. The dissociation

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**TABLE 7.3 Protein Structure and Choice of Metal Ion**

<table>
<thead>
<tr>
<th>Presence of Histidine or Tryptophan on “Surface” of Protein</th>
<th>Metal Ions Providing Adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>No His/Trp</td>
<td>---</td>
</tr>
<tr>
<td>One His</td>
<td>Cu$^{2+}$</td>
</tr>
<tr>
<td>More than one His</td>
<td>Cu$^{2+}$ (stronger adsorption), Ni$^{2+}$</td>
</tr>
<tr>
<td>Clusters of His</td>
<td>Cu$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, Co$^{2+}$</td>
</tr>
<tr>
<td>Several Trp, no His</td>
<td>Cu$^{2+}$</td>
</tr>
</tbody>
</table>
constant \( (K_d) \) at pH 7.0 was 33–37 \( \text{mM} \) for lysozyme and 3.5–6.8 \( \text{mM} \) for ovalbumin. The measured binding capacities \( (L_t) \) were 6.8 and 1.7 \( \mu \text{mol/mL gel} \) for the same proteins. See also the work of Hutchens and colleagues (84, 85).

### 7.3.3 Effect of pH, Type of Buffer, and Ionic Strength

There has been no comprehensive study of how buffers of different types affect sorption of proteins to \( \text{Me}^{n+}-\text{IDA} \) gels. Tables 7.4 and 7.5 give some indications of how conditions can be varied to give a suitable strength of adsorption.

\( \text{Fe}^{2+}-\text{IDA} \) gels present a special case as demonstrated by Andersson and Porath (22) and Muszynska and colleagues (86). They showed that phosphorylated proteins or peptides can be desorbed from such a gel by an ascending (sic!) pH gradient or by inclusion of phosphate in the elution buffer.

Sulkowski (87) extended the study to other types of proteins and also showed that selective desorption can be effected with an increasing concentration of sodium chloride.

It is customary to include sodium chloride, 0.1–1.0 \( \text{M} \), in buffers used in IMAC. One effect of this is to suppress ionic interactions between the sample and the gel. Sulkowski (55) has, however, shown that at least in some cases the ionic strength of the eluent affects the retention of proteins on \( \text{Cu}^{2+}-\text{IDA} \) columns. Cytochrome \( c \) from horse heart, with a pI of 10.6, eluted earlier in a pH gradient when ionic strength was high, but the opposite was observed with calmodulin (pI 4.1). The disparate behavior of these proteins was accounted for by assuming that the net charge of \( \text{Cu}^{2+}-\text{IDA} \) is negative.

High salt concentrations have also been used to suppress protein–protein interactions during IMAC (88–90). Such associations may make other chromatographic methods

### Table 7.4 Behavior of Cohn IV Fraction Proteins from Human Serum in IMAC

<table>
<thead>
<tr>
<th>Metal Ion</th>
<th>Buffers in Sample and Starting Buffer</th>
<th>Albumin</th>
<th>Transferrin</th>
<th>( \alpha_1 )-HS Glycoprotein</th>
<th>( \alpha_1 )-Lipoprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Zn}^{2+} )</td>
<td>50 mM Tris-HCl pH 8.0 150 mM NaCl</td>
<td>No retention</td>
<td>Retained</td>
<td>Retained</td>
<td>Retained</td>
</tr>
<tr>
<td>( \text{Cu}^{2+} )</td>
<td>100 mM Na acetate pH 7.7 500 mM NaCl</td>
<td>Complete retention</td>
<td>Retained</td>
<td>Retained</td>
<td>Retained</td>
</tr>
<tr>
<td>( \text{Cu}^{2+} )</td>
<td>20 mM phosphate pH 7.7 500 mM NaCl</td>
<td>Complete retention</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{Cu}^{2+} )</td>
<td>50 mM Tris-HCl pH 8.0 150 mM NaCl</td>
<td>Partial retention</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{Cu}^{2+} )</td>
<td>50 mM Tris-HCl pH 8.0 150 mM ( \text{NH}_4 \text{Cl} )</td>
<td>Major part unretained(^a)</td>
<td>Retained</td>
<td>Retained (partial?)</td>
<td>Retained</td>
</tr>
</tbody>
</table>

\(^a\)Albumin oligomers and minor part of albumin monomer were retained.

### Table 7.5 Qualitative Influence of Various Factors on the Binding of Proteins in IMAC

<table>
<thead>
<tr>
<th>System Parameter</th>
<th>Weaker Binding</th>
<th>Stronger Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stationary Phase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metal ion</td>
<td>( \text{Ca}^{2+} )</td>
<td>( \text{Co}^{2+} )</td>
</tr>
<tr>
<td><strong>Chelating Ligand</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type</td>
<td>TED</td>
<td></td>
</tr>
<tr>
<td>Amount bound</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td><strong>Mobile Phase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Buffer ions</td>
<td>(EDTA) citrate</td>
<td>Ammonia tris ethanolamine</td>
</tr>
</tbody>
</table>
such as ion exchange difficult if not impossible, and the use of IMAC as an early step allows ion exchange or gel filtration to be used later in the purification scheme.

Wunderwalt and colleagues (91) used $\alpha_2$-macroglobulin bound to Cu$^{2+}$-IDA Sepharose for the removal of endoproteinase from fluids in a “sandwich technique.” They discovered that much less bound $\alpha_2$-macroglobulin was removed from a column eluted with several column volumes of buffer of high ionic strength when sodium chloride was added to generate the ionic strength than when sodium phosphate was used.

7.3.4 Effect of Detergents and Other Additives

Detergents can, when necessary, be used with advantage in IMAC. Collagenase, a difficult enzyme to purify, was isolated with Zn$^{2+}$ IMAC as one step of a purification scheme. A detergent, Brij 35, was used in all buffers (92). Tween 80 was included in buffers used in a procedure for the isolation of plasminogen activator from human melanoma cells (93). The use of detergent gave better yields than were obtained in an earlier isolation of the activator from human uterine tissue (94). In both cases, Zn$^{2+}$ IMAC was used as one of the steps, desorption being effected by an imidazole gradient.

Grisshammer and Tucker found that a nonionic or a zwitterionic detergent could be used in the purification of a neurotensin receptor, but not the anionic detergent sodium dodecyl sulfate (82).

In the purification of human Factor XII, a trypsin inhibitor and surface binding inhibitors were used in all buffers (95). Human placenta mitochondrial membrane proteins could be fractionated using 0.4% octaethylene glycol dodecylether in the eluents (96). A 1:1 mixture of ethanol–water was used to remove colored contaminants before eluting the adsorbed protein in the purification of albumin from a Cohn IV extract (97).

In a study of metal-catalyzed oxidation, Krishnamurthy and colleagues found that lactate dehydrogenase was damaged by such oxidations during Cu-IDA IMAC when carried out in the presence of reducing agents such as ascorbate or the oxidant hydrogen peroxide (98).

7.4 CHROMATOGRAPHIC CONDITIONS

7.4.1 Planning of the Experiments

For a protein with unknown properties regarding its binding to metal chelate columns, it is advisable to use Cu$^{2+}$ and a neutral phosphate or acetate buffer containing 0.15–0.5 M NaCl for initial experiments. Ideally, total binding of the desired protein should occur with direct elution of contaminating proteins or the reverse. If inappropriate binding is obtained, a number of variations can be tried, as outlined in Table 7.5 can be tried.

A few experiments with a variety of metal ion, pH, and buffer compositions should be sufficient to establish suitable starting conditions. To vary the degree of substitution is more laborious. However, the amount of bound metal can be reduced by leaching the metal-primed column with a citrate buffer, at least when Cu$^{2+}$ is used (99).

The following section deals in some detail with practical aspects of IMAC for the optimization and systematization of experiments.

7.4.2 Loading of Metal Ion and Regeneration of the Column

Metal ions such as Cu$^{2+}$, Zn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, and Ca$^{2+}$ can be loaded in neutral or weakly acid solutions to avoid hydroxy precipitates with, for example, Cu$^{2+}$. For Cu$^{2+}$, the saturation of the column can be followed simply by observing the blue color of the column. When Zn$^{2+}$ is used, formation of a precipitate on addition of Na$_2$CO$_3$ to the eluent indicates saturation of the column.

Some bleeding of metal ions from the column is likely to occur during most IMAC experiments. In many cases this does not matter, but the leakage can be suppressed if necessary by washing the primed column with the final eluent before equilibration with the starting buffer (the buffer in which the sample will be applied). Alternatively, the column can be charged with metal ion to only 70–90% of its maximum capacity; this will cause migrating metal ions to be captured by chelating groups at the bottom of the column, equivalent to having an unloaded small column in series with the separation column, and to extracting metal ions from the eluate with a small amount of chelating gel after the chromatographic run.

As a general rule it may be advisable to strip columns of metal ions between runs using chelating agents, such as EDTA, and to recharge with metal ion before the next experiment. Stripping and recharging is essential when ions of weak complexing strength, such as Zn$^{2+}$ and Co$^{2+}$, are used with descending pH gradient protocols.

With Cu$^{2+}$- and Ni$^{2+}$-IDA columns, many chromatographic cycles can, at least under certain conditions, be performed without regeneration of the column. The experiment depicted in Figure 7.2c was repeated 20 times without regeneration of the column. The last chromatogram was indistinguishable from the first (K. A. Hansson, G. Moen, L. Kågedal, unpublished results).

7.4.3 Sample Application

One obvious advantage of IMAC is that it can be used to concentrate a protein from very dilute solutions. It may, however, be necessary to equilibrate the sample carefully with the starting buffer (e.g., by dialysis) to remove components such as amines that may interfere in the adsorption step.
on the properties of the samples one may wish to include salts, urea, detergents, glycol, and so on, in the sample and elution buffers. Such additives seem to have no or only minor influence on the adsorption of the protein but may serve to improve performance and yield. It is advisable to use 0.15–1.0 M NaCl or other salts in all buffers to achieve consistent results.

7.4.4 Elution Modes

After washing away unbound material, bound substances are recovered by changing the conditions to favor desorption. A gradient or stepwise reduction in pH to 3 or 4 is often suitable. Alternatively, competitive elution with a gradient of increasing concentration of, for example, ammonium chloride, glycine, histamine, histidine, or imidazole may be used. A combination of ascending gradients of pH and concentration of ammonium chloride was found to give the best result in one case (104). It has been pointed out (7) that when, for example, imidazole is used for elution, the columns should be saturated with imidazole prior to adsorption of the sample protein, and that imidazole should be included in the starting buffer. This is not always essential for good results, however, as shown in the purification of plasminogen activator (94).

A third method of elution is to include a chelating agent such as EDTA in the eluent. In this case, all adsorbed proteins will be eluted indiscriminately along with the metal ion. In all cases, high ionic strength should be maintained.

7.5 AREAS OF USE OF IMAC

The predominant use of IMAC to date has been in the isolation and purification of proteins and peptides, as discussed in this review. The chromatography of nucleotides, dinucleotides, and related compounds on metal chelates has also been shown to be possible (105, 106). Pyrimidines show little interaction with the metal ions, but purines are resolved on Cu²⁺ chelates. The technique is potentially very useful for the large scale purification of these compounds.

Of great interest is the demonstrated removal and inactivation of viruses in the IMAC process (107).

The technique discussed above has also been used in the study of the structure of proteins, where the exposure of certain amino-acid residues on the surface of the proteins can be explored, and for the immobilization of proteins to be used as active solid-phase enzymes (52, 108) or affinity adsorbents (91).

Table 7.6 lists some data from published purifications. Care has been taken to select examples exploiting various modifications of IMAC rather than showing only very typical procedures. The list, which is not exhaustive and does not include applications presented in Section 7.6, demonstrates that a wide range of adsorption and desorption buffers can be used.

7.6 APPLICATIONS

7.6.1 Purification of Copper, Zinc Superoxide Dismutase (Cu, Zn SOD) from Human Erythrocytes

A two-step purification protocol for the purification of Cu, Zn SOD has been designed by Weselake and colleagues (109). The first step is ion-exchange chromatography of a filtered lysate from human erythrocytes on a column of DEAE Sepharose CL-6B from which the Cu, Zn SOD-containing fraction is eluted in 10 mM potassium phosphate, pH 6.4, containing 100 mM NaCl.

An IDA Sepharose 6B gel was prepared according to Porath and colleagues (1). It contained 25–30 μmol of IDA/mL of sedimented gel as determined by titration of immobilized carboxyl groups of the immobilized IDA. A 4 × 5 cm (diameter) column was packed and charged to saturation with 50 mM copper sulfate. A second column, 2 × 5 cm, packed with uncharged IDA-gel was connected after the charged column to serve as a scavenger of any copper ions leached from the first column.

The columns were equilibrated with 10 mM potassium phosphate, pH 6.4, containing 100 mM NaCl and charged at a rate of 150 mL/h with the Cu, Zn SOD solution obtained from the ion-exchange step. The columns were then washed sequentially at a flow rate of 150 mL/h with the following:

- 10 mM potassium phosphate, 1.0 M NaCl, pH 6.4, 3500 mL
- 10 mM potassium phosphate, pH 6.4, 400 mL
- 10 mM sodium acetate, pH 5.0, 300 mL
- 20 mM sodium citrate, pH 5.0 (desorption of Cu, Zn SOD).

Fractions containing Cu, Zn SOD obtained with the citrate buffer were collected as shown in Figure 7.4, concentrated to 30 mL by pressure ultrafiltration, dialyzed against distilled water, freeze-dried, and stored dessicated at −20°C. The volume of the collected fraction was 365 mL.

The specific activity of the purified Cu, Zn SOD was 3800 U/mg. The overall purification factor was 2000 and the yield 58%. The purification in the IMAC step was 60-fold and the yield 63%. Attempts to use higher concentrations of citrate buffer and/or lower pH resulted in sharper elution profiles but with concomitant release of impurities. The purified Cu, Zn SOD was analyzed by SDS-gel electrophoresis, gel filtration, and isoelectric focusing and found to be essentially pure.
TABLE 7.6 Purification of Proteins by IMAC: Selected Examples

<table>
<thead>
<tr>
<th>Protein</th>
<th>Metal Ion(s)</th>
<th>Starting Buffer</th>
<th>Elution Buffer</th>
<th>Remarks</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma amyloid P component</td>
<td>Zn$^{2+}$</td>
<td>20 mM tris-HCl</td>
<td>Linear gradient of histidine, 0–30 mM, in starting buffer</td>
<td>Dialyzed sample from Ba$^{2+}$ citrate and (NH$_4$)$_2$SO$_4$ precipitation</td>
<td>100</td>
</tr>
<tr>
<td>Thiol proteinase inhibitor</td>
<td>Cu$^{2+}$</td>
<td>25 mM tris-HCl</td>
<td></td>
<td>Negative chromatography; flow-through fraction used in next step</td>
<td>101</td>
</tr>
<tr>
<td>Hu IFN-γ</td>
<td>Ni$^{2+}$</td>
<td>20 mM tris-HCl</td>
<td>100 mM Na acetate</td>
<td>22° gave better resolution and recovery; typical recovery &gt;90%; very dilute sample could be used</td>
<td>102</td>
</tr>
<tr>
<td>Granule proteins</td>
<td>Cu$^{2+}$</td>
<td>40 mM tris</td>
<td>Phosphate-acetic acid gradient pH 7.7–2.8 containing 500 mM NaCl</td>
<td>High ionic strength eliminates aggregation</td>
<td>89</td>
</tr>
<tr>
<td>Nucleoside di-phosphatase</td>
<td>Cu$^{2+}$</td>
<td>20 mM tris-HCl</td>
<td>10 mM L-histidine</td>
<td>Enzyme is active when bound to chelating gel</td>
<td>52</td>
</tr>
<tr>
<td>Dolichos biflorus seed lectin</td>
<td>Ca$^{2+}$</td>
<td>50 mM tris-acetate</td>
<td>10 mM EDTA in starting buffer</td>
<td>No desorption with 100 mM glycine–HCl, pH 2.2; subunit with carbohydrate binding activity binds to gel Cu$^{2+}$ and Ni$^{2+}$ failed</td>
<td>45</td>
</tr>
<tr>
<td>Collagenase (pig synovial)</td>
<td>Zn$^{2+}$</td>
<td>25 mM Na borate</td>
<td>50 mM Na acetate</td>
<td>Cu$^{2+}$ and Ni$^{2+}$ failed</td>
<td>92</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>Fe$^{2+}$</td>
<td>100 mM aceticate</td>
<td>200 mM NaCl</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>Pepsin</td>
<td>Fe$^{2+}$</td>
<td>100 mM Na acetate</td>
<td>100 mM Na acetate</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>Pituitary growth factor</td>
<td>Cu$^{2+}$</td>
<td>10 mM phosphate</td>
<td>Gradient with starting buffer</td>
<td></td>
<td>103</td>
</tr>
<tr>
<td>Human 68 kDa (U1) ribonucleoprotein antigen</td>
<td>Ni$^{2+}$</td>
<td>5 mM tris-acetate</td>
<td>pH step gradient, 6 M urea and tris-acetate, desorption in pH 5.0 buffer</td>
<td>Chelating group: IDA Histidine tail: (His)$_6$ at C-terminus</td>
<td>60</td>
</tr>
<tr>
<td>HIV reverse transcriptase</td>
<td>Ni$^{2+}$</td>
<td>20 mM tris, pH 8.0</td>
<td>Stepwise increase of imidazole concentration in starting buffer</td>
<td>Chelating group: IDA Histidine tail: Various histidine rich peptides at N-terminus</td>
<td>61</td>
</tr>
<tr>
<td>Upstream stimulatory factor</td>
<td>Ni$^{2+}$</td>
<td>20 mM tris-HCl, pH 7.9, with several additives</td>
<td>Increasing concentrations of imidazole and KCl</td>
<td>Chelating group: IDA Histidine tail: (His)$_6$ at C-terminus</td>
<td>62</td>
</tr>
<tr>
<td>TEM-β-lactamase</td>
<td>Zn$^{2+}$</td>
<td>50 mM phosphate</td>
<td>Stepwise reduction of pH in starting buffer</td>
<td>Chelating group: IDA Histidine rich tail: Angiotensin I</td>
<td>64</td>
</tr>
</tbody>
</table>
The authors concluded the following:

1. The release of SOD from the copper ion column was related to the chelating properties of the citrate ion.
2. Cu$^{2+}$-IMAC appears to be a simple and rapid procedure for purifying human erythrocyte Cu, Zn SOD, which avoids solvent- and heat-treatment steps.
3. The procedure did not appear to deactivate SOD by removing copper from the active site, as judged from the retained enzymatic activity.

### 7.6.2 Purification of Human Plasma αγ-Macroglobulin and αγ-Antitrypsin

A fine example of plasma protein purification by IMAC was given by Kurecki and colleagues in their preparation of αγ-macroglobulin ($\alpha_2$-M) and αγ-antitrypsin ($\alpha_1$-AT) (Table 7.7) (110). They used dialyzed samples obtained by fractional ammonium sulfate precipitation. Large amounts of $\alpha_2$-M bound tightly to a zinc column at pH 6, allowing removal of contaminating proteins. At pH 5, $\alpha_2$-M eluted in a sharp concentrated peak (Fig. 7.5). Their results show that a 2.5-fold purification of $\alpha_2$-M can be achieved. An electrophoretically homogeneous product was obtained in good yield by this mild two-step procedure.

$\alpha_1$-AT was isolated from the same batch of pooled human plasma (Fig. 7.6). Because the major contaminating plasma protein, albumin, was not retained by the zinc chelate at the pH of the starting buffer, pH 8, this step was very efficient, giving a 20-fold purification by elution at pH 6.5. Two ion-exchange steps were required in addition to IMAC to obtain homogeneous $\alpha_1$-AT. According to the authors, this method increases the yield of $\alpha_1$-AT by a factor of 2.5 compared to other methods.

### 7.6.3 Purification of Albumin from a Cohn IV Fraction of Human Plasma

While purifying albumin from the Cohn IV fraction, Hansson and colleagues carried out a study of the adsorption and desorption characteristics of albumin and contaminating proteins using zinc and copper chelates (97). The results, summarized in Table 7.4, show that, for a given metal ion, the choice of buffer and salt in the elution medium governs the behavior of the proteins.
Buffer constituents such as acetate and phosphate ions were found to favor the adsorption of the proteins, whereas the use of tris or ammonium salts caused direct elution of the protein. These findings were used to design the following purification procedure.

**Sample**

An extract (65 mL) of a Cohn IV fraction containing 0.64 g albumin and 0.76 g of other proteins, mainly transferrin.

**Buffers**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.1 M sodium acetate, 500 mM NaCl, pH 7.7</td>
</tr>
<tr>
<td>B</td>
<td>Ethanol–water 1:9</td>
</tr>
<tr>
<td>C</td>
<td>Ethanol–water 1:1</td>
</tr>
<tr>
<td>D</td>
<td>50 mM Tris-HCl, 150 mM NH₄Cl, pH 8.0</td>
</tr>
<tr>
<td>E</td>
<td>50 mM EDTA, 500 mM NaCl, pH 7.0</td>
</tr>
</tbody>
</table>

**Figure 7.5** Purification of α₂-macroglobulin (α₂-M) by Zn²⁺-IMAC. A 140-mL sample of α₂-M in 50 mM sodium phosphate, 20 mM NaCl, pH 6.0, prepared by (NH₄)₂SO₄ precipitation from 1 L of human plasma, was charged on a 2.5 × 14 cm Zn²⁺ chelate column equilibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 6.0, at a flow rate of 100 mL/h. Elution was then begun at 50 mL/h with the same buffer, and 20 mL fractions were collected. At fraction 30 the buffer was changed to 20 mM sodium cacodylate, 150 mM NaCl, pH 5.0, and 11 mL fractions were collected. Filled dots, A₂₈₀; crosses, α₂-M specific activity. The α₂-M material in fractions 36–38 was homogeneous in polyacrylamide gel electrophoresis. Reproduced from Reference 110 with permission.

**Figure 7.6** Purification of α₁-antitrypsin (α₁–AT) by Zn²⁺-IMAC. A 700-mL sample of α₁–AT in 50 mM sodium phosphate, 150 mM NaCl, pH 8.0, prepared from 1 L of human plasma by (NH₄)₂SO₄ precipitation was charged on a 2.5 × 90 cm Zn²⁺ chelate column equilibrated in the sample buffer at a flow rate of 100 mL/h. Elution was begun with the same buffer, and 22 mL fractions were collected. At fraction 65 (solid arrow), the buffer was changed to 50 mM sodium phosphate, 150 mM NaCl, pH 6.5, and 12-mL fractions were collected. The column was stripped with 50 mM EDTA, 500 mM NaCl, pH 7.0, at fraction 110 (dashed arrow). Filled circles, A₂₈₀; crosses, α₁–AT specific activity. The bar marks pooled fractions. Reproduced from Reference 110 with permission.

**TABLE 7.7** Purification of α₁-Antitrypsin and α₂-Macroglobulin

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein (mg)</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α₁-Antitrypsin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma (1000 mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium sulfate (50–80%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn²⁺-IDA gel</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DE-52, pH 6.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DE-52, pH 7.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₂-Macroglobulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma (1000 mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium sulfate (40–55%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*From Reference 110 with permission.*
Colored impurities were eluted with aqueous ethanol before desorption of the albumin with a tris buffer containing ammonium chloride.

Highly purified albumin was recovered with a 65% yield in one chromatographic step from the very crude and strongly discolored Cohn IV extract, Figure 7.7. Albumin in peak 3 (Fig. 7.7a) was 99.9% pure according to cellulose acetate electrophoresis.

### 7.6.4 Human γ-Interferon

Several types of interferons have been successfully purified by IMAC. The method is cheap and very efficient in isolating pure human fibroblast interferon (Hu IFN-γ). Papers by Edy and colleagues (111) and Heine and colleagues (112, 113) illustrate the methods. Using controlled porous glass in a batch procedure as a first step, they obtained interferon with an activity of $3.0 \times 10^5$ units/mg. From 44 mg of this they isolated $\sim 7 \mu g$ of homogeneous Hu IFN-γ in one IMAC step using a Zn$^{2+}$-chelate column. The specific activity was $1.7 \times 10^9$ units/mg and the yield in the IMAC step was 91.4%. Overall recovery was 52.6% (95).

Of methodological interest is the use of the end buffer to wash the Zn$^{2+}$ chelate column before equilibration with the starting buffer to remove excess Zn$^{2+}$.

The purification was performed at 4°C using a 1.5 × 16 cm column (K9/15, GE Healthcare) packed with IDA Sepharose 6B prepared by epoxy-coupling as described by Porath and colleagues (1). Before each run, the column was regenerated. The flow rate was 15–20 mL/h. During elution steps, 1 mL fractions were collected and protein content, pH, and interferon activity determined.

#### Buffers

A
20 mM phosphate, 1 M NaCl, 50 mM EDTA, pH 7.4

B
20 mM phosphate, 1 M NaCl, pH 7.4

C
100 mM sodium acetate, 1 M NaCl, pH 4.0

D
100 mM sodium acetate, 1 M NaCl, 1 mM ZnCl$_2$, pH 4.0

E
100 mM sodium acetate, 1 M NaCl, pH 4.2

F
100 mM sodium acetate, 1 M NaCl, pH 4.0

G
100 mM sodium acetate, 1 M NaCl, pH 5.9

#### Regeneration of the Column

1. Removal of Zn$^{2+}$ with A, five bed volumes.
2. Removal of EDTA with B, five bed volumes.
3. Equilibration with buffer C.
4. Introduction of Zn$^{2+}$ with buffer D. Saturation tested by the formation of a precipitate when a drop of the eluate was mixed with a Na$_2$CO$_3$ solution.
5. Removal of excess Zn$^{2+}$ with buffer C, five bed volumes.

---

**Figure 7.7** (a) Isolation of albumin from Cohn fraction IV extracts by Cu$^{2+}$-IMAC. The copper loaded column (K 16/20, GE Healthcare Life Sciences, Uppsala, Sweden, 65 mL gel bed) was charged with sample and eluted with eluents A–E as indicated in the chromatogram. See text for the composition of the sample and buffers. Fraction size was 11 mL, and the flow rate was 1.1 mL/min. (b) Polyacrylamide gel electrophoresis of fractions in panel a. Electrophoresis in a 4/30 gradient gel was carried out according to the manufacturers’ instructions (from GE Healthcare Life Sciences, Uppsala, Sweden) after concentration of fractions in Minicon™ concentration cells (Amicon, Lexington, MA, USA). Reproduced from Reference 97 with permission.
Sample Preparation

6. Prepurification by adsorption to porous glass and dialysis against B. Final volume 100 mL.

Chromatography

7. Application of dialyzed sample.
8. Wash with B, 1.5 bed volumes.
9. Wash with E, five bed volumes.
10. Elution with F, one bed volume.
11. Elution with G, two bed volumes.

The interferon peak fraction eluted at pH 5.2. Fractions with pH 5.6–4.2 contained interferon and were pooled.

Table 7.8 summarizes the results from one typical run (simplified from Reference 113).

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Volume (mL)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (units)</th>
<th>Specific Activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>100</td>
<td>44</td>
<td>13 x 10^6</td>
<td>3.0 x 10^5</td>
</tr>
<tr>
<td>Void + pH 7.4 wash</td>
<td>130</td>
<td>42</td>
<td>&lt;0.2 x 10^1</td>
<td>—</td>
</tr>
<tr>
<td>pH 5.9 wash</td>
<td>150</td>
<td>1.6</td>
<td>0.8 x 10^6</td>
<td>0.5 x 10^6</td>
</tr>
<tr>
<td>pH 5.6–4.2 eluate</td>
<td>6</td>
<td>0.0007</td>
<td>12 x 10^6</td>
<td>1.7 x 10^9</td>
</tr>
</tbody>
</table>

7.6.5 Lactoferrin from Human Milk

The use of high ionic strength buffers to eliminate any nonspecific interactions with the gel is common in IMAC. High salt concentrations were used to prevent the association of lactoferrin from human milk (88) and granulocytes (89) with other proteins during IMAC (Fig. 7.8).

7.6.6 Isolation of His-Tagged Glutathione S-Transferase Expressed in *Escherichia coli*

As discussed in Section 7.3.2 it has become a commonly used technique to introduce multiple histidine residues at either terminal of a protein to be expressed in a suitable microorganism. The high affinity of histidines to complexed metal ions is then exploited in an early IMAC purification step.

In the following example the target protein was glutathione S-transferase carrying six consecutive histidine residues at the C-terminal (GST-His<sub>6</sub>). It was expressed in *E. coli* and secreted into the cytoplasmatic compartment during culturing. The vector used was pGEX-5X-1 and the strain of *E. coli*, XL1-blue MR.

The sample was the supernatant obtained after centrifugation of the cell homogenate and filtering through a 0.45-μm filter. The column was HiTrap Chelating 1 mL, a

---

**Figure 7.8**  (a) Isolation of lactoferrin by Cu<sup>2+</sup>-IMAC. Sample: 70 mL defatted, casein-free human milk equilibrated with the starting buffer. Column: C 10/20. Bed height: 16.3 cm, charged with Cu<sup>2+</sup> to 10.3 cm from the top. Eluents: W = wash with starting buffer 50 mM tris-acetate pH 8.2, 500 mM NaCl, G = development to final buffer 50 mM tris-acetate, 500 mM NaCl, pH 2.8. Flow rate: 25 mL/h. Fraction size: 2.5 mL. (b) Gradient gel electrophoresis in SDS. Gel: PAA 4/30. Lane 1: LMW calibration kit; lane 2: defatted casein-free milk; lane 3: pooled material (P). (Work from GE Healthcare Life Sciences, Uppsala, Sweden.)
commercially available prepacked column (GE Healthcare) containing Chelating Sepharose High Performance, a 34-μm support carrying IDA groups. All operations were carried out at ambient temperature. The chromatographic instrument was an FPLC™ System operated at a flow rate of 2 mL/min (312 cm/h) in all chromatographic steps. Desorption of the bound GST-His<sub>6</sub> was in this case done by a direct change to a buffer containing 300 mM imidazole.

The starting material and collected fractions were analyzed by SDS electrophoresis using PhastSystem™ and PhastGel™ Gradient 10–15 (GE Healthcare). Silver staining was performed according to the manufacturer’s standard protocol.

**Buffers and Solutions**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer A</td>
<td>20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4</td>
</tr>
<tr>
<td>Buffer B</td>
<td>20 mM sodium phosphate, 0.5 M NaCl, 300 mM imidazole, pH 7.4</td>
</tr>
</tbody>
</table>

**Chromatography**

1. Washing of the column by elution with 5 mL distilled water.
2. Charging with Ni<sup>2+</sup> ions using 0.5 mL of the salt solution.
3. Washing by elution with 5 mL distilled water.
4. Equilibration of the column with 10 mL buffer A.
5. Application of 5 mL sample.
6. Removal of unbound or loosely bound material by elution with 10 mL buffer A.
7. Desorption of GST-His<sub>6</sub> with 7 mL buffer B.
8. Equilibration of the column with 5 mL buffer A.

It is evident from Figure 7.9 that a high degree of purity of GST-His<sub>6</sub> was reached in only one step.

### 7.6.7 Isolation of His-Tagged Green Fluorescent Protein from *E. coli* Extract

This example illustrates the isolation of His-tagged green fluorescent protein, GFP-(His)<sub>6</sub>, from a clarified *E. coli* extract (114). The chelating support, Ni Sepharose High Performance, is a product designed to afford very low levels of nickel in the purified protein.

The target protein, GFP-(His)<sub>6</sub>, was expressed in *E. coli* BL21. The column was HisTrap HP 1 mL, a commercially available prepacked column containing Ni Sepharose High Performance precharged with Ni<sup>2+</sup> (GE Healthcare). All operations were carried out at ambient temperature. The chromatographic instrument was an AKTA™ 100 system.
with a 2-mm UV-cell operated at a flow rate of 1 mL/min (150 cm/h) in all chromatographic steps. Desorption of the bound GST-(His)$_6$ was effected by an ascending linear imidazole gradient. Fractions containing GFP-(His)$_6$ as detected by the absorbance at 490 nm were pooled (16 mL).

The starting material and collected pool were analyzed by SDS electrophoresis using a Multiphor II electrophoresis unit and ExcelGel™ SDS Gradient 8–10 (GE Healthcare). Coomassie blue staining was performed according to the manufacturer’s standard protocol.

Buffers and Solutions

| Buffer A | 20 mM sodium phosphate, 0.5 M NaCl, 5 mM imidazole, pH 7.4 |
| Buffer B | 20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4 |
| Sample   | E. coli extract clarified by centrifugation (39,000g, 20 min, 4°C) and filtration through a 0.45-μm filter and containing 1.85 mg/mL of GFP-(His)$_6$, 0.5 M NaCl, and 5 mM imidazole |

Chromatography

1. Equilibration of the column with Buffer A, 5 mL
2. Sample application, 32 mL (~60 mg GFP-(His)$_6$)
3. Elution (wash) with Buffer A, 20 mL
4. Desorption of GFP-(His)$_6$ by a 25 mL linear gradient generated using Buffer A and Buffer B and going from 5 to 250 mM imidazole
5. Elution with Buffer B, 5 mL

The experiment demonstrates that a substantial amount of protein, in this case ~60 mg, can be purified to high purity on a quite small column (see Fig. 7.10a and b).

7.7 REFERENCES

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8

COVALENT CHROMATOGRAPHY

FRANCISCO BATISTA-VIERA
Ca´tedra de Bioquı´mica, Dpto.de Biociencias, Facultad de Quimica, Gral.Flores 2124. Casilla de Correo 1157, Montevideo, Uruguay

LARS RYDÉN
Centre for Sustainable Development (CSD) Uppsala, Uppsala University, Villavägen 16, SE-752 36 Uppsala, Sweden

JAN CARLSSON
Department of Physical & Analytical Chemistry, Uppsala University, Box 579, SE-751 23 Uppsala, Sweden

8.1 INTRODUCTION

Chromatographic techniques for the isolation of proteins and other biological macromolecules, such as ion-exchange and hydrophobic interaction chromatography, are based on noncovalent interactions between the molecules to be separated and the adsorbent. Covalent chromatography (1–7), however, as indicated by the name, involves the formation (and breaking) of covalent bonds between the solute and the stationary phase (Fig. 8.1). As is usually the case in the chromatography of biologically active proteins, specificity and mild conditions are required. These requirements can, as shown later, be fulfilled.
Potential sites for chemical reactions in proteins are primarily the amino-acid side chains with their various functional groups. In particular, the amino and carboxyl functions have been used for the immobilization of proteins to insoluble polymers. In these cases, the chemistry has been designed to give stable bonds, and it is in practice difficult to release the immobilized protein without destroying it. The only functional group for which conditions are available for the formation of a stable covalent bond that can also be split under mild conditions is the thiol group. Covalent chromatography, which has already been discussed as a potential technique, has thus, with a few exceptions (7), been applied to the isolation of thiol-containing substances.

Patchornik and co-workers (8, 9) have, however, designed methods for the reversible covalent attachment of methionyl and tryptophanyl side chains to activated polyacrylamide matrices. The chemistry involved requires long incubation times and low pH (dilute acetic acid) and the methods have not received general attention. They would probably be useful for the isolation of peptides containing methionine or tryptophan in special cases.

This chapter deals exclusively with covalent chromatography on thiol-activated solid phases provided with reactive disulfides or disulfide oxides. Thiol groups frequently occur in proteins and often participate directly in their functions as enzymes, hormones, receptors, and so on (10, 11). Thiol groups in proteins vary in reactivity and can also be introduced chemically. Both circumstances make covalent chromatography a widely applicable technique.

To exploit fully the possibilities and to avoid some pitfalls of covalent chromatography, a thorough knowledge of the chemical properties of the thiol group is necessary. This chapter, therefore, gives a short description of the main types of reactions in which thiols and some of their derivatives take part, with an eye to their relevance for the practical application of the technique. In particular, their reactions with the so-called reactive disulfides and the disulfide oxides utilized in covalent chromatography and reversible protein immobilization are treated in some detail.

This chapter also briefly describes some of the properties of thiol groups in proteins from various biological sources. Special emphasis is placed on how the reactivities of thiol groups change with the conditions used.

It is hoped that these introductory sections make the main part of the chapter—the properties of the gel derivatives used in covalent chromatography and the principles and practice of the chromatography itself—easy to follow. The chapter is concluded with descriptions of a few practical applications.

8.2 CHEMICAL PROPERTIES OF THIOL GROUPS

8.2.1 Ionization, Oxidation, Metal Ligation, and Alkylation

Thiol groups are normally the most reactive groups found in proteins and they can participate in a large number of reactions (10–14). This is due to the high nucleophilicity of the corresponding thiolate ions, which exist at reasonable concentrations at neutral to weakly alkaline pH values. Thiols easily oxidize to give disulfides (R–S–S–R), which are comparatively unreactive and important for the stabilization of the protein tertiary structure:

\[
2R–SH \rightarrow R–S–S–R \ (\text{disulfide}) + 2H^+ + 2e^- \quad (8.1)
\]

The rate of oxidation increases with the increasing concentration of thiolate ion, that is, with increasing pH. At low pH (pH < 5) and in the absence of strong oxidizing agents, the thiol group is stable and can tolerate several days of incubation even in the presence of air at room temperature.

Thiols are avid ligands for heavy metal ions. In some cases, as in the cupric ion complex, the thiolate oxidizes rapidly in the presence of oxygen. The presence of chelating agents, such as ethylenediaminetetraacetic acid (EDTA), is thus advisable in situations where oxidation is a potential problem.

Other thiol complexes, such as zinc, and even more so the mercury complex, are stable. The latter was used in some of the first applications of covalent chromatography (15), where the gel contained an immobilized organic mercury compound.

Thiols can dissociate into a proton and a thiolate anion:

\[
R–SH \rightarrow R–S^- + H^+ \quad (8.2)
\]

Their \(pK_a\) values generally lie in the range 8–10.5. Thiolate-dependent reactions thus proceed readily at pH 8 and above, and at moderate speed in the pH range 6–8. Some representative \(pK_a\) values are given in Table 8.1. The described pH dependence applies for low molecular weight aliphatic thiols and many protein-bound thiols. The latter,
however, occasionally shows a completely different rate pH dependence due to microenvironmental effects.

Thiols react with oxidizing agents via one of two routes (14): the monomeric oxidation pathway, which proceeds via two electron steps and turns the thiols into sulfenic (R–SOH), sulfinic (R–SO₂H), and sulfonic (R–SO₃H) acids, or the dimeric oxidation pathway, which converts the thiols to disulfides (R–S–S–R), which can then be further oxidized to disulfide oxides [thiolsulfinates (R–SO–S–R) and thiol-sulfonates (R–SO₂–S–R), respectively], and finally to sulfinic and sulfonic acids. The end products of the two routes are thus the same. Depending on the oxidizing agents used, the reactions take place by one of these two routes.

Alkylation that leads to a stable thioether can be used to block a thiol group permanently. In the most common procedure, iodoacetate or its amide is used and a carboxymethyl derivative is formed (16):

\[
I–CH₂–COO⁻ + R–SH \rightarrow R–S–CH₂–COO⁻ + HI
\]

(8.3)

This reaction, like other nucleophilic displacement reactions with thiols, involves the unprotonated form (i.e., the thiolate ion) and the rate therefore increases with increasing pH.

8.2.2 Thiol–Disulfide Exchange

Thiol–disulfide exchange is a special form of alkylation (S-alkylation). This reaction can easily be reversed and is used in covalent chromatography. It will therefore be discussed in some detail. The reaction is a two-step nucleophilic displacement in which a mixed disulfide is formed as an intermediate:

\[
R₁–S–S–R₂ + 2HO–CH₂CH₂–SH \rightarrow 2R₁–SH + HO(CH₂)₂–S–S(CH₂)₂OH
\]

(8.5)

When one of the two isomeric thiol-diols, dithioerythritol (DTE) or dithiothreitol (DTT), is used for the reduction, the situation is quite different. The mixed disulfides formed from these compounds and an aliphatic disulfide are unstable and undergo subsequent internal thiol–disulfide exchange, leading to the formation of an internal disulfide in the form of a stable six-membered ring, which drives the reaction toward completion. Thus DTE or DTT will reduce an aliphatic disulfide in equimolar amounts.

It should also be mentioned that thiol–disulfide exchange reactions play an important role in biochemical processes, such as the biosynthesis of proteins, the aggregation and polymerization of some proteins, possibly the regulation of the activities of some intracellular enzymes, effector–receptor interactions, and membrane transport.
8.2.3 Reactions with Reactive Disulfides

A particularly interesting thiol–disulfide exchange is that between an aliphatic thiol and a so-called reactive disulfide (Fig. 8.2). These are compounds in which the corresponding thiol forms are stabilized either by resonance or by thiol–thione tautomerism. This results in decreased nucleophilicity and a correspondingly low reactivity toward disulfides. Reactive disulfides can be either homogeneous or mixed. In the first case, the two halves of the molecule are identical. In the mixed types, one of the halves (R–S–S–) is derived from an aliphatic thiol (R–SH, where R denotes the aliphatic group).

An example is the reaction between an aliphatic thiol and the reactive disulfide 2,2'-dipyridyldisulfide (2-PDS):

\[
\text{RSH} + \text{R}_1\text{S} \quad \rightarrow \quad \text{RSSR}_1 + \text{HS} \quad \text{Eq. 8.7a}
\]

\[
\text{HS} \quad \rightarrow \quad \text{S} \quad \text{Eq. 8.7b}
\]

In contrast to the thiol–disulfide exchange reaction involving an aliphatic disulfide, where the equilibrium lies toward the “middle,” these reactions are driven essentially to completion by formation of the thione form (\(K_{eq}\) around \(3 \times 10^3\)). An aliphatic thiol can thus be quantitatively transformed into a mixed reactive disulfide concomitantly with the formation of equimolar amounts of thione.

If a mixed reactive disulfide is used, the disulfide formed will be an ordinary aliphatic one:

\[
\text{RSH} + \text{R}_1\text{S} \quad \rightarrow \quad \text{RSSR}_1 + \text{HS} \quad \text{Eq. 8.8a}
\]

\[
\text{Thiol-form} \quad \rightarrow \quad \text{Thione-form} \quad \text{Eq. 8.8b}
\]

Both reactions (Eq. 8.7 and Eq. 8.8), which involve 2-thiopyridyl compounds, proceed at pH values where aliphatic thiol–disulfide exchanges are slow or nonexistent. In fact, thiol–disulfide exchange with 2-pyridyldisulfides can be carried out at pH values in the range of 1–9. The low nucleophilicity of the aliphatic thiol at acidic pH is compensated for by an increased electrophilicity of the 2-pyridyldisulfide as a result of the protonation of its ring nitrogen (pK of \(\sim 3\)). It should be noted that in a mixed 2-pyridyldisulfide the sulfur atom on the aliphatic side is the more electrophilic and will thus react with the incoming thiol sulfur to form the new disulfide, whereas the 2-thiopyridyl structure will leave as a thione. These facts explain the advantageous properties of the 2-thiopyridyl structure as a leaving group and why the binding step in covalent chromatography can proceed at low pH.

The thiol–disulfide exchange described with a mixed pyridyldisulfide produces an aliphatic disulfide that is stable toward simple aliphatic monothiols in excess at low pH. However, if DTT is used in the exchange, the intermediate form will rearrange to form the corresponding cyclic disulfide (Eq. 8.6b) even at a low pH. The combined properties of the thiopyridyl compounds and DTT thus allow the formation of a new thiol over a wide pH range.

The properties of reactive disulfides have been exploited in a number of applications. The homogeneous types have been used for the titration of thiol groups in proteins. The released thiones can be quantified easily by spectrophotometry, as their absorption maxima lie above the region where ordinary proteins absorb, and the molar absorptivity is above 8000 (Figs. 8.2 and 8.3). The most important application of the mixed type of reactive disulfide is in covalent chromatography. This is discussed in greater detail below.

8.2.4 Reactions of Thiols with Disulfide Oxides

Thiols react specifically and quantitatively with aliphatic disulfide oxides (thiolsulfinates and thiolsulfonates) (14) at
neutral or slightly alkaline pH. The reaction is faster when the incubation pH is around and above the pK\textsubscript{a} value of the thiol group, as thiolate is the reacting species.

The comparatively stable thiosulfonate reacts with 1 mol of thiol to give a mixed disulfide and a sulfinic acid in a thiol–disulfide-like exchange reaction (Eq. 8.9a). Thiol-sulfinates, however, turn 2 mol of thiol per mole into two mixed disulfides when the sulfenic acid formed in a first reaction step oxidizes a second mole of thiol (17) (Eq. 8.9b).

\[
\begin{align*}
R{-}SH + R_1{-}SO_2{-}S{-}R_2 & \rightarrow R{-}S{-}S{-}R_2 + R_1{-}SO_2H \\
2R{-}SH + R_1{-}SO{-}S{-}R_2 & \rightarrow R{-}S{-}S{-}R_2 + R{-}S{-}S{-}R_1 + H_2O
\end{align*}
\]

These reactions also have practical applications. Aliphatic thiosulfonates (such as methylene-thiosulfonate, MMTS) have been widely used for the reversible blocking of thiol groups in thiol-dependent enzymes (18) and aromatic thiosulfonates for the titration of thiol groups in proteins (19).

8.3 THIOL-CONTAINING PROTEINS

8.3.1 Redox State in Biological Tissues

In tissues, the redox state within the cells is entirely different from that in the extracellular matrix and fluid. Inside cells a number of reactions are critically dependent on free thiol groups. Coenzyme A and lipoic acid are examples of essential thiol compounds. The intracellular environment must thus be kept in a reduced state. This is achieved by the cysteine-containing tripeptide glutathione (GSH) (Fig. 8.4), which accounts for about 90% of all thiols in the cell and is present in total concentrations in the millimolar range. Glutathione serves as a scavenger of free radicals, oxidizing compounds, and other molecules that react with thiol groups.

The high concentration of reduced glutathione in the cell is maintained by new production through glutathione synthetase and by reduction of the disulfide form (oxidized glutathione, GSSG). The reduction is catalyzed by the NADPH-requiring enzyme, glutathione reductase. In an erythrocyte the ratio GSH/GSSG is about 500.

However, the extracellular environment is rich in oxygen, which is incompatible with the presence of exposed thiol groups.

8.3.2 Intra- and Extracellular Proteins

An overview of intracellular proteins (10, 11), which have been studied with regard to their content of thiol groups, shows that most of them have exposed thiol groups, whereas very few, if any, contain disulfide bridges. In many instances the thiol groups participate in the catalytic reactions and the alkylation of these groups leads to complete inactivation of the enzyme. This is often the case for oxidoreductases and transferases. Sometimes the thiol groups serve as ligands to metal ions, as in alcohol dehydrogenase.

For extracellular proteins the reverse situation applies (10, 11). Thiol groups are exceptions and occur only when they are required for a special purpose, whereas disulfide bridges are common. Some examples are the extracellular hydrolases, such as the serine proteases trypsin, chymotrypsin, and several of the blood coagulation factors. All have several disulfide bridges and no thiol group. However, some intracellular proteases, such as the cathepsins, depend on thiols for their activity.

Small extracellular proteins, such as venom neurotoxins and the protease inhibitors, are especially rich in disulfide bridges, which are required for stabilization of the structure (20). Similarly, many peptide hormones contain disulfides but none has a thiol group.

Exceptions to the rule are the plant proteases ficin, bromelain, and papain, which are extracellular thiol-dependent proteases. In these the thiol group occurs in an active site pocket where it is somewhat protected from oxidation. The mammalian plasma protein albumin sometimes carries a free thiol group (mercaptalbumin). This thiol group can trap thiol-
disulfide-containing compounds in serum under the formation of mixed disulfides. It can also be converted to sulfenic acid by the action of oxidizing agents (e.g., hydrogen peroxide) (21).

### 8.3.3 Demasking Protein Thiol Groups

The reactivity of a protein thiol depends on the conditions. Many proteins contain thiols that are buried in a hydrophobic environment and denaturants are needed for reaction with a hydrophilic reagent. In native human hemoglobin, two thiols per molecule react, and an additional four are unreactive (22). The known three-dimensional structure of the protein indicates that these four are partly buried. In aldolase, one thiol per subunit reacts readily, but an additional three groups react if a small amount of detergent is added (23). Still higher concentrations of detergent are required to abolish the enzymatic activity. In the native form of the copper enzyme ceruloplasmin, present in mammalian serum, no thiol group is accessible, but a slight modification—apparently the “nicking” of a single peptide bond—exposes one thiol (24). This occurs without loss of activity.

In many cases, complete unfolding of the protein is necessary to allow all thiol groups to react. For example, in ceruloplasmin an additional three thiol groups are reactive in 8 M urea or 6 M guanidine containing EDTA to trap released copper (24). This is also an example of a protein in which the thiols are liganded to a metal ion.

### 8.3.4 Reduction of Protein Disulfides

Thiols can be created in proteins by reducing disulfide bonds. This is usually done under conditions where all disulfides present will be reduced (use of denaturants and large excess of reducing reagent), but sometimes one can find conditions under which one or a few disulfide links can be reduced selectively. This is particularly true for interchain disulfides. In the lectin ricin, consisting of two peptide chains, and the immunoglobulins, containing two identical halves each with two peptide chains, the interchain disulfide links can be reduced selectively (without destroying the tertiary structure of the proteins) by a reducing agent such as DTT (25, 26).

In some cases an internal disulfide can be reduced without destroying the gross conformation or even the activity of the protein. One of the disulfides in bovine pancreatic trypsin inhibitor was reduced specifically by an equimolar amount of DTT in the absence of urea (27). Most often one finds, however, that the reduction of intrachain bridges proceeds cooperatively in a zipper fashion.

Solid-phase reducing agents (e.g., highly substituted thiopropyl-agarose) can replace advantageously soluble reducing agents (such as DTT) as alternative reagents for the reduction of protein disulfides (28). The use of solid-phase reducing agents has many advantages over the use of soluble ones: no liberation of contaminant products, easy separation from the reduced protein, easy regeneration, and the ability to reuse it many times. Comparative studies on the reduction of disulfide groups in β-lactoglobulin, and in *Kluyveromyces lactis* and *Aspergillus oryzae* β-galactosidases, demonstrated that optimal conditions for the reduction process (micromoles of SH of reductant/milligram of protein, presence of denaturing agents, incubation time) using DTT or thiopropyl-agarose were different, depending on the location of the disulfide bridges within the protein molecules (29). The matrix of the solid-phase reducing agent generates a steric effect, allowing the reduction of only disulfide bonds exposed on the surface. This property will be very useful for the selective reduction of proteins containing both superficial and internal disulfide bonds, which is difficult to control with soluble reducing agents.

### 8.3.5 Introduction of Thiol Groups

One can introduce thiol groups into proteins, thereby making them available to covalent chromatography. Several reagents are available for these thiolation reactions. The most commonly used is *N*-acetylhomocysteine thiolactone, which reacts with protein amino groups (30). Reagents have also been designed for the introduction of protected thiol groups. One of them is *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) (31).

\[
\text{Protein} \longrightarrow \text{NH}_2 + \text{SPDP} \longrightarrow \text{O} \quad (8.10)
\]

The 2-pyridyldisulfide introduced by this reagent is a mixed reactive disulfide and can be reduced under mild conditions, under which protein–disulfides are not split, by the use of an equimolar amount of DTT (an aliphatic monothiol is not suitable, as this leads to the formation of a mixed disulfide). The 2-thiopyridone liberated can be determined spectrophotometrically. The pyridyldisulfide-substituted protein can also be immobilized on a thiol gel.

Advances in molecular biology techniques have provided an opportunity to introduce specific sites of attachment on a particular protein. The most useful technique in that regard includes site-directed mutagenesis to introduce unique amino-acid residues (e.g., a single cysteine). Site-directed mutagenesis can be used to introduce a free cysteine at a suitable position on the protein, which can be used...
subsequently for immobilization by a disulfide exchange reaction.

Thus native glucose dehydrogenase from *Bacillus subtilis*, a tetrameric enzyme catalyzing the oxidation of glucose to glucono-δ-lactone, lacks cysteine residues and does not react with thiol-reactive adsorbents. A cysteine residue has been introduced in glucose dehydrogenase at position 44, using site-directed mutagenesis. This single cysteine residue was used as an “affinity tag” to simplify the purification procedure as well as for site-specific immobilization of the enzyme on activated thiopropyl-Sepharose, via the formation of a disulfide bond. The enzyme was purified to homogeneity with a final recovery of 65% (32). Two important advantages of this approach are that immobilization and purification of the genetically engineered enzyme can be achieved in a single step, and covalent attachment occurs via an amino acid that is not essential for enzymatic activity.

8.3.6 Derivatization of Thiol Groups

Thiol groups can be converted either to a blocked form (e.g., by alkylation) or to a reactive form by treatment with a reactive homogeneous disulfide. This chapter describes the latter of these possibilities, as it has been used to activate thiol proteins for covalent chromatography on a thiol matrix (see below).

The reaction that one wants to achieve is illustrated in Equation 8.7. It is important to use an excess of 2,2'-dipyridyl disulfide as otherwise the newly activated thiol groups on the protein might react with neighboring thiols to form disulfide links, either internally or intermolecularly, to give dimers or polymers of the protein.

The reaction can be performed either in ordinary buffers or in the presence of strong denaturants, in which case all thiols will become activated. The pH and other conditions are as described earlier. The reaction with a thiolated gel is discussed in a later section.

8.4 GELS FOR COVALENT CHROMATOGRAPHY

8.4.1 Principles

The most important application of the mixed reactive disulfides is as reactive groups in covalent chromatography. If a solid phase with a mixed reactive disulfide is incubated with a thiol-containing molecule, the latter becomes attached to the solid phase as a result of the thiol–disulfide exchange reaction. The disulfides shown in Figure 8.2 have been used with success for this purpose. Several others are likely to work as well. However, in most of the work reported so far, 2-pyridyldisulfide-substituted solid phases have been used. The principle of this type of chromatography is shown in Figure 8.5.
affinity methods, beaded agarose gels are often preferred. They can be derivatized without changing their excellent properties for the chromatography of proteins. They can also be transferred into nonpolar solvents without shrinking, which is a valuable property in some instances.

8.4.2 Introduction of Reactive Disulfides or Disulfide Oxides into Gels

This is usually done by first introducing thiol groups into agarose gels and subsequently converting the gel-bound thiols into 2-pyridyl disulfide or thiosulfinate/thiolsulfonate groups. An early method for the introduction of thiol groups into agarose gels was the coupling (1–5, 36) of glutathione to a cyanogen bromide-activated agarose gel:

\[
\text{Activated matrix} \quad \begin{array}{c}
\text{NH}_2^+ \\
\end{array} \quad \text{glutathione} \quad \text{SH} \\
\]

The presence of two carboxylate functions on the glutathione tripeptide (Fig. 8.4) makes the "arm" that links the thiol to the matrix negatively charged.

A second way to obtain a thiol-substituted agarose starts with the introduction of oxirane structures into the gel by the use of epichlorohydrin or bisepoxides (37). The oxirane structures (the three-membered rings) are reacted with thiosulfate to form a Bunte salt derivative (R–S–SO\(_3^2\)), which is subsequently reduced to a thiol by an excess of a low molecular weight thiol.

In contrast to glutathione, the hydroxypropyl spacer contains no charged structures. The methods described can also be used to introduce thiol groups into a number of other hydroxyl group-containing matrices. Thiol gels prepared as described earlier can be activated in different ways, as follows:

1. Converting gel-bound thiol groups into a mixed reactive disulfide, for example, by reacting them with an excess of 2,2'-dipyridyl disulfide (Eq. 8.15).

\[
\text{Thiol agarose} \quad \begin{array}{c}
\text{Disulfide intermediate} \\
\end{array} \quad \text{H}_2\text{O}_2 \\
\]

2. Creating disulfide oxide moieties. Oxidation of thio propyl-agarose with hydrogen peroxide at acidic pH for 20–30 h converts thiol groups on the support (via disulfide and thiosulfinate) into thiosulfonate moieties (34) (Fig. 8.7).

If gel-bound thiosulfinates are preferred, the thio propyl-agarose should be subjected to a two-step oxidation procedure (35). The gel is first reacted with potassium ferricyanide at neutral pH. The formed disulfide moieties are then converted into gel-bound thiosulfinate groups by controlled oxidation with a stoichiometric amount of monoperoxyphtalate at pH 5–7 (Fig. 8.8).

Figure 8.7 Preparation of thiosulfonate agarose.
The stability of thiolsulfinate/thiolsulfonate groups toward hydrolysis, especially at neutral and weakly acidic pH, is very high. In fact, solid phases containing these groups can be stored as suspensions at pH 5 and 4°C for at least six months, without a decrease in their thiol-binding capacity.

Contrary to the reactive disulfides, solid-phase disulfide oxides do not react with sodium azide in the concentrations of 0.1% often used to prevent bacterial growth in gels.

### 8.4.3 Degree of Substitution and Practical Capacity of Gels

The degree of substitution of solid-phase, thiol-reactive groups obtained can differ considerably (Table 8.2) from one derivative to another and depends, to a large extent, on the original thiol content of the solid phase. Solid phases with both low and high thiol content can be prepared with the oxirane approach, depending on what excess of oxirane compound is used, whereas only a relatively low degree of thiol substitution can be obtained through the cyanogen bromide-based method. As the solid-phase bound thiol groups can be more or less quantitatively converted into reactive disulfides (7) or disulfide oxide groups (34, 35), the final content of thiol-reactive groups is dependent on what excess of low molecular weight thiol compound is used. This, however, does not imply that the thiol-binding capacities of the solid phases will differ by the same factor. The figure for the theoretical thiol-binding capacity of a certain derivative is obtained by incubating an aliquot of it with a small excess of a low molecular weight thiol compound (e.g., glutathione) and, after thorough washing, quantifying the amount immobilized. This can be done easily by direct determination of the amount of released 2-thiopyridone by spectrophotometry for 2-pyridyldisulfide-based gels or by back titration (e.g., with 2,2'-dithiopyridine of unreacted free thiol in the case of disulfide oxide-containing solid phases).

The practical capacity obtained in chromatography is in general much lower than the degree of substitution of thiol-reactive groups, especially for a highly substituted gel (Table 8.2). This is due to the fact that the binding of high molecular weight substances such as proteins is limited by the space available on the polymer rather than by the concentration of active groups. The amount of gel needed for a specific application should ideally be worked out in a pilot experiment.

### 8.4.4 Comparison of 2-Pyridyldisulfide and Disulfide Oxides as Solid-Phase Reactive Groups

Agarose-bound thiolsulfinate (disulfide monoxide) and thiolsulfonate (disulfide dioxide) groups show, as does the 2-pyridyldisulfide group, very high specificity for reaction with thiols (33–35).

Their reactivity if the groups are available is high. Mercaptoalbumin, which contains a buried SH group, does not bind at all on thiolsulfonate-agarose, but does to some degree to thiolsulfinate-agarose (34, 35).

This is different from 2-pyridyl disulfide-based agarose gels, which bind virtually all thiol compounds capable of reaction with low molecular weight thiol-titrating reagents in solution. This property of thiolsulfonate-agarose can possibly be used for the separation of proteins with exposed thiol groups from those with buried thiols.
At high ionic strength the 2-pyridyldisulfide gels bind some proteins lacking thiol groups, especially immunoglobulins, through a noncovalent interaction. This has been ascribed to the so-called thiophilic interaction and has been used in chromatography (39).

Disulfide oxides do not exhibit such thiophilic adsorption properties.

Perhaps the most important difference between disulfide oxides and 2-pyridyldisulfide is that when the former structures are used as solid-phase reactive groups, there is no release of any low molecular weight compound as a result of the immobilization of a thiol, as the formed sulfenic or sulfenic groups remain bound to the matrix. The reaction of thiol compounds with agarose-bound 2-pyridyldisulfide groups, however, leads to the release of 2-thiopyridone. A disulfide oxide gel is thus the obvious choice in applications where unwanted thiols are to be removed from a solution.

However, the course of immobilization on disulfide oxide gels cannot be followed spectrophotometrically, as no chromophore is released.

Solid-phase disulfide oxides have about the same stability as a function of pH as the corresponding 2-pyridyldisulfide derivatives. An additional advantage is that thiolsulfinate and thiolsulfonate gels are also stable in the presence of sodium azide.

The thiolsulfinate-agarose, as the 2-pyridyldisulfide-agarose, can, at least in theory, be regenerated an unlimited number of times. This is not possible with thiolsulfonate-agarose due to the formation of gel-bound, nonreducible sulfinate groups. This gel loses about 50% of its thiol-binding capacity after each cycle of use and regeneration and can thus in practice only be used a few times.

### 8.5 CHROMATOGRAPHIC TECHNIQUES

The recommendations and protocols presented in this section are focused on 2-pyridyldisulfide agarose derivatives as chromatographic material, but should also be applicable for the disulfide oxide agarose derivatives after adaptation to the special characteristics of these derivatives, which are discussed in Section 8.4.4 (see also Refs. 33–35).

#### 8.5.1 Preparatory Experiments

The authors recommend analyzing the thiol content of the sample for covalent chromatography in advance. This is to make sure that the capacity of the gel is not exceeded. Very often, biological samples contain low molecular weight thiols such as glutathione. These should be removed by dialysis or gel filtration before the covalent chromatography is performed.

Thiol titration is conveniently done by spectrophotometric determination of the 2-thiopyridone released when a small amount of the sample (1–5 mg in 1–3 mL) reacts with 2,2′-dipyridyldisulfide (0.1–0.2 mM). The conditions can be chosen to suit the sample in question. Buffers between pH 3 and 8 (formate, acetate, phosphate, and Tris) in the concentration range from 0.05 to 0.4 M, with or without strong denaturants such as 8 M urea or 6 M guanidine-HCl, can be used. Under standard conditions (pH 7.5), a reaction time of a few minutes is usually enough for complete reaction. The addition of EDTA (5 mM) is recommended to trap transition metal ions, which might catalyze oxidation of the thiol groups.

A similar sample can also be reacted with the thiol-activated gel. In the case of activated thiopropyl agarose, 0.3 g of a swollen and equilibrated gel dried by aspiration on a filter is incubated batchwise with the same amount of protein as described earlier in 2–3 mL in a small tube that is closed and rotated end over end for the prescribed time (~1 h). The tube is then centrifuged, and a spectrum is run on the supernatant. The amount of protein thiols bound by the gel can be calculated from the absorbance at 343 nm after the background level has been subtracted. It is important to run a spectrum and not just to read at a single wavelength because the background absorbance is sometimes considerable.

A small amount of the thiopyridone liberated upon incubation of the activated gel is sometimes released by mechanisms other than the binding of protein (40). This “leakage” is of the order of 0.02% of the active structures per hour in a buffer without denaturants, but is higher when high concentrations of urea or guanidine are present. The release corresponds to an absorbance at 343 nm of 0.004 per hour at pH 4.

The results of the preliminary experiments are used to determine proper conditions for the chromatography and the practical binding capacity of the gel. In many cases, especially with high molecular weight thiol proteins, this is not more than ~1% of the active structures present in a highly substituted 2-pyridyldisulfide-agarose gel (Table 8.2).

#### 8.5.2 Binding of Sample Proteins

The coupling of the sample to the gel can be performed either batchwise (i.e., by suspending the gel in the sample solution) or columnwise (i.e., by letting the sample pass through a column packed with the gel equilibrated with the chosen buffer). The packing and dimensions of the column are not critical. It is, however, often better to use a long, thin column than a short, wide one and to adjust the flow rate such that the sample is in contact with the gel for the time chosen (at least 1 h in the standard procedure). The absorbance at 343 nm of the effluent can be used to estimate the amount of thiol groups that have reacted with the gel. If the reading at 280 nm is used to estimate the amount of non-bound protein, the contribution from released thiopyridone
(Fig. 8.3) has to be subtracted. The absorbance of 2-thiopyridone at 280 and 343 nm is approximately equal.

### 8.5.3 Washing

Unbound and nonspecifically adsorbed proteins should be washed off the column. The choice of washing buffers depends on the stability and intended use of the sample that is covalently bound to the column. Normally, a high ionic strength buffer is recommended to neutralize charge interactions (buffer containing 0.1–0.3 M NaCl). In the simplest case the same buffer is used for application, washing, and elution. If necessary, washing with detergents such as Triton and Tween can also be included. The washing should be monitored by measuring the absorbance of the effluent; 1 to 2 column volumes are usually sufficient. The washing operation is completed by equilibrating the column with the buffer to be used for the reductive elution.

If the coupling is done batchwise, the washing can be performed either on a glass filter or in a column; the latter procedure being most convenient for a small amount of gel.

### 8.5.4 Reductive Elution

Reductive elution is normally done at pH 8. The low molecular weight thiol used to reduce the bound sample, as well as residual thiopyridyl structures, is mostly either 10–25 mM DTT or 25–50 mM 2-mercaptoethanol. If cysteine is used, it must be remembered that its oxidized form, cystine, is less soluble than its reduced form, and may easily precipitate in the column after some time. As described previously, the elution can be followed by measuring the absorbance of the effluent at 343 nm.

Because the thiopyridyl groups are reduced much more easily, it is possible to avoid the contamination of eluted proteins by thiopyridone released from nonused groups on the gel by carrying out two separate reductive elutions. In the first, the residual thiopyridyl structures are removed by an equimolar amount of reducing agent. After appropriate washing, the bound protein is then released by an excess of thiol. The first step can be performed at either alkaline or acidic pH.

In some cases, a series of solutions of thiols of different reducing power has been used to achieve the specific release of bound proteins (see Section 8.6.3) (41).

In the case of solid-phase disulfide oxides, recovery of the immobilized thiol molecule can be performed by reductive elution with aliphatic low molecular weight thiols (e.g., 50–100 mM DTT in 0.1 M sodium phosphate buffer pH 8.0).

### 8.5.5 Recovery of Thiol Proteins

When disulfide oxide agarose has been used as the chromatographic material, the bound thiol protein is the only compound released from the gel when it is treated with an excess of a low molecular weight thiol, as the simultaneously formed sulfenic or sulfonic acid groups are bound to the gel (Section 8.4.4). The released protein is eluted together with the excess of reducing agent (and possibly a small amount of corresponding disulfide). In the case of 2-pyridyl disulfide agarose, a considerable amount of 2-thiopyridone emanating from excess gel-bound reactive groups (if not removed in a separate step as described above) is also found in the eluate. The low molecular weight compounds should preferably be separated from the proteins before further handling, such as derivatization of thiol groups, activity measurement, and so on. This is done most easily by a gel filtration step. If it is possible to use a low pH buffer, the risk of oxidation of thiols is minimized. If the solutes are low molecular weight peptides rather than proteins, desalting is still possible by the use of a slightly hydrophobic gel, such as Sephadex LH-20, at an acidic pH. The peptides will then elute in the void volume, whereas thiopyridone, mercaptoethanol disulfide, and salts are retarded (Fig. 8.9) (40).

The eluted material can also be recovered by lyophilization if appropriate volatile buffers are used. This is convenient if the eluted sample is recovered in a large volume (which is often the case). When lyophilization is to be performed, the authors recommend removing the unused thiopyridyl structures in a special reductive elution step as described earlier.

**Figure 8.9** Chromatography of 20 mL of eluate from the reductive elution of an 11-mL bed of activated thiopropyl-agarose containing coupled thiol peptides from human ceruloplasmin on a column (3.2 × 27 cm) of Sephadex LH-20. Fractions of 7.3 mL were collected and analyzed for absorption at 230 nm (dots), 343 nm (open circles), and conductivity (crosses). The fractions in the first peak (elution volume, 100 mL), which contained the thiol peptides, were pooled and lyophilized. The Tris buffer eluted at about 150 mL, and the components with elution volumes of about 200, 240, and 290 mL are believed to be mercaptoethanol, mercaptoethanol disulfide, and thiopyridone, respectively. Reproduced from Reference 40 with permission.
If 2-mercaptoethanol and a volatile buffer salt such as ammonium acetate are used, all low molecular weight compounds will evaporate in the lyophilization step.

8.5.6 Reactivation of Thiol Gels

After chromatography, the gel is in its thiol form and has to be reactivated before it can be reused. This is best done in a batchwise fashion, after washing the gel on a glass filter funnel. It is first incubated for 45 min with a 5 mM solution of DTT in 0.1 M sodium phosphate, pH 7.5, to reduce all aliphatic disulfides, which might have been formed in the elution step, to thiols. Oxidized and excess reduced DTT and other low molecular weight thiols are then removed by washing with buffer.

The following steps differ depending on whether the thiol-reactive structure is a 2-pyridyldisulfide or a disulfide oxide. In the former case, the gel is incubated for 45 min with a saturated (1.5 mM) solution of 2,2'-dipyridyl disulfide in 0.1 M phosphate buffer, pH 8.0. With high capacity gels (thiol content > 100 µmol/g dry derivative), it is necessary to use a 20 mM solution of 2,2'-dipyridyldisulfide to obtain complete reactivation. In this case, the reaction and the subsequent washing are done in buffer containing 20–30% ethanol in order to ensure that the reagent is dissolved. Excess reagent is finally removed by extensive washing. The reactivated gel can be stored in the same way as the fresh gel.

The presence of DTT in the eluates is conveniently checked by thiol titration with reagents such as 2,2'-dipyridyl disulfide or 5,5'-dithiobis(2-nitrobenzoate). Similarly, the 2,2'-dipyridyldisulfide can be assayed by adding a small amount of thiol compound followed by absorbance measurement at 343 nm.

In the case of a thiosulfonate gel, it is practical to reactivate it only a few times (as mentioned earlier), at least twice, however, depending on the thiol group content of the starting thiopropyl-agarose. Thiosulfinate-agarose, however, can be regenerated, at least in theory, an unlimited number of times. The recommended procedures for the original first synthesis and the reactivation of the reactive disulfide oxide gels (in all cases starting from the thiol gels) are as follows.

For the thiosulfonate gel, suction-dried thiol-agarose (15 g) is suspended in 0.2 M sodium acetate, pH 5.0 (45 mL). Hydrogen peroxide (30%) is added in aliquots under continuous shaking. 1.8 mL initially and 2.2 mL each after 30, 90, and 150 min. Incubation is then continued to give a total reaction time of 30 h. The oxidized gel is transferred to a sintered glass filter and washed with 0.1 M acetic acid until it is free from hydrogen peroxide. The activated gel is then equilibrated and stored in 0.2 M sodium acetate, pH 5.0.

For the thiosulfinate gel, 15 g of suction-dried thiol-agarose is suspended in 30 mL 0.1 M sodium phosphate buffer, pH 7.0, and 0.1 M potassium ferricyanide is added in 1.0-mL aliquots while shaking until the yellow color persists for at least 30 min. The gel is then thoroughly washed on a sintered glass filter with buffer: 1 M NaCl and 0.2 M sodium acetate, pH 5.0. The gel is then suspended in 30 mL of pH 5.0 buffer containing the required stoichiometric amount of dissolved magnesium monoperophthalate (0.5 mol/mol –S–S– groups, 0.5 mol of the magnesium salt corresponds to 1 mol of monoperophthalate).

The suspension is incubated while shaking for 2 h at room temperature. The gel derivative is then thoroughly washed with 50 mM sodium acetate buffer, pH 5.0, 0.1 M acetic acid, and 0.2 M sodium acetate buffer, pH 5.0, and is finally stored as a suspension in this buffer.

8.5.7 Chromatography of Activated Proteins or Peptides

Covalent chromatography can also be carried out in the reverse fashion to that just described. The sample is then treated with a homogeneous reactive disulfide and subsequently run on a gel containing immobilized thiol groups. In this approach it is important to remove excess reagent (e.g., by gel filtration) after the activation step.

The coupling should be done with a low-capacity gel to minimize unwanted thiol–disulfide exchange reactions on the gel, which could lead to immediate release of the newly linked protein. Such side reactions can be diminished by performing the coupling at a slightly acidic pH where aliphatic thiol–disulfide exchange is minimized. The ensuing steps in the procedure are the same as in the conventional approach.

This reversed covalent chromatography is particularly useful for the isolation of peptides obtained by the proteolytic digestion of large proteins (see Section 8.6.4).

8.6 APPLICATIONS

Covalent chromatography has found its most important use in the separation of thiol-containing molecules from non-thiols at an early stage in the fractionation of complex protein mixtures. Under certain conditions, a higher degree of specificity can be obtained, sometimes even allowing different thiol-containing molecules to be separated from each other. Moreover, the technique can be used to concentrate thiols from solutions in which they are present at very low concentrations. Another important application of thiol-reactive adsorbents is for the reversible immobilization of enzymes via disulfide bonds. Thiol enzymes containing exposed thiol groups can be immobilized directly, but if the thiol groups are buried or absent they can be provided through mild thiolation procedures (e.g., by use of SPDP, see earlier discussion).

These applications and others are illustrated by the following examples.
8.6.1 Isolation of Urease from Jack Bean (42, 43)

Urease is an enzyme that catalyzes the hydrolysis of urea into ammonia and carbon dioxide. It is a thiol-rich protein consisting of six identical subunits noncovalently associated to an aggregate of molecular weight 500,000. Many of the thiol groups are nonessential and can be modified without loss of the urease activity.

The starting material was Jack bean meal, which is commercially available. The meal (60 g) was mixed with 300 mL of 0.05 M Tris-HCl buffer containing 36% ethanol, 0.1 M KCl, and 1 mM EDTA, pH 7.2. The mixture was stirred for 5 min at 28°C and filtered. The filtrate was centrifuged (500g, 20 mm). The supernatant (about 210 mL) was diluted to 300 mL with 0.05 M Tris-HCl buffer, pH 7.2, and the pH was adjusted to 7.2 by 0.5 M NaOH.

A column with a total volume of 6.3 mL (1 x 8 cm) was prepared from activated thiopropyl-agarose, a high-capacity gel of the type described in Table 8.2. The column was equilibrated with 0.05 M Tris-HCl buffer, pH 7.2, containing 0.1 M KCl and 1 mM EDTA. The Jack bean meal extract (250 mL) was passed through the column at a flow rate of 20 mL/h. Most of the UV-absorbing material passed through the column unretained. The first 150 mL of eluate contained no urease activity. The activity gradually increased to that of the applied sample within the next 100 mL of eluate. The column was washed with the Tris-HCl buffer until the absorbance at 280 nm of the eluate was less than 0.04. The urease activity was released by eluting the column with 20 mM dithiothreitol (20 mL) dissolved in 0.05 M Tris-HCl buffer, pH 8, containing 0.1 M KCl and 1 mM EDTA.

The specific urease activity (units/mg dry material) of the eluted material (after the removal of low molecular weight substances on Sephadex G-25) was 167 times that of the starting material. The activity increased to 280 after an additional gel filtration on Sepharose 6B, which removed some high molecular weight material of low specific activity.

The capacity of the activated thiopropyl-agarose to bind urease active material was 5.1 mg/mL gel. The purified urease preparation was stable for several weeks when stored at 4°C. Before the reductive elution step the column could hydrolyze urea very efficiently when a solution of the substrate was passed through it. The column was thus an effective urease reactor.

8.6.2 Purification of Papain (36)

Papain is a protease that occurs in the latex of the tropical fruit Carica papaya. It is a single-chain protein with a molecular weight of about 23,500. Papain has a single thiol group that is essential for its activity. The following procedure worked both with a commercial crystallized papain preparation and an ammonium sulfate precipitate of dissolved dried papaya latex.

In the preparation based on the commercial enzyme, 200 mg of papain was dissolved in 0.1 M Tris-HCl, pH 8, containing 5 mM DTT. The reduction activated the enzyme by converting blocked active-site cysteines to the thiol form. Excess DTT was then removed on Sephadex G-25 equilibrated with 0.1 M Tris-HCl at pH 8 or 0.1 M sodium acetate at pH 4, both containing 0.3 M NaCl and 1 mM EDTA. The void material (usually containing 0.4–0.6 mol of thiol per mole of protein) in 100 mL of the Tris or acetate buffer was used as the sample for covalent chromatography. The preparation based on crude papain was obtained by dissolving 100 g of dried papaya latex containing 0.1–0.2 mol of thiol per mole protein in about 200 mL of either of the previously mentioned buffers.

Either sample was applied on a column (1.8 x 30 cm) of activated thiol-agarose (a low capacity gel, see Table 8.2). The column was eluted with the application buffer until the absorbance of the eluate at both 280 and 343 nm was less than 0.03. The gel was then equilibrated with 0.1 M Tris-HCl, pH 8, containing 0.3 M NaCl and 1 mM EDTA. The papain was eluted from the column with 50 mM L-cysteine in the same buffer. Fractions were read at 280 and 343 nm and tested for esterolytic activity toward N-benzoyl-L-arginine ethyl ester (BAEE). The fractions corresponding to the activity peak were pooled, and the protein was precipitated by the addition of 30 g of (NH₄)₂SO₄ per 100 mL of solution. The precipitate was dissolved in 0.1 M Tris-HCl, pH 8, and the protein was separated from low molecular weight material by chromatography on Sephadex G-25 in 0.1 M KCl containing 1 mM EDTA. To prevent the formation of papain-L-cysteine mixed disulfide during the gel filtration, DTT was added immediately before application to the Sephadex G-25 column to a final concentration of 5 mM. Using these conditions, ~100 mg of pure papain with a thiol content of 1 mol per mole of protein was obtained.

The chromatography described earlier at pH 8 is an example of the separation of proteins on the basis of the presence of an exposed thiol. The specificity is even more pronounced at pH 4 when, due to microenvironmental effects, the thiol group of native papain reacts much faster with 2-pyridyldisulfide groups than does the thiol of denatured papain or low molecular weight compounds (36). When a mixture of fully active papain (0.1 mM) and L-cysteine (up to ~5 mM) was subjected to covalent chromatography at pH 4, all of the papain reacted with the mixed disulfide gel and essentially all of the L-cysteine passed through the column.

8.6.3 Sequential Elution Covalent Chromatography (41)

Hillson succeeded in separating the two enzymes—protein disulfide isomerase (PDI) and protein disulfide oxidoreductase, also called glutathione-insulin transhydrogenase
(GIT)—involved in the in vivo formation of protein disulfides by the reductive sequential elution of a thiopropyl-agarose column on which they had been immobilized. The starting material was a sample of partially purified protein disulfide isomerase from beef liver, which, apart from PDI, also contained several thiol-oxidoreductase activities. Samples were prepared by two different techniques, one involving ion-exchange chromatography (partially purified preparation) and the other ammonium sulfate precipitation (crude preparation).

The protein samples (25–125 mg at 10 mg/mL) were pretreated with 0.1 mM dithiothreitol in 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl2/1.25 mM EDTA/0.1 M NaCl (TKM/EDTA/NaCl buffer) at 30°C for 30 min. This gentle reduction unmasks any buried thiol groups that may be present as mixed disulfide. After reduction the sample was centrifuged and separated from DTT on a Sephadex G-25 column (2 × 25 cm) using the previously mentioned buffer. The reduced protein was then applied directly on a column of activated thiopropyl-agarose (30–45 g wet weight of high capacity gel). The elution was then interrupted for 30–60 min at 30°C to allow binding to occur.

Alternatively, the sample was incubated batchwise with the gel for 16 h with gentle shaking, after which the gel was poured into a column. Batch incubation gave a higher level of coupling and is therefore the preferred sample loading method.

After application the column was cooled to 4°C and washed with TKM/EDTA/NaCl buffer to remove unbound and nonspecifically adsorbed protein. Bound proteins were then displaced from the gel by successive elution with different low molecular weight thiol compounds used in order of increasing reducing power: 20 mM L-cysteine, 50 mM glutathione, and 20 mM DTT, each in TKM/EDTA/NaCl buffer, pH 7.5. In each step one void volume of the reducing buffer was run into the column, which was then incubated at 30°C for 30 min to allow reaction to occur. Elution was then continued at 4°C at flow rates of 2–10 mL/h, followed by a wash with TKM/EDTA/NaCl buffer to rinse the column. Fractions of 5 mL were collected and monitored for protein at 280 nm and for displaced 2-thiopropiridone at 343 nm.

In each step, fractions containing protein were pooled and solid DTT was added to a final concentration of 5 mM. The pooled fractions were incubated at 25°C for 30 min to reduce any mixed disulfide formed between protein and eluent, and the solution was then dialyzed extensively against TKM buffer, pH 7.5, at 4°C. The procedure resulted in preparations of four protein fractions (i.e., unbound protein washed through the column and material displaced by L-cysteine, glutathione, and DTT, respectively.

The breakthrough peak contained both PDI and GTI, probably due to overloading or incomplete reaction. PDI activity was found only in the cysteine fraction, with no associated GTI activity. The glutathione fraction showed no detectable activity except for a small amount of GTI in one run, and finally the DTT fraction contained GTI activity but no detectable PDI activity.

The degree of purification and the yields differed significantly, depending on whether the starting material was a partially purified (ion exchange) or a crude protein mixture (ammonium sulfate precipitate). In the latter case, the percentage yields of enzyme activities in the bound fractions were 70–98% of PDI activity in the cysteine fraction and 89–100% of GTI in the DTT fraction. Sequential elution covalent chromatography proved to be a powerful tool for the rapid isolation and separation of protein disulfide isomerase and protein disulfide oxidoreductase, which had not been achieved previously.

8.6.4 Purification of Thiol Peptides (44–48)

Covalent chromatography affords facile group-specific isolation of thiol peptides from protein digests. Svensson and co-workers isolated peptides from papain digests of reduced ribonuclease and of mercaptoalbumin (44). Their approach was to react the protein with 2,2’-dipyridyldisulfide before digestion and then to apply the activated peptide mixture to a column of reduced thiopropyl-agarose. The coupling to the column was carried out with 0.2 M ammonium acetate, pH 8.0. The reductive elution was done with the same buffer containing 50 mM 2-mercaptoethanol. The eluted peptide mixture was lyophilized, as only volatile buffer substances and mercaptoethanol had been used. The single thiol peptide from albumin was obtained directly in pure form.

In the procedure of Rydén and Norder (40), the protein, human ceruloplasmin, was immobilized to the column via its thiol groups before the protease was added. This allowed the use of large amounts of protease and permitted the use of two proteases (pepsin and trypsin) sequentially to obtain subfragments of the original peptides. In these experiments, the reaction with the column was carried out at pH 4.0 in sodium acetate buffer and elution was carried out with Tris-HCl, pH 8.0, containing 50 mM mercaptoethanol. The thiol peptides eluted were purified further by gel filtration and HPLC. The recoveries in these experiments were ~70% for the coupling step.

Covalent chromatography is clearly the method of choice for the rapid isolation of thiol peptides from large proteins, which normally give very complex peptide mixtures upon digestion (45). Serum albumin contains 589 and human ceruloplasmin 1046 residues.

The M1 protein of influenza virus is a highly hydrophobic polypeptide containing three cysteinyl residues, which is resistant to enzyme cleavage during its incubation in water solutions (46). To overcome this problem, M1 protein was immobilized on activated thiopropyl Sepharose-6B as a way to facilitate its proteolytic fragmentation. In fact, after the specific reversible covalent attachment of M1 protein to the solid phase to stretch it, it became susceptible to trypsin
hydrolysis. After tryptic digestion, noncysteine-containing peptides of M1 were removed by washing the support, while cysteine-containing ones were detached from the solid phase by reduction. As a result, 24 unique tryptic peptides of M1 protein were clearly separated by reversed-phase chromatography and further characterized. This method opened a new way to the investigation of functional properties of distinct domains of viral thiol proteins.

A procedure to perform quantitative proteomics has been described in which cysteine-containing peptides from tryptic digests of complex protein mixtures are selected by covalent chromatography based on thiol–disulfide exchange, identified by mass spectrometry, and quantified by differential isotope labeling (47, 48).

A 10–30 mg protein sample from a cell lysate was reduced with DTT at a 40 molar excess in 6 M guanidine-HCl at alkaline pH. The reduced sample was applied to an RPLC column packed with Poros 50 R2 (Applied Biosystems). After thoroughly washing the column with water containing 0.1% tetrahydrofuran (TFA), 1–2 mM 2,2'-dipyridyldisulfide in 0.1 M sodium acetate (pH 5.0) containing 50 mM EDTA was passed through the column continuously for more than 2 h. Excess 2,2'-dipyridyldisulfide was then eluted from the column with water containing 0.1% TFA until the absorbance at both 280 and 343 nm decreased to a baseline level. Activated proteins were eluted from the reversed-phase column with 70% acetonitrile containing 0.1% TFA in water. Solvent was evaporated and the proteins were used for tryptic digestion in the following step. Following initial treatment of a protein mixture with 2,2'-dipyridyl disulfide reagent (pH 5), the protein thiols are activated for thiol–disulfide interchange by converting them into 2-pyridyl disulfide groups. After proteins were derivatized with 2,2’-dipyridyl disulfide, the mixture was dissolved in a Tris buffer (pH 8.0) containing 2 M urea and trypsin was added at a 1:50 (w/w) ratio relative to protein content. Digestion was allowed to proceed over 2 M urea and trypsin was added at a 1:50 (w/w) ratio. Digestion was finished, the gel was washed thoroughly to eliminate nonspecific binding. Captured cysteine-containing peptides were released from the gel with 25 mM dithiothreitol (pH 7.5) containing 1 mM EDTA. The released peptides were then alkylated with iodoacetic acid, followed by fractionation by reversed-phase liquid chromatography. It was concluded that by selecting cysteine-containing peptides, the complexity of protein digest could be reduced and database searches greatly simplified (47, 48).

### 8.6.5 Reversible Immobilization of β-Galactosidase (49–53)

β-Galactosidase (lactase) catalyses (among other reactions) the hydrolysis of lactose, producing an isomolecular mixture of glucose and galactose, a reaction of great importance from a nutritional and technological point of view. The enzyme is used in the dairy industry to hydrolyse whey lactose, thereby solving a waste-handling problem. The glucose and galactose formed can then be isomerized to fructose and tagatose, respectively, to produce a natural sweetener. Alternatively, glucose can be fermented into other useful products such as ethanol.

Furthermore, due to its transglycosidase activity, β-galactosidase can also be applied with synthetic purposes, such as the preparation of galactooligosaccharides. However, all these applications are limited because of economic considerations, unless it is used in an immobilized form, allowing for the reuse of the enzymatic bed. Thus, reversible immobilization of neutral β-galactosidases (from E. coli and K. lactis) onto thiol-reactive supports were performed.

E. coli β-galactosidase is an oligomeric enzyme with a high content of cysteine (64 cysteine residues per tetramer). About one-fourth of them react with thiol reagents without affecting the enzymatic activity. It has been found that E. coli β-galactosidase can be immobilized on thiol sulfonate-agarose or thiol sulfinate-agarose under mild conditions through its nonessential, exposed thiol groups (49, 50).

In the following experiment, β-galactosidase grade VIII from E. coli (Sigma, St. Louis, MO) and thiol sulfinate-agarose (20 μmol of thiol-reactive structures per gram of suction-dried gel) were used. An amount of 1 g of suction-dried thiol sulfinate-agarose was incubated batchwise with 2.3 mL of β-galactosidase solution (62 EU/mL and 0.89 mg/mL of protein) in 0.1 M sodium phosphate buffer pH 7.0, containing 3 mM magnesium chloride (coupling buffer). The mixture was gently agitated at room temperature overnight. The derivative was then exhaustively washed with coupling buffer to desorb noncovalently bound protein. Enzymatic activity was assayed with o-nitrophenyl-β-D-galactopyranoside in 0.1 M potassium phosphate, pH 7.5, and 3 mM MgCl2 (activity buffer). Interestingly, thiol sulfonate- and thiol sulfinate-gels displayed high selectivity towards the E. coli enzyme and, therefore, the immobilization proceeded with a concomitant purification. Thus, immobilization of the enzyme on thiol sulfinate-agarose proceeded with a yield of 85% and a 2.1-fold increase in specific activity. The residual solid-phase thiol-reactive groups could be reacted with various low molecular weight thiol compounds, with each compound giving rise to a specific microenvironment for the immobilized enzyme. Thus, blocking with glutathione led to improvements in the thermal stability for the immobilized β-galactosidase derivatives. These derivatives also showed excellent long term stabilities after
storage at 4°C in 0.1 M potassium phosphate, pH 7.0. The immobilized enzyme was quantitatively released with 50 mM DTT in 0.1 M sodium phosphate, pH 8.5, which confirmed that the binding was due to disulfide formation.

The β-galactosidase from *K. lactis* is an oligomeric enzyme, containing 14 cysteine residues per dimer. It is the preferred neutral-pH lactase for processing by-products from cheese manufacturing (whey, whey permeates) because of the GRAS (Generally Recognized As Safe) status of *Kluyveromyces* yeasts. Although it has no exposed thiol groups (so that no covalent reversible immobilization could be achieved in its native form), a previous reduction step allowed its immobilization onto thiol sulfinate or thiol sulfonate supports (51, 52). Thus, when reduction was performed with 50 mM DTT in 0.1 M phosphate buffer pH 8.5, a threefold increase in SH content was achieved. The immobilization process involves the formation of disulfide bonds between exposed thiol groups of the reduced enzyme and thiol-reactive structures on the support and proceeded in high yields and almost full preservation of the activity. In order to avoid a gel-filtration step after enzyme reduction with soluble reducing agents (e.g., DTT), a batchwise reduction process for *K. lactis* β-galactosidase using solid-phase reducing agents such as highly substituted thioalkyl-agarose was optimized (29).

Very recently, a continuous solid-phase process for its reduction and thiol-dependent immobilization was developed (53). Basically, the process involves two fixed-bed mini-reactors connected in tandem, one packed with thioalkyl-agarose and the other with thiol sulfinate-agarose. For higher performance, recirculation of the enzyme solution through both reactors was required.

Native enzyme solution (3.0 mL, containing 8.5 mg protein/mL, 640 EU/mL) in 50 mM potassium bicarbonate buffer pH 8.5, 3 mM MgCl₂, was pumped through the reducing mini-reactor (a column with 9.0 mL packed thioalkyl-agarose) at a flow rate of 2.0 mL/min. The resulting reduced enzyme fed the second mini-reactor (a thiol sulfinate-agarose column, 9.0 mL of packed gel). Then the percolate was reincirculated for 16 h at 22°C through both mini-reactors. Finally, the mini-reactors were sequentially washed with potassium bicarbonate buffer, with and without 0.5 M NaCl (in order to elute nonspecifically bound proteins), and disconnected. The mini-reactor was equilibrated in activity buffer and thus was ready for lactolysis applications.

8.6.6 Characterization of Subunit Proteins (54, 55)

The covalent chromatography of thymidylate synthase on activated thiopropyl-Sepharose 6B has been described (54). This enzyme is in its native form, a dimer with identical subunits, each containing a cysteine residue at the active site. The cysteine thiol groups did not react directly with activated thiol Sepharose 4B, but immobilization was performed via a splittable mixed disulfide bond introduced between the thiol-reactive solid phase and the catalytic thiol group(s) of the enzyme. The immobilization procedure allowed the isolation of enzymes with high specific activity from pools of pure thymidylate synthase, the activity of which had declined during storage. The decrease in activity took place even when pure thymidylate synthase was stored in the presence of 2-mercaptoethanol. By using the covalent chromatography procedure, it was possible to separate the protein into two fractions, one with higher and the other with lower thymidylate synthase activity. The two fractions also showed distinct biochemical and biophysical properties. The ability of the native enzyme to bind to the thiol-reactive adsorbent through more than one mixed disulfide bond was also studied. By restricting the length of the incubation time of the immobilization reaction, it was possible to limit the proportion of enzyme binding through more than one thiol group. Following this strategy, native thymidylate synthase could be immobilized via only one of its subunits, thus leaving the remaining active site thiol on the other subunit free and available for reaction with N-ethylmaleimide. The heterodimer was then eluted from the solid phase with 50 mM 2-mercaptoethanol. This novel approach, using covalent chromatography and selective chemical modification, is proposed as a new tool to study the subunit interactions involved in the catalytic and regulatory mechanisms of certain oligomeric proteins (55).

8.6.7 Purification of the Nicotinic Acetylcholine Receptor using Reversibly Immobilized α-Toxin (56)

A new method of affinity chromatography purification of the detergent-solubilized nicotinic acetylcholine receptor protein (nAChR) was reported, based on the reversible coupling of a chemically monomodified α-toxin from *Naja nigrin collis* to a resin (56).

The α-toxin was monothiolated on the ε-amino group of its lysine-15 by reaction with *N*-succinimidyl-3-(2-pyriddyl dithio)propionate and was covalently linked in a reversible manner to a thiopropyl-activated agarose resin by thiol-disulfide exchange. It was found that 50% of the immobilized toxin molecules were effective for purifying nAChR, indicating a high accessibility of resin-bound toxins to their binding sites on the receptor protein. Purified α-toxin/nAChR complexes were eluted with nearly 100% recovery by reduction of disulfide bonds with DTT. High purity solutions of nAChR were obtained, as shown by polyacrylamide gel electrophoresis.

8.7 REFERENCES

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FRANCISCO BATISTA-VIERA
Cátedra de Bioquímica, Dpto.de Biociencias, Facultad de Química, Gral. Flores 2124. Casilla de Correo 1157, Montevideo, Uruguay

JAN-CHRISTER JANSON AND JAN CARLSSON
Department of Physical & Analytical Chemistry, Uppsala University, Box 579, SE-751 23 Uppsala, Sweden

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9.1 INTRODUCTION

All biological processes depend on specific interactions between molecules. These interactions might occur between a protein and low molecular weight substances (e.g., between substrates or regulatory compounds and enzymes; between bioinformative molecules—hormones, transmitters, etc.—and receptors, etc.), but biospecific interactions occur even more often between two or several biopolymers, particularly proteins. Examples can be found from all areas of structural and physiological biochemistry, such as in multimolecular assemblies, effector–receptor interactions, DNA–protein interactions, and antigen–antibody binding. Affinity chromatography (see References 1–4 for general and earlier references) owes its name to the exploitation of these various biological affinities for adsorption to a solid phase. One of the members of the pair in the interaction, the ligand, is immobilized on the solid phase, while the other, the counterligand (most often a protein), is adsorbed from the extract that is passing through the column. Examples of such affinity systems are listed in Table 9.1.

In many cases, affinity chromatography is a very powerful method. This is particularly true when the protein of interest is a minor component of a complex mixture. The extraction of the vitamin B12 transport protein transcobalamin II from blood serum is given as an impressive example of the purification of 10 mg of active protein from 40 kg of plasma in a simple two-step procedure using a column with immobilized cobalamin (vitamin B12) as the ligand (5).

The term affinity chromatography has been given quite different connotations by different authors. Sometimes it is very broad, including all kinds of adsorption chromatographies based on nontraditional ligands, in the extreme all chromatographies except ion exchange. Often it is meant to include immobilized metal ion-affinity chromatography (IMAC), covalent chromatography, hydrophobic interaction chromatography, and so on. In other cases it refers only to ligands based on biologically functional pairs, such as enzyme–inhibitor complexes. This chapter uses the term not only to include functional pairs but also the so-called biomimetic ligands, particularly dyes, whose binding apparently often occurs to active sites of functional enzymes, although the dye molecules themselves of course do not exist in the functional context of the cell.

Because affinity chromatography proper relies on functional properties, active and inactive forms can often be separated. This is, however, not unique to affinity methods. Covalent chromatography (Chapter 8) can do the same thing when the activity depends on a functional thiol group in the protein. By affinity elution, ion-exchange chromatography (Chapter 4) is also able to separate according to functional properties. These are, however, exceptions to what is a rule for the affinity methods.

Affinity chromatography has proved to be of great value also in the fractionation of nucleic acids, where complementary base sequences can be used as ligands, and in the separation of cells, where cell surface receptors are the basis of

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Counterligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody Inhibitor</td>
<td>Antigen, virus, cell</td>
</tr>
<tr>
<td>Lectin</td>
<td>Enzyme (ligands are often substrate analogs or cofactor analogs)</td>
</tr>
<tr>
<td>Nucleic acid</td>
<td>Polysaccharide, glycoprotein, cell surface receptor, membrane protein, cell</td>
</tr>
<tr>
<td>Hormone, vitamin</td>
<td>Nucleic acid-binding protein (enzyme or histone)</td>
</tr>
<tr>
<td>Sugar</td>
<td>Receptor, carrier protein</td>
</tr>
<tr>
<td></td>
<td>Lectin, enzyme, or other sugar binding protein</td>
</tr>
</tbody>
</table>
9.2 AFFINITY INTERACTIONS

A good affinity ligand should possess the following characteristics:

- The ligand must be able to form reversible complexes with the protein to be isolated or separated.
- The specificity must be appropriate for the planned application.
- The complex constant should be high enough for the formation of stable complexes or to give sufficient retardation in the chromatographic procedure.
- It should be easy to dissociate the complex by a simple change in the medium, without irreversibly affecting the protein to be isolated or the ligand.
- It should have chemical properties that allow easy immobilization to a matrix.

For any particular protein intended to be purified by affinity chromatography, there is often a choice of several different ligands. In addition to the obvious choice of using a monoclonal antibody of appropriate affinity, which is generally applicable to all immunogenic solutes, one may look for components of naturally occurring biospecific pairs such as enzyme–substrate (analogs), enzyme–cofactor (analogs), and enzyme–inhibitor complexes. For glycoproteins, there is the possibility of using immobilized lectins, and the latter are often isolated by adsorption to immobilized carbohydrates. Considerable interest has been focused on immobilized biomimetic dyes, which show a wide variety of specificities applicable to several groups of enzymes, plasma proteins, and other proteins such as interferons.

To adsorb a protein counterligand to an affinity gel, the binding constant $K_A$ for the interaction needs, for most practical purposes, to exceed or be equal to $10^{-3} - 10^6$ M (corresponding to a dissociation constant $K_D$ of $1 - 10$ μM; the $K_D$ is equal to the inverse of $K_A$). However, interactions in the order of millimolar to micromolar ($K_A = 10^3 - 10^6$ M) will also, with a reasonable ligand density, cause retardation of the interacting protein. In these cases, isocratic elution chromatography with small sample volumes can sometimes be very useful. Note that heterogeneous immunoassays such as enzyme-linked immunosorbent assay (ELISA) require higher association constants, and thus other detection methods are needed in these cases. This is why it is a risk to lose monoclonal antibodies that have lower affinities, but that are very useful for purification purposes. The high affinity interactions often require drastic and sometimes denaturing conditions for elution (i.e., decrease of binding constant). Interactions with binding constants exceeding $10^{10} - 10^{11}$ M are sometimes impossible to use as the conditions required to dissociate the complex are often the same as those that unfold the protein.

Generally, ligands may be classified as either monospecific or group-specific, each of which in turn may be divided into low molecular weight or macromolecular.

9.2.1 Monospecific Low Molecular Weight Ligands

This group includes ligands such as steroid hormones, vitamins, and certain enzyme inhibitors. The term monospecific refers to the fact that these ligands bind to a single or a very
small number of proteins in any particular cell extract or body fluid. Thus, lysine binds only plasminogen from blood plasma samples (6) and vitamin B₁₂ will bind only its transport proteins: intrinsic factor from pure gastric juices and transcobalamin II from plasma (5).

Despite the high specificity, nonspecific adsorption may occur. This can be due to interaction with the ligand or with residues from the immobilization reaction or the spacer arm. One way to cope with this problem is to make a second adsorbent lacking only the ligand itself and to allow the desorbed material from the ligand-containing adsorbent to pass this under identical conditions. Another way is to use a specific displacer (e.g., the ligand itself in soluble form) (see Section 9.5.4.3), followed by a more harsh, general displacement agent for the regeneration of the adsorbent.

Generally, monospecific ligands bind more strongly and require harsher eluents than group-specific ligands, which can usually be eluted under mild conditions. Examples of extremely strong binding are the steroids and steroid receptors, which have association constants in the range 10⁸–10¹⁰ M. Here, it is often impossible to find elution conditions that allow the protein to be recovered in native form. One possibility is to use steroid analogs that have lower binding constants as ligands. Another example of a monospecific low molecular weight ligand with a very high binding constant to its counterligand is biotin, which binds avidin (7) with a $K_A$ of 10¹⁵.

### 9.2.2 Group-Specific Low Molecular Weight Ligands

This is the largest group of ligands containing a wide variety of enzyme cofactors and their analogs. This group also includes biomimetic dyes, boronic acid derivatives, and a number of amino acids and vitamins. A representative list of group-specific ligands and their target proteins is given in Tables 9.2 and 9.3. The target proteins are most often enzymes and the most thoroughly studied are the NAD⁺- and NADP⁺-dependent dehydrogenases and kinases.

A large number of affinity chromatography adsorbents are based on group-specific ligands coupled to a variety of carrier matrices commercially available from several sources. Two of the most widely used adsorbents are N-(6-aminohexyl)-5’-AMP coupled to beaded 4% agarose and the biomimetic textile dye Cibacron Blue F3G-A coupled to crosslinked beaded 6% agarose (e.g., Blue Sepharose™ 6 Fast Flow). The 5’-AMP gel shows affinity for a variety of NAD⁺-dependent dehydrogenases with a binding capacity of ~10 mg enzyme/mL gel. Despite its relatively broad specificity, very high purification factors may be obtained using specific elution protocols with either soluble cofactors or by ternary complex formation using a combination of cofactor and substrate (11). Alternatively, when the ligand–enzyme association constants are sufficiently far apart, gradient elution with a soluble cofactor may result in adequate separation, as has been shown for lactate dehydrogenase (LDH) isoenzymes.

The blue dye ligand is an analog of adenylyl-containing cofactors. Consequently, the adsorbent can be used to purify a very wide range of enzymes requiring such cofactors (12), including both NAD⁺- and NADP⁺-dependent enzymes, although it shows some selectivity for NAD⁺-dependent enzymes. In this respect it resembles the 5’-AMP ligand. However, the blue ligand binds a wider range of proteins and has been used for the isolation of several quite disparate proteins, as shown in Table 9.3.

A red dye, Procion Red HE-3B, coupled to Sepharose CL-6B has been used for the purification of a variety of NADP⁺-dependent enzymes and a number of other unrelated proteins such as interferon, inhibin, plasminogen, and dopamine-β-monoxygenase, suggesting that binding may depend not only on specific steric factors but also on ionic and hydrophobic interactions. A large variety of other dyes have been exploited as affinity ligands by Scopes (13). It is also possible to use chemical modification of the textile dye structures to improve their specificities and affinities. Lowe and colleagues (14) thus improved the interaction between horse liver alcohol dehydrogenase and Cibacron Blue F3G-A.

Another ligand type that appears to fit in the category of low molecular weight and group-specific is the one described by Porath as the T-gel (T = thiophilic) (15). It contains sulfone and thio ether groups and shows high selectivity for immunoglobulins.

### 9.2.3 Monospecific Macromolecular Ligands

Specific protein–protein interactions are common and essential in biology. Examples include subunit interactions in quaternary structures, interactions in multienzyme complexes,
and hormone–receptor protein interaction. Few of these have, however, been exploited in affinity chromatography. Exceptions include the binding of fibronectin to gelatine (16), of antithrombin to thrombin and heparin (17), and of the transferrin receptor to transferrin. The reader is referred to the relevant references for further information.

A group of specific protein–protein interactions that have large general significance comprises antibody–antigen binding, which is described in more detail below.

### 9.2.4 Immunoadsorbents

The high specificity of antibodies makes them extremely useful ligands for affinity chromatography, especially where the substance to be purified has no immediately apparent complementary-binding substance other than its antibody. Both antigens and antibodies can be used as affinity ligands, and the immobilized protein is known as an immunoadsorbent or immunosorbent. Immunoadsorbents can be used to purify soluble proteins and peptides, solubilized membrane proteins, viruses, and even whole cells.

The traditional immunoabsorbents based on polyclonal antibody preparations have largely been replaced by adsorbents based on monoclonal antibodies. Modern hybridoma technology allows highly specific antibodies to be obtained against a predefined antigen present at concentrations much less than 1% of total immunogen. By using suitable screening methods, it is possible to obtain rare hybridomas producing an antibody of virtually any desired specificity and affinity and which can be immobilized for use in purifying the antigen.

There are several advantages of using monoclonal antibodies for immunosorbents. For minor protein components, single-step purification factors of several 100s are possible. High to moderate affinities ($K_D$ of $10^{-5}–10^{-9}$ M) preserve the antigens (yields > 90%) and increase the life span of the adsorbent, often to several hundred cycles. A pH of 2–3 will normally displace the antigen, and the capacity for

<table>
<thead>
<tr>
<th>TABLE 9.3 Examples of Proteins with Affinity for Two Broadly Specific Dye Ligands, Cibacron Blue F3G-A and Procion Red HE-3B (from Reference 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cibacron Blue F3G-A</strong></td>
</tr>
<tr>
<td><strong>Kinases and Phosphatases</strong></td>
</tr>
<tr>
<td>Adenylate cyclase</td>
</tr>
<tr>
<td>Adenylate kinase</td>
</tr>
<tr>
<td>Amino acyl tRNA synthetase</td>
</tr>
<tr>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>Creatine kinase</td>
</tr>
<tr>
<td>DNA polymerase</td>
</tr>
<tr>
<td>Fructose diphosphatase</td>
</tr>
<tr>
<td>cGMP-dependent protein kinase</td>
</tr>
<tr>
<td>Nucleoside kinase</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
</tr>
<tr>
<td>Phosphorylase A</td>
</tr>
<tr>
<td>Protein kinase</td>
</tr>
<tr>
<td>Restriction endonucleases</td>
</tr>
<tr>
<td>Succinyl-CoA transferase</td>
</tr>
<tr>
<td><strong>Dehydrogenases</strong></td>
</tr>
<tr>
<td>Alcohol dehydrogenase (NAD$^+$)</td>
</tr>
<tr>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>Hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase (NAD$^+$)</td>
</tr>
<tr>
<td>Lactate dehydrogenase (NAD$^+$)</td>
</tr>
<tr>
<td>Malate dehydrogenase (NAD$^+$)</td>
</tr>
<tr>
<td>Phosphogluconate dehydrogenase</td>
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<tr>
<td></td>
</tr>
</tbody>
</table>
binding is normally at least 10-fold higher than that of an adsorbent based on polyclonal antibodies. The binding capacity would probably be still higher if all monoclonals were immobilized via their Fc moieties. Uniform binding of the antigens allows sharp desorption peaks and consequently a high concentration of antigen in the eluate.

In principle, monoclonal antibodies also allow a constant supply of a highly uniform antibody, which gives rise to high reproducibility from batch to batch of immunoadsorbent. The degree of substitution (ligand density) is an important optimization factor.

One serious disadvantage with monoclonal antibodies is their high cost, which makes the use of a relatively small column with repetitive operation almost mandatory. The next disadvantage, which is shared with all adsorbents based on immobilized proteins, is the high risk of fouling and irreversible chemical denaturation, notably proteolytic degradation. General fouling by nonspecific adsorption of various biopolymers and lipids is best prevented by a preliminary purification step. Thus, to also prevent proteolytic attack, crude extracts should never be applied directly to columns packed with adsorbents based on monoclonal antibodies.

Monoclonal antibodies may be purified using a variety of tools. Generic methods are based on immobilized staphylococcal protein A or streptococcal protein G and, to some extent on thiophilic adsorption (15). Combinations of cation-exchange chromatography, hydrophobic interaction chromatography, and gel filtration have proved to be useful in many cases. However, because of the very wide distribution of isoelectric points and relative hydrophobicities among monoclonals, one is forced to develop tailor-made purification procedures in each individual case. A most useful handbook on the subject has been published (18), and literature (including interactive software) is available from GE Healthcare Bio-Sciences (19).

Monoclonal antibodies have been covalently attached to several different matrices, using a variety of coupling methods. The use of 4% agarose (for process applications Sepharose 4 Fast Flow) and the NHS or CNBr method is normally recommended. The use of monoclonal antibodies in affinity chromatography has been thoroughly discussed by Goding (20).

Chase and colleagues (21) have shown that there is a linear relationship between the binding capacity of an immunoadsorbent and the amount of immobilized antibody. The ability of a particular immunoadsorbent to bind low concentrations of antigen depends on the dissociation constant $K_D$ of the immobilized antibody (Table 9.4). No effect on the antibody loading could be registered for the dissociation constant, but the kinetic properties of the adsorbent were substantially improved at low loadings. This means that the immunoadsorption experiments can be run at higher flow rates. However, the reduced capacity has to be compensated by using larger bed volumes, which leads to larger volumes of wash and eluent buffers, dilution of desorbed antigen, greater risk for nonspecific adsorption to the matrix, and higher matrix costs. This is why immunoadsorbents with low antibody loading are primarily suggested for immunosubtraction procedures (i.e., for the removal of low concentrations of known impurities from protein products).

Finally, it is appropriate to remember the most important general limitation of immunoadsorption as a tool for the isolation and purification of proteins: the immobilized antibody will only recognize and bind to the corresponding antigenic determinant of the actual protein. It will not discriminate between protein molecules that have been modified or partially degraded in other parts. This means that, in the majority of cases, other separation techniques have to be applied after the immunoadsorption step to remove molecules with possible immunogenic neodeterminant structures, irrespective of whether these molecules are biologically active or not.

### 9.2.5 Group-Specific Macromolecular Ligands: Lectins and IgG-Binding Proteins

This group includes several ligands that have found widespread popularity, including lectins such as concanavalin A (Con A) and lentil for the isolation of glycoproteins,

<table>
<thead>
<tr>
<th>$K_D$ of Immobilized Antibody, M</th>
<th>300</th>
<th>1000</th>
<th>3000</th>
<th>10,000</th>
<th>30,000</th>
<th>100,000</th>
<th>1,000,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-6}$</td>
<td>0.3</td>
<td>1</td>
<td>3</td>
<td>10</td>
<td>30</td>
<td>100</td>
<td>1000</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>0.03</td>
<td>0.1</td>
<td>0.3</td>
<td>1</td>
<td>3</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>0.003</td>
<td>0.01</td>
<td>0.03</td>
<td>0.1</td>
<td>0.3</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>$10^{-9}$</td>
<td>0.0003</td>
<td>0.001</td>
<td>0.003</td>
<td>0.01</td>
<td>0.03</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>$10^{-10}$</td>
<td>0.00003</td>
<td>0.0001</td>
<td>0.0003</td>
<td>0.001</td>
<td>0.003</td>
<td>0.01</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The table shows the concentration of antigen (µg/mL) that results in a 50% utilization of the adsorption capacity of an immunoadsorbent when in equilibrium with the sample solution.
staphylococcal protein A and streptococcal protein G for the purification of IgG, and calmodulin for the isolation of a wide variety of calcium-dependent enzymes. An important member of this group is the sulfated polysaccharide heparin, which is frequently used for the purification of several coagulation proteins and other plasma proteins, in addition to a variety of enzymes and other unrelated proteins such as steroid receptors and virus surface antigens (22). Table 9.5 lists examples of proteins that have been shown to have affinity to immobilized heparin.

The ability of immobilized lectins to interact specifically with sugars makes them excellent tools for purifying both soluble and membrane-derived glycoproteins and polysaccharides such as enzymes, hormones, blood plasma proteins, antigens, antibodies, and blood group substances. Table 9.6 lists the most commonly used lectins together with their specificities. Immobilized Con A has been the most widely used because of its specificity for the commonly occurring α-D-mannose and α-D-glucose and because the binding of soluble glycoproteins to the gel is easily reversed by the addition of low molecular weight sugars or sugar derivatives. Secretory glycoproteins that contain a large amount of N-acetylglucosamine are usually purified on immobilized wheat germ lectin. Immobilized lentil lectin has the same specificity as Con A, but with a lower binding constant. This makes it more suitable for the purification of membrane glycoproteins, which often have a very strong binding affinity to Con A.

### 9.2.6 Ligands Derived from Chemical and Biological Combinatorial Libraries

The rapid developments in combinatorial chemistry and biology (23, 24) offer a new approach for the design of affinity ligands (25). Because of the paramount importance of this novel approach, two new chapters (Chapters 10 and 11) with comprehensive information are included in this book.

### TABLE 9.5 Proteins with Affinity to Immobilized Heparin
(Adapted from Reference 22)

<table>
<thead>
<tr>
<th>Coagulation Proteins</th>
<th>Enzymes that Act on Nucleic Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin III</td>
<td>Restriction endonucleases</td>
</tr>
<tr>
<td>Factor VII</td>
<td>RNA polymerase</td>
</tr>
<tr>
<td>Factor IX</td>
<td>RNA polymerase I</td>
</tr>
<tr>
<td>Factor XI</td>
<td>RNA polymerase III</td>
</tr>
<tr>
<td>Factor XII, XIIa</td>
<td>DNA polymerase</td>
</tr>
<tr>
<td>Thrombin</td>
<td>DNA ligase</td>
</tr>
<tr>
<td></td>
<td>Polynucleotide kinase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other Plasma Proteins</th>
<th>Protein Synthesis Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Properdin</td>
<td>Initiation factors</td>
</tr>
<tr>
<td>Complement C1</td>
<td>Elongation factor (EF-1)</td>
</tr>
<tr>
<td>Complement factor B</td>
<td>Ribosomes</td>
</tr>
<tr>
<td>βIH</td>
<td></td>
</tr>
<tr>
<td>Complement C2</td>
<td>Receptors</td>
</tr>
<tr>
<td>Complement C3</td>
<td>Steroid receptors</td>
</tr>
<tr>
<td>Complement C4</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>C3b inactivator</td>
<td>Androgen receptors</td>
</tr>
<tr>
<td>Inter-α-trypsin inhibitor</td>
<td></td>
</tr>
<tr>
<td>Gc globulin</td>
<td></td>
</tr>
<tr>
<td>Protein HC</td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td></td>
</tr>
<tr>
<td>β2-Glycoprotein 1</td>
<td></td>
</tr>
<tr>
<td>C-reactive protein</td>
<td></td>
</tr>
<tr>
<td>Lipoprotein lipase</td>
<td></td>
</tr>
<tr>
<td>Hepatic triglyceride lipase</td>
<td></td>
</tr>
<tr>
<td>Lipases</td>
<td></td>
</tr>
<tr>
<td>Lipoprotein lipase</td>
<td></td>
</tr>
<tr>
<td>Hepatic triglyceride lipase</td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 9.6 Lectins and their Sugar Specificities

<table>
<thead>
<tr>
<th>Source of Lectin</th>
<th>English Name</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dolichos biflorus</td>
<td>(Anti A lectin; horse gram)</td>
<td>α-N-Acetyl-β-D-galactosamine</td>
</tr>
<tr>
<td>Bandeirea simplicifolia</td>
<td>(Sunn Hemp)</td>
<td>α-D-Galactose</td>
</tr>
<tr>
<td>Ricinus communis</td>
<td>(Castor bean)</td>
<td>β-D-Galactose, N-Acetyl-β-D-galactosamine</td>
</tr>
<tr>
<td>Canavalia ensiformis</td>
<td>(Con A)</td>
<td>α-D-Glucosamine, α-D-Mannose</td>
</tr>
<tr>
<td>Helix pomatia</td>
<td>(Snail)</td>
<td>N-Acetyl-β-D-galactosamine</td>
</tr>
<tr>
<td>Lens culinaris</td>
<td>(Lentil)</td>
<td>α-D-Mannose, α-D-Glucosamine</td>
</tr>
<tr>
<td>Pisum sativum</td>
<td>(Pea)</td>
<td>α-D-Mannose, α-D-Glucosamine</td>
</tr>
<tr>
<td>Arachis hypogea</td>
<td>(Peanut)</td>
<td>Galactose, β-1-3-N-Acetyl-β-D-galactosamine</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td>(Phytohaemagglutinin)</td>
<td>N-Acetyl-β-D-galactosamine</td>
</tr>
<tr>
<td>Glycine max</td>
<td>(Soybean)</td>
<td>N-Acetyl-β-D-galactosamine</td>
</tr>
<tr>
<td>Triticum vulgaris</td>
<td>(Wheat germ)</td>
<td>Tri-N-Acetyl-β-D-glucosamine</td>
</tr>
</tbody>
</table>
9.3 PREPARATION AND EVALUATION OF AFFINITY ADSORBENTS

A general description of gels used in chromatography is given in Chapter 2. A list of commercially available matrices intended for ligand immobilization is provided in Table 9.7.

9.3.1 Choice of Matrix

As in all adsorption chromatography, an adsorbent with a large surface area per unit column volume is desirable in order to maximize the capacity of the affinity adsorbent. Hydrophilic gels with a high surface-to-volume ratio (Chapter 2) are very suitable as matrices. For affinity chromatography applications, the ideal gel material should have the following characteristics.

- It should be macroporous, to accommodate the free interaction of large molecular weight proteins with ligands that could themselves be proteins or other macromolecules.
- It should be hydrophilic and neutral to prevent the proteins from interacting nonspecifically with the gel matrix itself.
- It should contain functional groups to allow derivatization by a wide variety of chemical reactions.
- It should be chemically stable to withstand harsh conditions during derivatization, regeneration and maintenance.
- It should be physically stable to withstand hydrodynamic stress in packed beds and, when applicable, sterilization by autoclaving.
- It should be readily available at low cost to facilitate industrial applications.

These characteristics point to gels based on polymers that are highly substituted with alcohol hydroxyls, thus polysaccharides. Among the latter, the spontaneously gel-forming galactan agarose indeed has most of the characteristics of an ideal matrix for affinity chromatography. The major weakness of native agarose is its chemical and physical instability; however, this has been largely compensated for by chemical crosslinking of the physically crosslinked so-called junction zones in the agarose gel structure (26) (see Chapter 2). Ever since its introduction in 1968 by Cuatrecasas, Wilchek and Anfinsen (27), 4% agarose has been the most popular matrix for affinity chromatography. A contributing reason for this popularity, in addition to its advantageous matrix properties, is that simple and convenient coupling methods were developed early on for agarose (see Section 9.4.1), as well as commercially available preactivated matrices (Table 9.7).

Less frequently used gel matrices for affinity chromatography include cellulose, crosslinked dextran, polyacrylamide, and silica. To this group also belong the potentially interesting and commercially available matrices made of mixtures of polyacrylamide and agarose (Ultrigel) and polymerized tris(hydroxymethyl)acrylamide (Trisacryl, Biosepra, France). Cellulose has found its niche as a carrier for ligands in the affinity chromatography of oligonucleotides and nucleic acids. This area has been thoroughly treated by Schott (28). Because cellulose is much more inexpensive than agarose, but used with the same immobilization methods, it is an alternative in large-scale industrial applications of affinity methods. Gels based on crosslinked dextran and polyacrylamide both suffer from the serious disadvantage of having too small pore diameters, which become still smaller after derivatization with affinity ligands. Also, fewer methods are available for immobilization on polyacrylamide.

In traditional low pressure affinity chromatography systems, beads with a diameter of ~100 μm are usually standard. However, beads with diameters in the range 5–30 μm are used in so-called high performance liquid affinity chromatography (HPLAC). In this type of affinity chromatography, higher pressure drops are often required, which also means a demand for higher gel rigidity. This is why the first HPLAC applications were based on modified and derivatized porous silica (29). The major reason for using smaller particles is to increase the chromatographic efficiency by decreasing the diffusion path lengths and increasing the interphase area between the stationary and mobile liquids. The most serious drawback with silica-based stationary phases is their solubility at pH values above 7.5, which prevents their regeneration and maintenance under alkaline conditions (30). An alternative to silica for HPLC applications, which has also proven useful in HPLAC, is small-diameter agarose beads (31).

Synthetic organic polymers, highly substituted with alcohol hydroxyls and with adequate porosity and rigidity, should also present interesting matrices. Some of these are now commercially available (Table 9.7).

Some notable differences between the different matrices exist with respect to their chemical properties. The majority of immobilization methods depend on the presence of hydroxyl groups on the matrix and have been adapted for use in aqueous solvents. Agarose, however, also retains its macroporosity in organic solvents. Thus activation procedures that require an organic milieu can be performed as well as coupling of ligands not soluble in water. As most organic chemistry is based on work in apolar solvents, this means that a wealth of ligand immobilization methods is potentially available for beaded agarose.

9.3.2 Properties of Ligand

For the preparation of the affinity adsorbent the ligand should have the following features.
<table>
<thead>
<tr>
<th>Ligand to be Coupled</th>
<th>Functional Group</th>
<th>Type of Gel</th>
<th>Name of Product</th>
<th>Name of Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins</td>
<td>−NH₂</td>
<td>Beaded agarose with cyanate ester groups</td>
<td>CNBr-activated Sepharose 4B</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>Proteins (peptides)</td>
<td>−NH₂</td>
<td>Beaded agarose with reactive ester on spacer</td>
<td>Activated CH–Sepharose 4B</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>−OH</td>
<td>Beaded agarose with epoxy (oxirane) groups on short spacer (low capacity)</td>
<td>Epoxi-activated Sepharose 6B</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>Thiol compounds (e.g., proteins)</td>
<td>−SH</td>
<td>Beaded agarose with reactive disulfide groups (short spacer) (high capacity)</td>
<td>Thiopropyl Sepharose 6B</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>Amines (peptides, proteins)</td>
<td>−NH₂</td>
<td>Beaded agarose with reactive sulfonic ester groups</td>
<td>Tresylactivated Sepharose 4B</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>Thiol compounds (thiol proteins and low molecular weight thiols)</td>
<td>−SH</td>
<td>Beaded agarose with adipic acid hydrazide groups</td>
<td>Agarose–adipic acid hydrazide</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>Amines and thiols (including proteins)</td>
<td>−NH₂</td>
<td>Beaded agarose with reactive disulfide groups</td>
<td>Reacti-Gel (GX) Pierce</td>
<td></td>
</tr>
<tr>
<td>Aldehydes (low and high molecular weight)</td>
<td>−CHO</td>
<td>Beaded agarose with adipic acid hydrazide groups</td>
<td>Reacti-Gel (HW-65F) Pierce</td>
<td></td>
</tr>
<tr>
<td>Amines (esp. low molecular weight)</td>
<td>−NH₂</td>
<td>1,1’-carboxyldimidazole activated 6% crosslinked beaded agarose, part. diam. 45–165 μm</td>
<td>Reacti-Gel (25DF) Pierce</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,1’-carboxyldimidazole activated Fractogel TSK, part. diam. 32–65 μm, frac. range: 50,000–500,000 M_w</td>
<td>Reacti-Gel (25DF) Pierce</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,1’-carboxyldimidazole activated beaded crosslinked dextran. Dry part. diam. 20–80 μm, frac. range: 1000–5000 M_w</td>
<td>Reacti-Gel (GF-2000) Pierce</td>
<td></td>
</tr>
<tr>
<td>Amines (esp. proteins)</td>
<td>−NH₂</td>
<td>Gluteraldehyde-activated Ultrogel (2% polyacrylamide, 2% agarose), Part. diam. 60–140 μm. Exd. limit: 3 × 10⁶ Da</td>
<td>Act-Ultrogel ACA 22 Biosepra</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Derivatized crosslinked agarose gel containing N-hydroxysuccinimide ester groups</td>
<td>AfGel 10 Gel BioRad</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Possibly silica-based oxirane derivative groups part. diam. 32–63 μm</td>
<td>AfGel 15 Gel BioRad</td>
<td></td>
</tr>
<tr>
<td>Amines</td>
<td>−NH₂</td>
<td>Possibly silica-based derivative with imidazoyl-carbonate groups, part. size: 32–63 μm</td>
<td>AF-CDI 650 Fractogel TSK Merck</td>
<td></td>
</tr>
<tr>
<td>Thiol compounds (in principle also carbohydrates but matrix will hydrolyze under the harsh conditions necessary)</td>
<td>−SH</td>
<td>Beaded agarose (4%) with (6 carbon) aminospacer</td>
<td>AH-Sepharose 4B GE Healthcare</td>
<td></td>
</tr>
<tr>
<td>Amines (esp. low molecular weight)</td>
<td>−NH₂</td>
<td>Beaded agarose (4%) with amino terminal (6-carbon) spacer attached to matrix via highly stable ether bond thus more stable for leakage than AH-variety</td>
<td>EAH-Sepharose 4B GE Healthcare</td>
<td></td>
</tr>
</tbody>
</table>

(Continued)
It should be compatible with the solvents used during the coupling procedure.

- It should have at least one functional group by which it can be immobilized to the matrix. [Commonly used groups include –NH₂ (amino), –COOH (carboxyl), –CHO (aldehyde), –SH (thiol), and –OH (hydroxyl).]
- It should have a functional group for coupling that is nonessential for its binding properties (i.e., the binding properties of the ligand should not be adversely affected as a result of its immobilization).

Ligands of a high molecular weight type (e.g., proteins) with a large number of suitable functional groups can normally be immobilized without adversely influencing structure or function. In low molecular weight ligands the coupling, of course, results in a relatively large change in the molecule. If the affinity interaction decreases, a chemical modification of the ligand may be necessary, to provide it with an appropriate functional group for immobilization.

The functional group used should permit the formation of a stable covalent bond so that the ligand is not released from the matrix. This is particularly important for small ligands where “single-point attachment” is often the case. For proteins, “multipoint attachment” between the ligand and matrix is rather common. In such affinity adsorbents, the stability of each individual bond is less critical.

It is, of course, also essential that the ligand remains intact during the immobilization procedure and that it is sufficiently stable to allow the planned affinity chromatography to be carried out. This might be a problem when proteins are coupled at high pH. It is essential that the ligand reagent is as pure as possible and, in particular, does not contain substances with functional groups that can react competitively in the immobilization. Proteins should be subjected to gel filtration to remove low molecular weight substances such as ammonium sulfate.

### 9.3.3 Choice of Spacer Arm

Occasionally, an affinity adsorbent might show poor function due to low steric availability of the ligand. This rarely happens with high molecular weight ligands but may occur with low molecular weight ligands. The use of a “spacer arm” in many cases solves this problem. Commonly used spacer arms are aliphatic, linear hydrocarbon chains with two functional groups located at each end of the chain. One of the groups (often a primary amine, –NH₂) is attached to the matrix, whereas the group at the other end is selected on the basis of the ligand to be bound. The latter group, which is also called the terminal group, is usually a carboxyl (–COOH) or amino group (–NH₂).

The most common spacers are 6-aminohexanoic acid (H₂N–(CH₂)₅–COOH), hexamethylene diamine (H₂N–(CH₂)₆–NH₂), and 1,7-diamino-4-azaheptane (3,3-diaminodipropylamine) [32–34]. The spacer arm is introduced into the matrix by the same immobilization methods that are described below for ligands.

Longer spacer arms can be introduced by first immobilizing a spacer arm with a terminal primary amine and then increasing the length of the arm by reaction with succinic anhydride (33). Another possibility is to immobilize a
spacer arm with a terminal carboxyl group and then increase its length by reaction with 1,7-diamino-4-azaheptane with the aid of a condensation reagent (see Section 9.4.1.5).

A drawback with hydrophobic arms, especially the longer ones, is that they can give rise to unwanted nonspecific interactions. Polypeptides, particularly glycine oligomers, are examples of hydrophilic spacers. These, however, might bind proteins by nonspecific ionic interactions.

Sometimes it is stated that a spacer should be used for ligands with a molecular weight of less than 5000, but because of the risk of introducing nonspecific binding sites by side reactions in the gel during coupling, by the arm itself, or by both, the authors recommend first trying to prepare an affinity adsorbent by direct coupling of the ligand to the matrix. It should also be remembered that in several of the ligand immobilization methods described later, such as the bisepoxirane and glutaraldehyde method, the ligand will automatically be provided with a spacer as a result of its immobilization (35, 36).

### 9.3.4 Evaluation of the Prepared Affinity Adsorbent

Before an attempt is made to use the prepared adsorbent in affinity chromatography, one should always make sure that the ligand immobilization has succeeded and, if possible, determine the ligand density (degree of substitution as micro-

The analysis can be carried out in several ways. A simple method is "indirect evaluation" (i.e., the amount of immobilized ligand is calculated as the difference between the amount of ligand originally added to the matrix and the amount of ligand recovered in the liquid phase and pooled washings after finished coupling). If the ligand absorbs light of a suitable wavelength (λ<sub>max</sub> > 250 nm) with an acceptable molar absorptivity (ε > 5000 cm M<sup>-1</sup>), this analysis can simply be carried out with a photometer. This method, however, often gives erroneous values and should only be used for a rough estimation.

A very useful method is elemental analysis. This technique can be used if the ligand contains elements such as nitrogen, sulfur, halogen, or phosphorus, provided that these elements are not present in the matrix or become introduced into the matrix as a result of the activation and coupling procedures. Peptide and protein ligands can, after hydrolysis, be quantitatively determined by amino-acid analysis.

Another possibility is to label the ligand with a suitable gamma-emitting radioisotope before coupling. In the case of small ligands with carboxyl or amino groups, acid–base titration is sometimes an easy method for determining the ligand density.

Finally, activity determination for enzyme ligands can be used. It must be taken into account that immobilized enzymes often have changed kinetic properties due to steric and diffusional restrictions arising from the interaction with the substrate (i.e., comparison with free enzyme might not be valid). An analysis of the adsorption characteristics of the affinity gel should also be performed.

The capacity of an affinity adsorbent is defined as milligrams or micromoles of counterligand that can be adsorbed per milliliter of sedimented gel. In a 90-μm average diameter beaded 4% agarose gel, the total surface area is ≈ 5 m<sup>2</sup>/mL bed volume, of which only about 8 cm<sup>2</sup> refers to the outer particle surface (the external matrix surface area of a 4% agarose bead is only 2% of the surface area of a corresponding solid sphere). The maximum theoretical binding capacity of a 60,000-molecular-weight protein should thus be ≈ 80 mg/mL. This value cannot be achieved for several reasons.

It is appropriate to distinguish between static and dynamic binding capacities. The static capacity is measured in batch experiments, which allow ample time for equilibrium to become established. The static capacity depends on the density of the immobilized ligand and its availability for interaction with a particular protein. Some of the immobilized ligands might be inaccessible to a particular protein as a result of steric exclusion due to their location within the gel matrix. This is particularly true when small ligands are used for the binding of high molecular weight proteins. Thus, the functional binding capacity is often much lower than the nominal binding capacity as calculated from the measured ligand density.

The dynamic capacity of the affinity adsorbent is the binding capacity under operating conditions (i.e., in the packed affinity chromatography column during sample application and washing procedures). Factors that influence the dynamic binding capacity are discussed in Chapter 2.

For affinity systems based on low molecular weight ligands that bind high molecular weight proteins, the matrix-bound affinity complexes sometimes stericly shield neighboring ligands from interacting with unbound protein molecules. In such cases, adequate binding capacity can be achieved at a substantially lower ligand substitution. In fact, a high degree of substitution should be avoided as it may cause undesired nonspecific adsorption. When the ligand and corresponding binding protein are of approximately the same size or, more unusually, when the ligand is much larger than the protein to be isolated, the problem of gel porosity primarily concerns the immobilized ligand. An alternative way to achieve a large surface area besides using beads of high porosity is to use smaller beads, as in HPLAC.

### 9.3.5 Storage of Affinity Adsorbents

The conditions for storage of the prepared affinity adsorbents of course depend on the stability of the matrix, the ligand, and the covalent bond by which the ligand is attached to the matrix.

Polysaccharide matrices such as beaded agarose hydrolyze at a significant rate at acidic pH (<4) and oxidize with the...
formation of matrix-bound carboxyls at high pH (>9). Protein ligands may change their conformation and lose their activity as a result of exposure to extreme pH, high temperatures, and denaturing agents (organic solvents, urea, etc.). The commonly employed CNBr method leads to the formation of an isourea linkage (37), which is split at a rather high rate through hydrolysis and aminolysis at alkaline pH (>8).

The immobilization procedure might decrease or increase the stability of the system. Several procedures, notably CNBr and epichlorohydrin coupling (38), lead to the introduction of covalent crosslinkages into the matrix and thus render it more stable. Certain ligands may also be stabilized as a result of their immobilization. Thus, the aggregation and autodigestion that occur in solution with proteases, for example, might be prevented.

Normally, the affinity adsorbent can be stored as a suspension in an appropriate buffer at physiological pH at 4°C for long periods of time. It is, however, advisable to add an antimicrobial substance such as sodium azide to prevent bacterial growth. For very labile adsorbents, lyophilization can be used to increase the storage time. To make sure that the beads will re-swell properly on reconstitution with water or buffer, it is necessary to prevent them from irreversibly collapsing as a result of the lyophilization. Dextran or polyethyleneglycol (PEG) is then added before lyophilization and, after reconstitution of the beads, washed away on a glass filter.

9.4 IMMOBILIZATION TECHNIQUES

In general, the immobilization procedure consists of three steps:

1. Activation of the matrix to make it reactive toward the functional group of the ligand.
2. Coupling of the ligand.
3. Deactivation or blocking of residual active groups by a large excess of a suitable low molecular weight substance such as ethanolamine.

The activation normally consists of the introduction of an electrophilic group into the matrix. This group later reacts with nucleophilic groups such as –NH₂ (amino), –SH (thiol), and –OH (hydroxyl) in the ligand. Alternatively, a matrix with nucleophilic groups can be used to immobilize a ligand containing an electrophilic group, although such an approach is less common (see Section 9.4.1.6). The activated structure is sometimes stable enough for the activated matrix to be isolated and stored until coupling of the ligand is performed. In other cases the coupling procedure has to be performed immediately after activation.

The ligand is either coupled directly to the activated matrix or the matrix is first provided with a spacer arm to which the ligand is subsequently attached. Coupling to the spacer arm is often performed in a one-step procedure by use of a condensation reagent that forms amide bonds between the carboxyl and amino functions present in the ligand and spacer arm, but spacer arms containing a terminal carboxyl group can also be activated in a separate step.

When affinity adsorbents are prepared, a bond as stable as possible should be established between the matrix and the ligand to prevent leakage of the ligand. In certain cases, however, it may be useful to have the ligand attached through a bond that is stable but can be cleaved when desired. An example of such a bond is the aliphatic disulfide, which can be both formed and split under mild conditions by thiol–disulfide exchange reactions. Procedures for carrying out such reversible covalent immobilizations of ligands are described in Chapter 8.

An overview of the various immobilization methods to be presented is found in Table 9.8. The more useful of these are described according to matrix (agarose, polyacrylamide, and silica) and properties of the ligands. Methods used for agarose matrices can be used equally for other polysaccharide matrices, with the exception of methods requiring organic solvents.

In Section 9.6, several applications of the techniques are given in some detail.

9.4.1 Methods for Agarose and Other Polysaccharide Matrices

9.4.1.1 CNBr and CDAP Cyanylating Procedures These methods are suitable for –NH₂-containing ligands, especially polypeptides and proteins.

The original CNBr technique as developed by Axén and colleagues (39, 40) is a classical two-step method with activation and coupling. A water suspension of a polysaccharide (e.g., beaded agarose) is reacted with CNBr at a high pH (11, 12), which leads to the introduction of cyanate ester and imidocarbonate groups into the matrix. The activation of Sepharose with cyanogen bromide can be monitored with a colorimetric method (41).

\[
\text{Matrix} + \text{CNBr} \rightarrow \text{O} \quad \text{C} \quad \text{N} \quad \text{O} \quad \text{or} \quad \text{O} \quad \text{C} \quad \text{NH}
\]

(9.1)

The relative amounts of the two groups depend on the type of polysaccharide used (e.g., the relative amount of cyanate esters is higher for agarose than for crosslinked dextran in which imidocarbonate is predominant). If the right conditions are used, the activated matrix can be stored for a long time without significant loss of reactive groups either as a
<table>
<thead>
<tr>
<th>Reagent for Activation and Coupling</th>
<th>Matrix</th>
<th>Functional Group in Matrix</th>
<th>Activation Conditions</th>
<th>Activated Structure</th>
<th>Ligand</th>
<th>Functional Group in Ligand</th>
<th>Coupling Conditions</th>
<th>Ligand Matrix Bond</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNBr (titration)</td>
<td>Polyols (esp. polysacch.)</td>
<td>–OH</td>
<td>Aq. pH 11–12</td>
<td>Eq. 9.1</td>
<td>Amines (esp. proteins)</td>
<td>–NH$_2$</td>
<td>Aq. pH 7–8.5</td>
<td>Isourea</td>
<td>33, 35</td>
</tr>
<tr>
<td>CNBr (buffer)</td>
<td>Polyols (esp. polysacch.)</td>
<td>–OH</td>
<td>Aq./buffer</td>
<td>Eq. 9.1</td>
<td>Amines (esp. proteins)</td>
<td>–NH$_2$</td>
<td>Aq. pH 7–8.5</td>
<td>Isourea</td>
<td>34</td>
</tr>
<tr>
<td>CDAP</td>
<td>Polyols (esp. polysacch.)</td>
<td>–OH</td>
<td>Org./aq.</td>
<td>Eq. 9.4</td>
<td>Amines (esp. proteins)</td>
<td>–NH$_2$</td>
<td>Aq. pH 7–8.5</td>
<td>Isourea</td>
<td>35</td>
</tr>
<tr>
<td>DSC [(N,N′-disuccinimidylcarbonate)]</td>
<td>(Esp. agarose)</td>
<td>–OH</td>
<td>Organic</td>
<td>Eq. 9.12</td>
<td>Amines (esp. proteins)</td>
<td>–NH$_2$</td>
<td>pH 6–8</td>
<td>Carbamate</td>
<td>38</td>
</tr>
<tr>
<td>CDI (Carbonyldiimidazole)</td>
<td>(Esp. agarose)</td>
<td>–OH</td>
<td>Organic</td>
<td>Eq. 9.11</td>
<td>Amines, thiols</td>
<td>–NH$_2$, –SH</td>
<td>pH 9–10</td>
<td>Sec. amine throether</td>
<td>37</td>
</tr>
<tr>
<td>Tosyl chloride</td>
<td>(Esp. agarose)</td>
<td>–OH</td>
<td>Organic</td>
<td>Eq. 9.1</td>
<td>Amines, e.g., proteins thiols</td>
<td>–NH$_2$, –SH</td>
<td>pH 8–9</td>
<td>Sec. amine throether</td>
<td>37</td>
</tr>
<tr>
<td>Treosyl chloride</td>
<td>(Esp. agarose)</td>
<td>–OH</td>
<td>Organic</td>
<td>Eq. 9.1</td>
<td>Amines, e.g., proteins thiols</td>
<td>–NH$_2$, –SH</td>
<td>pH 8–10</td>
<td>Ether, sec. amine throether</td>
<td>27</td>
</tr>
<tr>
<td>Bisoxiranes</td>
<td>Polyols</td>
<td>–OH</td>
<td>Aq. pH 13–14</td>
<td>Eq. 9.7</td>
<td>Carbohydrates, amines, thiols</td>
<td>–OH</td>
<td>pH 11.5–13</td>
<td>Ether, throether</td>
<td>36</td>
</tr>
<tr>
<td>Epichlorohydrine</td>
<td>Polyols</td>
<td>–OH</td>
<td>Aq. pH 13–14</td>
<td>Eq. 9.9</td>
<td>Carbohydrates, amines, thiols</td>
<td>–OH</td>
<td>pH 11.5–13</td>
<td>Ether, throether</td>
<td>36</td>
</tr>
<tr>
<td>DVS (Divinylsulfone)</td>
<td>Polyols</td>
<td>–OH</td>
<td>Aq. pH 13–14</td>
<td>Eq. 9.10</td>
<td>Carbohydrates, amines, thiols</td>
<td>–OH</td>
<td>pH 10.5–12</td>
<td>Ether, throether</td>
<td>31</td>
</tr>
<tr>
<td>Carboximidicesters</td>
<td>Polyols</td>
<td>–COOH, –NH$_2$</td>
<td>Aq.</td>
<td>Eq. 9.13</td>
<td>Amines carboxylates</td>
<td>–NH$_2$, –COOH</td>
<td>pH 5</td>
<td>Amides</td>
<td>9, 41–44</td>
</tr>
<tr>
<td>Esteractivated carboxyl Reaction</td>
<td>Polyols</td>
<td>–COOH</td>
<td>Organic</td>
<td>Eq. 9.14</td>
<td>Amines (esp. proteins)</td>
<td>–NH$_2$</td>
<td>pH 5–9</td>
<td>Amides</td>
<td>45</td>
</tr>
<tr>
<td>with matrix thiol</td>
<td>Polyols</td>
<td>–SH</td>
<td>Aq.</td>
<td>Eq. 9.13</td>
<td>Amines carboxylates</td>
<td>–NH$_2$, –COOH</td>
<td>pH 5</td>
<td>Amides</td>
<td>9, 41–44</td>
</tr>
<tr>
<td>Thiol–disulfide exchange</td>
<td>Polyols</td>
<td>–SH</td>
<td>Aq.</td>
<td>Eq. 9.13</td>
<td>Amines carboxylates</td>
<td>–NH$_2$, –COOH</td>
<td>pH 5</td>
<td>Amides</td>
<td>45</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>Polyamide</td>
<td>–CO NH$_2$</td>
<td>Aq.</td>
<td>Eq. 9.16</td>
<td>Amines (esp. proteins)</td>
<td>–NH$_2$</td>
<td>pH 7</td>
<td>Prob. sec. amine</td>
<td>52</td>
</tr>
<tr>
<td>Hydrazine (acylazide)</td>
<td>Polyamide</td>
<td>–CO NH$_2$</td>
<td>NaNO$_2$ in HCl</td>
<td>Fig. 9.2</td>
<td>Amines (esp. low molecular weight)</td>
<td>–NH$_2$</td>
<td>pH 7–9</td>
<td>Amide</td>
<td>53</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>Polyamide</td>
<td>–CO NH$_2$</td>
<td>NaNO$_2$ in HCl</td>
<td>Fig. 9.2</td>
<td>Amines (esp. low molecular weight)</td>
<td>–NH$_2$</td>
<td>pH 7–9</td>
<td>Amide</td>
<td>53</td>
</tr>
<tr>
<td>Oxirane via silanization</td>
<td>Silica, glass</td>
<td>–Si–OH</td>
<td>Fig. 9.3</td>
<td>Variety of compounds</td>
<td>–NH$_2$, –SH</td>
<td>pH 8</td>
<td>Sec. amine, throether</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>Isocyanide</td>
<td>Various</td>
<td>–COOH, –NH$_2$, –COOH, –CO, –NC</td>
<td>Aq. pH 6.5</td>
<td>Eq. 9.15</td>
<td>Variety of comp.</td>
<td>–COOH, –NH$_2$, –COH, =CO</td>
<td>pH 6.5</td>
<td>Amide</td>
<td>49, 51</td>
</tr>
</tbody>
</table>
suspension or lyophilized. The cyanate esters are hydrolyzed at alkaline pH, whereas the imidocarbonates are converted to carbonates at acidic pH. Activated agarose is commercially available (Table 9.7).

Amino-containing ligands are covalently linked to the activated matrix in an aqueous medium at close to physiological pH, 7–8.

\[
\text{OH} + \text{H}_2\text{N} \rightarrow \text{NH}^+ \\
\text{OH} \text{O} \rightarrow \text{C} \text{N} \text{H}_2 \\
\text{OH} \text{O} \rightarrow \text{C} \text{H}_2 \\
\text{OH} \text{O} \rightarrow \text{C} \text{N} \\
\]

The bonds formed between the ligand and the matrix are mainly of the isourea type. When the ligand reacts with the imidocarbonates, the products are \(N\)-substituted imidocarbonates as well as isourea derivatives. \(N\)-Substituted carbamates also occur when the ligand reacts with cyclic carbonate (Eq. 9.2c), formed by the hydrolysis of the \(N\)-substituted imidocarbonates.

The simplicity of the method, the fact that it works so well in combination with beaded agarose (a matrix with excellent chromatographic properties), and that it is mild enough for binding sensitive ligands such as proteins like antibodies and enzymes, have made the CNBr technique by far the most used technique for the preparation of affinity adsorbents.

In the activation step the pH is kept constant either by the addition of strong sodium hydroxide or by the use of a buffer (40, 42). The high pH is needed to deprotonate the polysaccharide hydroxyl groups (\(pK = 12\)) to the corresponding alkoxide ions, which are sufficiently nucleophilic to react with the CNBr. The basic reaction medium causes the hydrolysis of CNBr to inert cyanate ions \(OCN^-\), thus consuming more than 90% of the initially added amount of CNBr (Fig. 9.1). The formed cyanate esters, to a great extent, also hydrolyze to inert carbamate groups and react with matrix hydroxyls to form less active imidocarbonates (43). This of course decreases the capacity of the activated matrix to bind ligand and introduces possible sites for nonspecific interactions in the affinity adsorbent.

![Figure 9.1](image.png)  
*Figure 9.1* Reactions occurring when a polysaccharide matrix is activated by the classic CNBr method at pH 11–12 (from Reference 43).
In some cases, the imidocarbonates (as well as the carbonates formed as a result of hydrolysis, at acidic pH, of the imidocarbonates) also act as covalent crosslinkages and stabilize the matrix mechanically and chemically without significantly changing the porosity. This is particularly useful when non-crosslinked agarose is used. Despite the side reactions, the original CNBr method and varieties of it have been widely used in many successful applications of affinity chromatography, which show that by performing the activation and coupling in an accurate way, reproducible results can be obtained. The yield in the reaction is poor. Less than 2% of the CNBr forms useful reactive groups. This does not present an economic problem, at least not on a small scale, as CNBr is an inexpensive chemical. However, more serious are the health hazards arising from dealing with large quantities of CNBr. Because of its toxicity and high vapor pressure, all work with CNBr should be carried out in properly ventilated fume hoods.

Kohn and Wilchek (43) have devised a method to increase the electrophilicity of CNBr by forming a so-called cyano-transfer complex by CNBr and certain bases such as triethylamine (TEA) and dimethylamino-pyridine (DAP). The cyano transfer complex formed with TEA is not stable, but the one formed with DAP can be isolated as 1-cyano-4(dimethylamino)pyridinium bromide.

These cyano transfer complexes are far more electrophilic than CNBr and are thus able to cyanylate the matrix hydroxyl groups to cyanate esters at a much lower pH than used in the conventional procedure. The hydrolysis of CNBr is thus avoided, as is the transformation of cyanate esters to other products. As a result, the yield of cyanate esters improves dramatically to 20–80% depending on the conditions.

However, the activation reaction requires that a mixed organic solvent–water system is used (e.g., acetone–water = 6:4); in a pure water system, irreproducible and low yields result. A low temperature (0°C) typically also gives better results than room temperature.

The cyano transfer reaction is best suited for agarose beads that can be transferred to mixed solvents without shrinking. Note that the matrices will not be reinforced by covalent crosslinkages to the same extent as with the original CNBr method discussed earlier. Several cyano transfer complexes have been described. One of the more useful reagents is 1-cyano-(4-dimethylamino)pyridinium tetrafluoroborate (CDAP) (43), which is also available commercially.

[Chemical structure]

CDAP is a quite stable and nonhygroscopic salt that can be safely handled on the laboratory bench in open vessels without health hazards. It can be stored as a solid at room temperature for long periods of time, or can be dissolved in acetonitrile at −20°C for weeks. It hydrolyzes rather slowly in 0.1 M HCl, but complete hydrolysis occurs in a few hours at pH 7.

In the coupling step, regardless of the activation method used, ligand amino groups react with the matrix-bound cyanate esters to form isourea bonds (Eq. 9.2a). In the case of crosslinked dextran and cellulose, which are most conveniently activated by the conventional CNBr procedure, the majority of the activated groups are imidocarbonates, which, in the coupling step, can be converted to both N-substituted isourea structures and N-substituted imido-carbonates, as mentioned earlier.

Isourea derivatives have pK values of ~9.5 and are therefore positively charged at neutral pH. The adsorbent thus becomes a weak anion exchanger. This does not usually present a problem. More serious is the fact that the reaction is reversible and the isourea bond can be cleaved [e.g., by hydrolysis at weakly alkaline pH and by aminolysis with low molecular weight amines (37)]. It can, in fact, be demonstrated that single-point attached ligands are released at a significant rate.

Thus, the CNBr technique is not the ideal immobilization method for such ligands. For ligands bound through multiple
points, such as polypeptides, the rate of release is not greater than for other commonly used immobilization methods and is more dependent on the stability of the matrix used.

9.4.1.2 Bisepoxirane, Epichlorohydrin, and Divinylsulfone Methods Activations based on bisepoxiranes permit the immobilization of ligands containing hydroxyl, amine, and thiol groups (35). Especially useful is the possibility of coupling sugar ligands (e.g., mono- and oligosaccharides). An interesting characteristic is that the ligands will be provided automatically with a hydrophilic spacer arm. The reactions are described in Equation 9.7.

\[
\text{Bisepoxirane: } O + \text{CH} - \text{CH} - (\text{CH}_3) - \text{CH} - \text{CH}_2 \\
\text{Activated matrix: } O - \text{CH} - \text{CH} - (\text{CH}_3) - \text{CH} - \text{CH}_2 + \text{H}_2\text{N} - \text{Ligand} \\
\text{Epichlorohydrin: } O - \text{CH} - \text{CH} - (\text{CH}_3) - \text{CH} - \text{CH}_2 + \text{H}_2\text{N} - \text{Ligand}
\]

This activation also introduces covalent crosslinkages in the matrix. Although this might lead to a certain decrease in porosity, it is, at least in the case of agarose, advantageous as it increases the stability (e.g., thermostability) and rigidity of the gel.

The oxirane group is rather stable at pH values below 8. The activated matrix can therefore be stored as a suspension for prolonged periods of time until used. This, however, also means that rather high values of pH have to be used to couple hydroxyl (pH 11–12) and amino ligands (preferably pH > 9). The method is therefore not suitable for unstable ligands (e.g., many proteins). It should, however, be possible to couple thiol-containing proteins at lower pH values as the thiol (thiolate ion) is a better nucleophile than the other two functional groups. The poor reactivity of the oxirane groups also makes it difficult to eliminate residual activity (remaining groups) after coupling in the blocking step under conditions tolerable for the coupled ligand. Reactions with the commonly used deactivation reagent ethanolamine have to be performed at a rather high pH to be efficient. Thiol reagents such as mercaptopropanol will work at a lower pH, but can only be used if the ligand does not contain easily reducible disulfide bonds. Oxirane groups can be determined easily by reaction with sodium thiosulfate (44). The sodium hydroxide formed is simply tittered with acid.

\[
\text{O} - \text{CH} - \text{CH} + \text{Na}_2\text{S}_2\text{O}_3 \\
\text{S} - \text{SO}_3^- + \text{OH}^- + 2\text{Na}^+
\]

Typically, a degree of substitution of 50 µmol of active groups per milliliter of gel can be obtained on beaded 6% agarose by varying excess bisepoxirane used in the activation (35). The most commonly used bisepoxiranes are 1,4-butanediol-bis(epoxypropylether), ethyleneglycol-bis(epoxypropylether), and 1,2,3,4-diepoxybutane. The activation and coupling procedures with epichlorohydrin on polysaccharide gels are very similar to those used for bisepoxiranes (45).

\[
\text{O} - \text{CH} - \text{CH} - \text{CH}_2 \\
\text{Activated matrix}
\]

Activation with epichlorohydrin leads to the introduction of gel-bound oxirane groups and of crosslinkages in the matrix. The properties of the activated gel are thus very similar to those obtained with the bisepoxiranes, except for the fact that the spacer introduced is shorter.

As with bisepoxiranes and epichlorohydrin, divinylsulfone (DVS) can also be used for the immobilization of amino-, hydroxyl-, and thiol-containing ligands to hydroxyl-containing matrices (38).
9.4.1.3 Organic Sulfonyl Chlorides: Tosyl and Tresylchloride Methods

These methods are most suitable for the immobilization of amino- and thiol-containing ligands to beaded agarose (46). The reactions are described in Equation 9.11:

\[
\begin{align*}
\text{Toxylchloride} & \quad \text{Activated matrix} \\
\text{OH} + \text{Cl} & \rightarrow \text{O} \\
\end{align*}
\]

(9.11a)

\[
\begin{align*}
\text{O} & \quad \text{CH}_3 + \text{H}_2\text{N} \quad \text{Ligand} \\
\end{align*}
\]

(9.11b)

Organic sulfonyl chlorides react with hydroxyl groups to form good leaving groups, sulfonates, that allow binding of nucleophiles directly to the hydroxyl carbon. This principle can be utilized for the coupling of low molecular weight ligands, as well as high molecular weight ligands such as proteins, to hydroxyl-group-carrying matrices. The method works with both amino- and thiol-containing ligands that become immobilized to the matrix through stable \(-\text{CH}_2-\text{NH}-\) and \(-\text{CH}_2-\text{S}-\) linkages.

The activation has to be performed in nonaqueous solvents, preferably acetone. Thus, only matrices that swell in the solvents can be activated. Beaded agarose is most often used, but cellulose and silica derivatives containing hydroxyl groups have also been activated successfully and used for the immobilization of a variety of ligands. Activated matrices can be stored for several weeks as suspensions in 1 mM HCl without losing their coupling capacity.

The coupling can be performed both in an aqueous solvent and in an organic solvent such as dimethylformamide (DMF). The latter is used when the ligand is not soluble in water.

The reactivity of the sulfonate ester formed is strongly influenced by the R group. Tosylates (\(R=\text{CH}_2\text{C}_6\text{H}_5\)) and especially tresylates (\(R=\text{CF}_3\text{CH}_2\)) seem to be the most suitable for the immobilization of ligands (46). In fact, tresylates allow efficient immobilization even at neutral pH and at 4°C. Tosylated matrices, however, are less reactive and require coupling at pH 9 to 10.5 and are thus used with ligands that can tolerate such conditions. Apart from being less expensive than tresylchloride, tosylchloride also has the advantage of releasing a chromophore upon reaction. This means that the coupling reaction can be followed photometrically.

9.4.1.4 Methods Using \(N,N^0\)-Disuccinimidyl-Carbonate (DSC) or Carbonyldiimidazol (CDI)

A two-step method, using \(N,N^0\)-disuccinimidyl-carbonate (DSC), as the activating agent for the immobilization of amino-containing ligands to beaded agarose, has been described by Wilchek and Miron (47).
Hydroxysuccinimide carbonate groups are first introduced into the gel by reaction of its hydroxyl groups (especially primary ones) with DSC in organic milieu with a base catalyst such as TEA. The carbonate subsequently reacts with amines under the formation of carbamates. The coupling reaction runs both in aqueous systems under mild conditions (pH 6–8) and in organic solvent (if a base catalyst such as TEA is used). It can be used to immobilize amino-containing ligands of both high molecular weight (such as sensitive proteins) and low molecular weight types. Unlike the isourea bond formed in the CNBr methods, the carbamate bond is very stable and noncharged under conditions usually applied for affinity chromatography.

In aqueous systems the hydroxysuccinimide carbonate groups rapidly decompose by hydrolysis with regeneration of the gel hydroxyl groups and release of \( \text{N-hydroxysuccinimide} \). The hydrolysis is faster at higher pH but proceeds at an appreciable rate even at neutral and weakly acidic pH. The activated gel should therefore be protected from water before it is mixed with the ligand solution. \( \text{N-Hydroxysuccinimide} \) has a \( \lambda_{\text{max}} \) at 261 nm with a molar extinction coefficient of 10,000 M cm.

The degree of activation of the gel can thus be determined photometrically after complete hydrolysis of the hydroxysuccinimide carbonate groups at high pH or after aminolysis with hydroxylamine. The hydroxysuccinimide groups also react to some extent with neighboring hydroxyl groups on the gel under formation of gel-bound carbonate groups. This seems to occur in conditions favoring a high degree of substitution (large excess of DSC). Despite their low reactivity, the carbonate groups formed might cause problems later on when the gel derivative is used as affinity adsorbent by inadvertently immobilizing reactive substances in the sample. The carbonate groups in the gel can be eliminated by prolonged exposure to alkaline pH (\(~11\) ), conditions that might be detrimental to the ligand.

The CDI reagent can also be used to activate hydroxyl-containing matrices (48). The imidazoyl carbonate groups thus introduced into the matrix react with amino-containing ligands with the formation of carbamates. The activation has to be performed in an organic solvent, but the coupling can be run in aqueous systems. The imidazoyl carbonate groups are not as reactive as the groups introduced in the similar DSC method. Thus the ligand coupling has to be performed at a higher pH. The degree of activation (moles of imidazoylcarbonate groups per milliliter of gel) can be determined by keeping the activated gel at pH 3 for 4 h. This treatment leads to hydrolysis of the reactive groups and to the release of imidazol, which can be determined by titration between pH 9 and 4 (49).

\subsection{9.4.1.5 Condensation Methods Based on Carbodiimides}

For a long time the carbodiimides have been used for the synthesis of peptides. They were also among the first reagents to be used in the synthesis of affinity chromatography adsorbents and are still among the most widely used. Using these reagents, stable amide bonds can be formed between a ligand that contains an amino group and a carboxyl-containing matrix (or vice versa) in a one-step procedure.

The reaction is performed by mixing the ligand with the matrix together with the reagent at slightly acidic pH (\(~5\) ) (pH adjustment with HCl and buffer).

\begin{equation}
\text{Carboxyl-containing gel} \quad \text{Carbodiimide} \quad \text{Isourea ester} \quad (9.13a)
\end{equation}

\begin{equation}
\text{C} \quad \text{NH} \quad \text{R'} \quad \text{H}_2\text{N} \quad \text{Ligand} \quad (9.13b)
\end{equation}

The first step in condensation is the addition of the carboxylate to either \( C=\text{N} \) bond of the diimide, yielding the highly reactive and unstable \( O\)-acylurea (isourea ester). This, in turn, reacts chiefly with the amine to produce an amide and \( \text{N,N'-dialkylurea} \). The major side reaction, the intramolecular rearrangement of the \( O\)-acylurea and formation of a stable \( N\)-acylurea, can be minimized if a large excess of amine-containing ligand is used. If that cannot be achieved, some of the resulting \( N\)-acylurea will be bound to the matrix (33).

The reaction time is usually several hours and it is necessary to adjust the pH in the suspension during the first hour. Carbodiimides are relatively unstable compounds and must be handled with care because of their toxicity. The method is usually used to couple ligands to spacer arms with carboxyl or amino groups as terminal groups, but can of course also be used to immobilize ligands to any matrix containing amino or carboxyl groups.

Among those that have been used in the preparation of derivatives for affinity chromatography are the water-soluble type such as 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-p-toluenesulfonate (CMCL) and 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide hydrochloride (EDCL), and those soluble in organic solvents such as dicyclohexyl-carbodiimide (DCC) (50–53).

It has been shown that the mild performance of carbodiimide coupling procedures requires the presence of very high nucleophile concentrations (e.g., unprotoned amino
compounds) in the reaction medium (e.g., slightly acidic, pH 5.0–6.0) in which carboxyl groups are being activated by carbodiimide. However, most of the commercially available aminated supports (e.g., AH-Sepharose) contain a low concentration of aliphatic amino groups with very high pK values.

Because of that, agarose gels provided with a high density of primary amino groups with low pK values have been designed and developed (54). Thus in monoaminoethyl-N-aminoethyl-agarose (MANA-gel), the primary amino group has a surprisingly low pK value (6.8), because it is very close to a secondary amino bond. These two facts together make MANA gels suitable for mild carbodiimide activation of carboxyl ligands and their efficient coupling to yield highly substituted affinity adsorbents.

An alternative way to activate carboxyl-containing matrices is to convert the carboxyl groups to reactive esters by reaction with N-hydroxysuccinimide in anhydrous medium in the presence of a carbodiimide (DCC) (55). The matrix-bound N-hydroxysuccinimide ester reacts easily at pH 5–8 with amines with the formation of an amide and release of N-hydroxysuccinimide (9:14a).

The ester is labile and undergoes hydrolysis in aqueous solutions, especially at a pH above 6. The activated gel should therefore be stored as a suspension in an anhydrous medium (e.g., dioxane). For shorter periods (minutes to a few hours) it can be stored in water at pH 5–6, provided that there are no amines present. As the technique is more laborious than those based on a direct use of a condensation reagent, it is only recommended for ligands containing both primary amines and carboxyl functions (e.g., proteins), where the use of a condensation reagent in the coupling would lead to unwanted inter- and intramolecular crosslinkages.

9.4.1.6 Methods for Thiol-Containing Matrices So far, two main principles for the immobilization of ligands to matrices have been discussed:

- introduction of electrophilic groups into the matrix that subsequently react with nucleophilic groups in the ligand
- use of condensation agents to establish amide bonds between amino groups in the ligand and carboxyl groups in the matrix (or vice versa).

Another possibility is to immobilize a ligand by means of an electrophilic group (either already present or introduced prior to the immobilization) that reacts with a nucleophilic group in the matrix. Although not widely used, this approach has several merits. It is most easily applied to thiol-containing matrices. Thus, reaction of a thiol-containing matrix with alkyl or aryl halides and ligands containing C=O, and under certain conditions C=C bonds, will lead to stable thio-ether derivatives through nucleophilic displacement (in the first case) and addition (in the second case). Unsaturated compounds such as testosterone and estradiol have been attached to beaded thiol agarose using γ radiation (56). Heavy metal ion-containing ligands can also be bound to a matrix via thiol groups by mercaptide formation (57). Activated halides with the halogen α to a carbonyl group (as in iodoacetic acid) react smoothly at weakly alkaline pH with thiols. These reactions usually take place in aqueous or polar organic solvents under rather mild conditions, but for unactivated halides higher pH values have to be used. Estradiol and testosterone, mentioned earlier, require higher pH values.

Most matrices suitable for affinity chromatography can be provided with aliphatic thiol groups by simple organic chemistry. Several methods for the thiolation of polysaccharides, especially beaded agarose, are described in detail in Chapter 8 on covalent chromatography. Porous glass and silica are also easily substituted with thiol groups by their silanization with γ-mercaptopropyl-trimethoxysilane (58).

9.4.1.7 The Isocyanide Method Most of the ligand immobilization methods described earlier require that a reactive amino group is present in the ligand, although in some cases they also work with hydroxyl or thiol compounds. Much more flexible is the so-called isocyanide (isonitrile) method, which, despite the fact that it was described a long time ago, has not attracted much interest (59). The method is based on the four-component condensation of amine, carboxyl, isonitrile, and carbonyl compounds originally
discovered and examined by Ugi and colleagues (60). The principle is outlined in Equation 9.15:

\[
R^1 - C - OH + H_2N - R^2 \rightarrow R^1 - C - N - CH - CONH - R^2
\]

An ammonium ion structure is formed from an aldehyde or ketone and an amine. With the isocyanide, this structure forms a highly reactive intermediate that is very susceptible to addition reactions with nucleophiles, such as carboxylic and hydroxylic ions. A stable amide is finally produced by intramolecular rearrangement. Although the reaction appears complicated, the technique is, in practice, very simple to use for immobilization purposes.

The ligand to be attached may contain amino, carboxyl, aldehyde, ketone, or isocyanide functions. The matrix may contain any of the others. The two remaining functional groups are added to the reaction mixture as low molecular weight substances (e.g., aliphatic amines, carboxylic acids, aldehydes, ketones, and isonitriles).

The reaction occurs in aqueous medium at pH 5–6. Matrices that have been used include crosslinked dextran and beaded agarose containing carboxyl groups, polyacrylamide, agarose substituted with amino groups or carboxyl groups, and a large number of insoluble polymers containing isonitrile groups (59, 61). Immobilized substances include proteins, peptides, amino acids, biotin, and steroids (which have been used as carboxyl compounds).

The coupling of a protein can be directed toward its amino or carboxyl groups using an excess of low molecular weight carboxyl or amine compound, respectively.

### 9.4.2 Methods for Polyacrylamide Matrices

The glutaraldehyde and hydrazine methods are suitable for matrices having amide groups, such as polyacrylamide, and for the immobilization of amino-containing ligands (35, 62). These methods do not work on polysaccharide matrices. The mechanism of the activation and coupling is not

---

**Figure 9.2** Immobilization of an amino group containing ligand to a polyacrylamide matrix by the hydrazine method.
completely understood, but is supposed to follow the scheme outlined in Equation 9.16:

\[
\begin{align*}
\text{Activated matrix} & \quad + \quad \text{ligand} \\
\end{align*}
\]

Hydrazine is a particularly useful reagent in combination with amide-containing matrices. When polyacrylamide is heated with hydrazine, hydrazide acrylamide (or polyacrylhydrazide) is formed. The hydrazide groups can be converted to reactive acylazides by treatment of the matrix with sodium nitrite in hydrochloric acid (63). Amino-containing ligands can then be coupled to the activated matrix by the formation of stable amide bonds (Fig. 9.2). It has been claimed that yields when coupling high molecular weight ligands are usually low, possibly because of a combination of instability of the acylazide group and steric hindrance. For low molecular weight ligands the technique seems to work well.

The hydrazide matrix can also be used for the immobilization of ligands containing aldehyde and ketone groups (64). This reaction occurs at low pH (~5) with formation of a hydrazone, which is then stabilized by reduction with alkaline sodium borohydride (pH of ~9) (Fig. 9.2). Although ligand immobilization by means of hydrazines is most simply performed on amide-containing matrices, it can also be used in combination with other matrices such as agarose and porous glass, provided they are modified in a suitable way (64, 65).

### 9.4.3 Methods for Silica Gels and Porous Glass Matrices

Although silica and porous glass beads show good dimensional stability and thus can cope with the often rather high pressures used in HPLAC, they do not fulfill several of the other requirements of an ideal affinity support (Section 9.3.1). Their hydrophobic character and content of negatively charged silanol groups can be changed by chemical modification. In addition to making the matrix surface more hydrophilic and masking the acidic silanol groups, the modification should provide the matrix with functional groups suitable for the coupling of ligands either directly or after activation.

A commonly used procedure (Fig. 9.3) is silanization with reagents such as \(\gamma\)-aminopropylsilane and \(\gamma\)-glycidoxypropylsilane (28):

\[
\begin{align*}
\text{Activated matrix} & \quad + \quad \text{ligand} \\
\end{align*}
\]

Reaction with the first of these reagents gives an amino group functionalized matrix to which many affinity ligands are easily attached either directly by use of the condensation reagents discussed earlier or after further derivatization. The matrix may be converted to a carboxyl-group-containing matrix by reaction of the amines with a suitable anhydride. Unfortunately, these reactions turn the silica or glass into an ion exchanger.

Silanization with the \(\gamma\)-glycidoxypropylsilane leads to the introduction of oxirane groups into the silica or porous glass (28). As discussed previously, this group reacts with nucleophiles with the formation of stable bonds and allows the coupling of ligands containing amino, hydroxyl, or thiol groups to the activated matrix. Because of steric shielding, only a small fraction of the oxirane groups are used up in the ligand immobilization. The excess groups may be hydrolyzed to diols by treatment with acid. These diols give the silica surface a hydrophilic character and decrease its tendency for unwanted protein binding.
9.5 CHROMATOGRAPHIC TECHNIQUES

Having immobilized the ligand or selected the ready-made affinity gel, the next step is chromatography. A typical separation by affinity chromatography consists of four stages: adsorption, washing, elution, and column regeneration. The general practical aspects for affinity chromatography are described in this section. For detailed practical recommendations on individual adsorbents, reference should also be made to the manufacturer’s instructions.

9.5.1 Sample Preparation: Prefractionation

Many affinity separations constitute one-step purification procedures. However, it may be advantageous to include a preliminary step using precipitation or ion-exchange chromatography (see Chapter 1), as this removes some of the major contaminants, reduces the amount of material that must subsequently be processed, and improves the resolution and concentrating effects of the affinity step. This preliminary separation step is recommended when large amounts of sample are to be processed or when the substance of interest is in the presence of very large amounts of precipitating contaminants, such as lipoproteins and clotting factors. When a protein ligand is used in the affinity step, there is also a risk of causing proteolysis by proteolytic enzymes in the extract. This is diminished by prefractionation.

For efficient adsorption, the conditions (pH, ionic strength, etc.) of the sample and the column must allow efficient binding and preferably be optimal for binding. This can be achieved by dialysis of the sample, by desalting and exchange of the buffer by gel filtration, or by adding chemicals or adjustment of pH.

When the starting material consists of tissue, cell culture, fermentation product, or plant material, other steps, such as solubilization, homogenization, extraction, filtering, and/or

Figure 9.3 Immobilization of an amino group containing ligand to a silica matrix using activation with γ-glycidoxypropylsilane.
centrifugation, are included in the fractionation scheme, as described in Chapter 1.

9.5.2 Column Operation

The column size is usually not a critical parameter. Rather, the column size is governed by the capacity of the adsorbent and the amount of substance to be purified. Capacities of affinity gels are generally high, and short, wide columns are often used to obtain rapid separations on beds of usually 1–10 mL of gel.

Typical capacities are 25 mg immunoglobulin/mL gel on Protein A Sepharose CL-4B, 10 mg lactate dehydrogenase/mL gel on AMP Sepharose, and more than 1 mg fibronectin/mL gel on Gelatin Sepharose 4B.

If the binding affinity of the ligand is markedly low (\(K_D > 10^{-4} \text{M}\)) the protein to be purified will not bind but will be retarded on the column (isocratic elution), and the separation of the desired protein from the unbound contaminants will become dependent on the column length. Longer columns are recommended. It is also advisable to use small sample volumes (e.g., 5% of the total bed volume). Ligand density should be maximized and flow rates reduced in isocratic elutions.

Column packing should follow the usual precautions for chromatographic techniques. The recommended flow rates should be used, and packing should be carried out evenly to ensure straight bands.

To achieve binding equilibrium, several approaches apply. Sometimes, batchwise procedures are preferred with gentle stirring during both adsorption and desorption or only during the adsorption step. When columns are used they can be left with reduced or stopped flow to make prolonged contact possible to achieve adequate adsorption and desorption, respectively.

This chapter only gives general recommendations with respect to flow rates. The researcher has to compromise between the acceptable level of resolution, the time required for the separation, and the utilization of available adsorption capacity.

However, for sharp elution peaks and maximum recovery with minimal dilution of the purified protein, the lowest flow rate acceptable from a practical point of view should be used. This is especially important for adsorbents that bind several proteins and when competitive elution is used. Here, the flow rate used is governed by the rate of dissociation from the ligand.

For standard low pressure affinity chromatography, flow rates of 50 cm/h and higher have been used successfully. In HPLAC (see later), the improved mass transfer properties, giving higher dynamic capacities, allow the separation to be performed at much higher linear flow rates, with sharper elution peaks giving smaller elution volumes. Typical flow rates used in HPLAC are between 50 and 125 cm/h. In some cases, flow rates in excess of 200 cm/h have been used successfully in industrial-scale applications.

9.5.3 Adsorption of the Sample: Washing

For efficient adsorption the column and sample must be equilibrated with a buffer reflecting the conditions optimal for binding. The volume in which the sample is applied is not critical, provided that the ligand binds specifically and effectively and that the total capacity of the column is not exceeded.

After sample application the column must be washed with several volumes of the starting buffer to remove all unbound material. The chromatography is usually monitored by UV absorbance, and the washing step is finished when the original baseline is reached. It is recommended to wash with high ionic strength buffers when nonspecific ionic binding is suspected. Nonspecific hydrophobic binding is less easily handled as the specific binding often depends also on hydrophobic interactions.

9.5.4 Desorption

The principle of desorption is to change the binding equilibrium for the adsorbed substance from the stationary to the mobile phase. This can be achieved specifically or nonspecifically.

Ligand-protein interaction is often based on a combination of electrostatic, hydrophobic, and hydrogen bonds. Agents that weaken such interactions might be expected to function as effective nonspecific eluents. Careful consideration of the relative importance of these three types of interaction, as well as the degree of stability of the bound protein, will help in the choice of a suitable eluent. A compromise may have to be made between the harshness of the eluent (or the combination of eluents) required for effective elution and the risk of denaturing the proteins to be purified. Elution can be the most difficult stage of affinity chromatography, especially if the dissociation constant of the ligand is very low.

Broadening of desorption peaks is often a problem. This can be due to slow diffusion mass transport or to slow equilibration kinetics or a wide range of binding affinities in the system. The diffusion is usually not limiting in the HPLAC gels but might be so in the larger standard gel beads. Slow dissociation from the ligand can be a problem regardless of the gel. Peak shapes are often improved by reversing the direction of flow during desorption, as the distance in the column covered by the desorbed protein is minimized. This is called reversed elution.

Gradient elution often gives excellent results in affinity chromatography. A heterogeneity in the sample with regard to binding to the column is then used. For example, iso-enzymes can often be separated by gradient elution (66).
and glycoproteins can be separated according to affinity on immobilized lectins by a sugar gradient (67).

9.5.4.1 Change in pH or Ionic Strength The most frequently used method for eluting strongly bound substances nonspecifically is by decreasing the pH of the buffer (68, 69); sometimes an increase in pH can also be effective (70, 71). The chemical stability of the matrix, the ligand, and the adsorbed substance determine how low the pH can go. This is usually \( \sim \) pH 2–4. It is important to neutralize the fractions as soon as possible after elution. This is most easily done by pipetting a small volume of buffer into each tube of the fraction collector in advance.

An increase in the ionic strength of the buffer elutes proteins bound by predominantly electrostatic interactions (72, 73). Such interactions typically dominate binding to dye columns. Usually 1 M NaCl is sufficient, but occasionally 2 or 3 M salt is required (74). Continuous or stepwise salt gradients can resolve different proteins adsorbed to a particular dye column (75).

9.5.4.2 Change in Polarity When the binding is very strong and dominated by hydrophobic interactions, rather drastic methods of elution have to be used, such as reducing the polarity or including a chaotropic salt or denaturing agent in the buffer. This type of elution is typical for immunosorbents based on immobilized polyclonal antibodies (76–78).

Commonly used chaotropic salts are KSCN, KCNO, and KI in the concentration range 1–3 M; urea and guanidine HCl in moderate concentrations (4–6 M) are sometimes preferred. The polarity can often be decreased enough to promote elution by including 20–40% ethylene glycol in the buffer, although sometimes much higher values are required (79). It is effective as well as being mild and less likely to denature proteins.

When eluting most hydrophobic proteins, such as membrane proteins, decreasing the hydrophobic interactions with detergents is often preferred. Useful detergents are Lubrol, Nonidet P-40, or octylglycosides; Triton X-100 is disadvantageous because of its UV adsorption. The concentrations used are just below the critical micelle concentration (CMC).

A low concentration of a detergent is sometimes included during the entire purification process (see Chapter 1 and Reference 80). Thus, nonspecific hydrophobic adsorption, as well as aggregation, can be suppressed, particularly in antigen–antibody purification or when handling membrane proteins. The detergents can also be left in the buffer when eluting with, for example, a moderate change in ionic strength or pH. An alternative is to increase the detergent concentration to achieve elution.

One example of an affinity system with very strong interactions is rat biotin-binding protein–biotin-AH-Sepharose (81). This protein binds very tightly to the adsorbent and does not elute with a saturating concentration of biotin (4 mM) or with other desorption methods such as elevated temperature (40°C), 8 M urea, 8 M KI, or low pH (3.6). However, the protein could be eluted by 3 M guanidine-HCl or by 2 M urea plus 4 mM biotin in the equilibration buffer.

9.5.4.3 Specific Elution In specific elution, bound proteins are desorbed from the ligand by the competitive binding of the eluting agent either to the ligand or to the protein. Specific eluents are most frequently used with group-specific adsorbents as the selectivity is greatly increased in the elution step. Glycoproteins can be desorbed from lectin columns by elution with competing carbohydrates (67, 82). Desorption of a lectin from an immobilized carbohydrate with a competing carbohydrate is described in Section 9.6.2.1 and in Reference 83. Specific elution has also proved to be effective when isolating receptors (84).

Elution generally occurs at rather low concentrations of eluent (5–100 mM). A concentration of a single eluent or pulses of several different eluents can be used as well as gradients. Specific elution is often performed at neutral pH and is thus a mild desorption method causing little or no denaturation. If the eluting agent is bound to the protein, it can be dissociated by desalting on a gel filtration column or, when the binding is stronger, by dialysis.

9.5.5 Regeneration of Adsorbent All affinity adsorbents should be reusable. The exact extent of reuse depends on the nature of the sample and the stability of the ligand and matrix with respect to the elution and cleaning conditions used. The most important aspect in regeneration is to remove any material still bound to the adsorbent. In most cases it is sufficient to re-equilibrate with several column volumes of starting buffer. If necessary, one can wash with buffers of alternately high and low pH and even include detergents and denaturing agents, depending on the stability of the ligand. For ready-made adsorbents the manufacturer’s instruction should be followed.

9.5.6 High-Performance Liquid Affinity Chromatography High performance liquid affinity chromatography (HPLAC) (for a review, see Reference 28) combines the high specificity and selectivity of affinity chromatography with the speed of HPLC. Mechanically stable particles, provided with a noncharged, hydrophilic surface, which can withstand prolonged use in aqueous buffers at moderate or high pressures, are used as supports. The particles are usually considerably smaller than the ones used in standard low pressure affinity chromatography. This is because smaller particles lead to improved efficiency due to shorter diffusion distances (see Chapter 2). Solid beads offer optimum conditions in this respect.
However, to get an adequate surface area, average particle diameters of $<5 \mu m$ are required.

HPLAC was introduced by Mosbach and colleagues (66) in 1978 by demonstrating separations on derivatized $10-90 \mu m$ macroporous silica beads. Since then, other noncompressible porous particles such as highly crosslinked agarose, methacrylate, copolymers of ethylene glycol and methacrylate, and methacrylate vinyl copolymers have been used as support materials. Supports substituted with a number of standard affinity chromatography ligands have been described for HPLAC. To provide maximum flexibility of application, supports with reactive or activatable groups, to which desired ligands can be covalently bound, have also been prepared (85).

HPLAC is often performed using prepacked columns. Such columns containing supports with various immobilized ligands can be purchased from several manufacturers. Examples are Selecti-Spher-$10^{TM}$ concanavalin A, Selecti-Spher-$10^{TM}$ Protein A, and Selecti-Spher-$10^{TM}$ Protein G (Pierce Chemical Company), and Protein A Superose$^{R}$, Protein G Superose$^{R}$, and Chelating Superose$^{R}$ (GE Healthcare). HPLAC columns with several ligands such as biotin, Con A, and heparin are available from Showa Denko K. K., Durasphere (silica 7 $\mu m$). Silica substituted with Cibachrome blue F3GA, Con A, protein G, protein A, mellitin, and heparin are available from Alltech. TSK 5PW and Toyopearl (ethylene glycol/methacrylate copolymers, 10 and 40–90 $\mu m$, respectively) substituted with Cibachrome blue F3GA, aminophenyl boronate, heparin, and protein A can be purchased from TosoHaas. SigmaChrom affinity media (methacrylate, 2 $\mu m$) are available substituted with lysine, $N$-acetylgalactosamine, avidin, Cibachrome blue, Procion red, glutathione, Con A, lentil lectin, ricin lectin, wheat germ lectin, $5'$-adenosine monophosphate, heparin, pepstatin, protein A, and protein G.

Also commercially available are prepacked preactivated columns, such as NHS-activated Superose$^{R}$ (GE Healthcare), Selecti-Spher-$10^{TM}$ activated tresyl (Pierce Chemical Company) and Ultrafiafinity$^{TM}$ EP columns (Beckman Instruments), epoxy and divinylsulfon-activated Durasphere, TSK 5PW and Toyopearl with tresyl and epoxy groups, and Affy-prep (Biorad) with $N$-hydroxysuccinimide. With these products the users can easily perform in situ immobilization of the desired ligand.

It is also possible to prepare HPLC adsorbents from, for example, silica and crosslinked agarose with the batch activation and immobilization methods described in Sections 9.2 and 9.3. The user should, however, be aware of the technical difficulties of obtaining well-packed HPLC columns from small diameter particle suspensions (see Chapter 2).

Small ($<4 \mu m$) nonporous particles have been used as an alternative to the larger (10–90 $\mu m$ diameter) macroporous particles (86). Thus, amino functions were introduced into nonporous, monodisperse, polystyrene beads (average particle diameter, 3.7 $\mu m$) by nitration and subsequent reduction. Two affinity adsorbents were prepared by covalent coupling of the ligands $p$-aminophenyl-$\beta$-$D$-glucopyranoside and $p$-aminobenzamidine using the crosslinker hexamethylene-diisocyanate and aminated polystyrene particles. The adsorbents were used for HPLC of concanavalin A and trypsin, respectively (86).

HPLAC has so far been used mainly in micropreparative and analytical applications. Thus, cardiac myosin and actin have been purified in one step using salicylate immobilized to a preactivated Ultrafiafinity$^{TM}$ EP column (87). Desorption was achieved by specific competitive elution and by increased ionic strength.

Human IgG oligosaccharides have been resolved by serial affinity chromatography on lectin columns (88). This application demonstrated nicely the resolution of peaks obtained with small differences in $k'$ during isocratic elution. Extremely fast (20 sec or less) analytical separations have been obtained on affinity packings using minicolumns (<2 cm length) (89). One example of an analytical application of HPLAC is the determination of plasmin and plasminogen in human blood using Toyopearl (a hydrophilic vinyl polymer resin) substituted with the trypsin inhibitor $p$-aminobenzamidine as the affinity medium (8). The plasmin/plasminogen system plays an important role in fibrinolysis and its monitoring is therefore important in diagnosis, prevention, and therapy of various disorders such as thrombosis and myocardial infarction (8).

The following considerations should be kept in mind when selecting a support to prepare a HPLAC adsorbent: When a suitable commercial HPLAC prepacked column or adsorbent cannot be found for a particular separation problem, it is always possible to prepare an adsorbent from an unsubstituted support. Thus, a large variety of well characterized silica, agarose, and resin base matrices are available commercially, as are a number of reagents for their derivatization (see earlier discussion). As previously pointed out, the major disadvantage of silica particles is their instability in alkali, which leads to degradation and ligand leakage.

Crosslinked polystyrene particles are available commercially as both solid and porous beads and are much more chemically stable than silica. However, these particles are very hydrophobic and their surface has to be modified to be more biocompatible to prevent denaturation or unspecific binding of proteins. The modification and introduction of the desired ligands can also be a rather lengthy and difficult task for individuals with ordinary skill in organic preparative chemistry.

The agarose-based beads (e.g., Superose and Sepharose High Performance) are reasonably biocompatible, and a number of easy and straightforward synthetic routes exist for their substitution with a large variety of ligands. However, even the most extensively crosslinked agarose beads are softer than both polystyrene and silica, which
of biomolecules. The monoliths are prepared as single blocks with through-pores and mesopores (5–25 nm) by a sol–gel process. Because of the high hydrophobicity and strong nonspecific adsorption with biomolecules, their applications in bioseparations are scarce. Organic polymer-based monolithic counterparts have found their use mainly in bioseparation. Polyacrylamide and polymethacrylate are the most frequently used organic polymers for casting of monoliths for separation of biomolecules.

9.5.7 High-Performance Monolithic Affinity Chromatography

Macroporous monoliths constitute a new class of stationary phases for different HPLC formats, including μHPLC and large-scale HPLC. The first polymethacrylate monolith was suggested by Tennikova and colleagues (90), who realized the idea of a combination of advantages of convective mass transport in continuous bed support with a short length column. Monoliths can be used in reversed-phase, ion-exchange, hydrophobic interaction, and affinity chromatography. A monolith can be considered a chromatographic material that is cast in a single block and inserted into a chromatography housing (91). It represent a single body and lacks void volume. Although diffusion limits mass transport in particle columns and the pores are utilized only partially, interphase mass transfer in monoliths is governed by convection and the total pore volume is used. This allows a dramatic reduction in the time required for mass exchange between the mobile and stationary phases. Separation times are consequently at least an order of magnitude faster in monoliths (92).

Monoliths have become an interesting alternative to columns packed with beads, and have been successfully employed for separations involving large biological molecules such as proteins, plasmid DNA, and viruses. Polymeric monoliths consist of a solid continuous phase permeated by a continuous network of through-pores and can be distinguished depending on their shape, backbone material, porosity, or pore size. Despite their differences they all are characterized by low mass transfer. Because the flow of the liquid within the channels is driven by the pressure difference, the molecules to be separated are transported to the active sites located on the surface of the channels by convection. Nowadays, these phases are produced from synthetic polymers (polymethacrylate, polyacrylamide, polystyrene) or from inorganic (silica) base. Continuous superporous agarose beds have also been proposed (93); however, polysaccharide supports suffer from weak mechanical stability. Silica monoliths are prepared as single blocks with through-pores (1–2 μm) and mesopores (5–25 nm) by a sol–gel process. Because of the high hydrophobicity and strong nonspecific adsorption with biomolecules, their applications in bioseparations are scarce. Organic polymer-based monolithic counterparts have found their use mainly in bioseparation. Polyacrylamide and polymethacrylate are the most frequently used organic polymers for casting of monoliths for separation of biomolecules.

Cryogels are produced by crosslinking water-soluble polyacrylamide at subzero temperatures. The resulting polymer bed is hydrophilic, spongy, and elastic, and has macropores that are typically 10–100 μm in diameter. Cryogels have been used to separate a variety of different cells, including T-cells and B-cells, to separate bacteria and also to process crude feedstock such as Escherichia coli lysate. The elution can be performed by using a mechanical force to compress the separation media.

However, the most frequently used affinity chromatography monoliths so far are those based on the macroporous copolymer of glycidyl methacrylate and ethylene dimethacrylate (GMA-EDMA). The polymerization of these two monomers to polymethacrylate is a strong exothermal process that is induced thermally or by radiation in the presence of porogens and initiator. Upon polymerization, the extended poly-methacrylate chains become insoluble and precipitate to form small globules that form the backbone of the monolith. It is therefore clear that all parameters influencing the solubility of the polymethacrylate chains will affect their porosity. These parameters are the solvent, additives, pressure, and temperature. The resulting monolith (GMA-EDMA copolymer) contains macropores (1–2 μm) and epoxy groups that can be used directly for ligand immobilization (94). In addition, the diol groups that can be generated on this material tend to give a support with low nonspecific binding for many biological agents, as has been noted with other affinity supports.

Among the different designs of monolithic supports offered at present in the world market, the short GMA-EDMA beds seem to be more convenient for affinity separations. In fact, so far better and more significant results on affinity separations are obtained by using this kind of monolithic support. The high speed of the separation across the flat monolithic disks facilitates product recovery, because the exposure to punitive denaturing influences such as solvents, temperature, and contact time is dramatically reduced. The open channel-like morphology of disk-shaped solid phases allows the immobilization to be carried out in a single-step reaction between the epoxy groups of the sorbent and amino groups of the ligand to be immobilized, under very gentle biocompatible conditions (95).

Convective interaction media (CIM) monoliths, such as CIM disks (short columns) and CIM tubes that have high binding capacities for large molecules and even for particles, enable high flow rates with low pressure drop. These so-called “short columns” are currently produced and distributed worldwide by BIA Separations, Ljubljana, Slovenia, under the trade name CIM® disks. Standard CIM® disks have a diameter of 12 mm and a thickness (bed length) of 3 mm. Mini-CIM® disks, with a diameter of 6 mm and thickness of 2 mm, are also available.

Short monolithic columns are suitable for very fast separation of proteins. With protein A and protein G ligands, these
columns have the potential for rapidly processing large volumes of complex biological mixtures containing antibodies. It is also possible to stack two or more monolithic disks with different ligands into one cartridge. This separation modality has been named *conjoint liquid chromatography*.

The great interest for monoliths over the last decade can be explained by their performance advantages over particle-based media. One significant advantage is their simple synthesis. Polymer monoliths can be formed *in situ* into any shape as large as 8 L to as small as a few nanoliters in the channel of a microfluidic chip. This feature is particularly important for micro- and nanoscale devices where the packing of particulate sorbents is difficult and may be followed by poor reproducibility (96). Despite the many advantages of polymer monoliths, they present lower surface area per volume than porous particle media, possibly limiting their capacity per cycle in some applications. However, their high flow rates compensate, so productivity per unit time is as good, or often better, than particle-based media.

High performance monolithic chromatography (HPMC) offers possibilities for protein separations based on different types of interactions with adsorptive surface of stationary phases. The principle suggested is realized not by the use of conventional beads, but by monolithic macroporous layers shaped as flat disks or tubes. HPMC combines a high capacity and selectivity with low backpressure and, as a consequence, high speed and a short process time. Moreover, the affinity mode of HPMC provides an excellent opportunity to study the properties of *in vitro* constructed biological pairs. Such investigations provide more information about biological events taking place *in vivo* as well as developing optimized affinity separation media for the production of valuable biological substances. Furthermore, the pore size of CIM monolithic supports has been adjusted to accommodate even the largest molecules (like viruses and DNA) without compromising the mechanical stability of the support and are optimized for very high binding capacities at the highest flow rates.

Their use for preparative separation of biomolecules has evolved over the past decade. Polymethacrylate monolithic columns up to 8 L in size are already commercially available for large scale separations of biological macromolecules (94). Monoliths with high porosity and large pore size have also been tailored for cell separations. Thus, an epoxy-based monolith, substituted with polymyxin B as affinity ligand, has been applied successfully in affinity capturing of gram-negative bacterial cells (e.g., *E. coli*) (97). This separation method was effective, enabling cell capture, enrichment, and recovery. The excellent recovery, purity, and viability of the cells separated by the affinity monolithic column, in combination with a high enrichment factor, proved that this method is ideally suited as a pre-enrichment step for many cellular analyses (97).

### 9.6 APPLICATIONS

#### 9.6.1 Immobilization of Ligands

The following sections present the experimental details of a number of commonly used procedures for the immobilization of different ligands to various matrices. Although most of the methods have been developed for beaded agarose as a matrix, they can often be used in combination with other polyol matrices, provided that they retain their structure in the solvents used during the activation and coupling steps.

**9.6.1.1 Some General Advice** Before and after activation and after coupling, the matrix is usually washed and equilibrated with different buffer solutions. These washing steps are most easily performed on a glass filter funnel fitted on a Buchner flask (connected to a vacuum suction device). The matrix is suspended in a suitable volume of the desired medium, which is subsequently filtered off, or the reaction mixture is poured into the filter and the liquid with soluble components is sucked off. The matrix is resuspended in a new aliquot of medium and the liquid is again removed by filtration. This procedure is repeated until the unwanted soluble components are removed or when equilibrating the matrix with a new medium. Allow ample time in each step for the diffusion of soluble reagents and reaction products in and out of the beads. Often the filtration can be performed just by gravity flow or at a very small negative pressure. After each filtration a glass rod can be used to resuspend the matrix in a new portion of buffer.

Never let the matrix dry out completely on the filter during the filtration process (leave a few millimeters of liquid on top of the matrix surface) as this might lead to unwanted aggregation of the beads. This is especially critical when agarose is transferred from an aqueous to an organic medium such as acetone.

To obtain fast kinetics and efficient reactions, the activation, coupling, and deactivation should be carried out by suspending the nonreacted, activated, or coupled matrix in a minimum volume of liquid containing the reagent, ligand, and deactivation compound, respectively, to get a slurry that is dense but can still be agitated. As most beads, particularly agarose beads, are mechanically fragile, agitation with stirrers, fleas, and other harsh conditions should be avoided to minimize disintegration. For small volumes (up to 20 mL of suspension), end-over-end rotation can usually be applied by using sealed-off tubes as reaction vessels (provided that there is no gas evolved as a result of the reaction). A shaking board can also be used.

When the coupling reactions are performed in a buffered medium, it is essential to select buffers that do not react with the activated matrix in competition with the ligand to be immobilized. Thus, amino-containing buffers should be avoided when the ligand is coupled through an
aminofunction, carboxylic acids should not be used as buffers when the ligand is to be coupled via a carboxyl-group, and so on. When condensation agents such as carbodimides are used, neither amines nor carboxyl-containing buffers can be used. In most cases the reagents used for activation are highly toxic and often volatile.

It is therefore recommended that all work be performed in a ventilated fume hood until the washed and drained activated gel is incubated with the ligand. After coupling of the ligand, residual activated groups should be deactivated by reaction with an excess of a suitable low molecular weight compound (ethanolamine, β-mercaptoethanol, etc.) under the same conditions used for coupling the ligand.

The coupled matrix should then be washed very thoroughly to remove all noncovalently bound material. If possible, both alkaline and acidic buffers with varying ionic strength should be used. A tentative washing protocol might include the following:

- 0.1 M sodium phosphate buffer, pH 8
- the same buffer with 0.5 M NaCl
- 0.1 M sodium acetate buffer, pH 5
- the same buffer with 0.5 M NaCl
- the buffer in which the adsorbent is to be stored or used in the affinity chromatography.

The procedure should, of course, be modified according to the special properties of the ligands. Thus, if an organic solvent has been used, the washing should start with this solvent. If necessary, an appropriate detergent can also be included in the washing buffers.

Until used, the prepared affinity adsorbents are best stored as suspensions at 4°C, the medium depending on the stability of the ligand, matrix, and covalent bond used. The suspension should also contain an antibacterial substance, such as 0.02% sodium azide or Merthiolate.

9.6.1.2 The CNBr Method

The method described is essentially that according to Kohn and Wilchek (43), which is a modification of the original CNBr buffer procedure originally presented by Porath and colleagues (42).

9.6.1.2.1 Activation Step

The agarose gel (e.g., Sepharose 4B) is washed with distilled water. The drained gel (10 g) is then mixed with 10 mL distilled water and 20 mL of a 2 M sodium (or potassium) carbonate solution. The obtained suspension is cooled to 0°C using an ice bath. An ~10 M solution of CNBr in acetonitrile, DMF, or N-methylpyrrolidone is prepared by dissolving 1 g of CNBr in 1 mL of organic solvent. This solution is added, all at once, to the gel suspension under vigorous agitation. After exactly 2 min the reaction mixture is transferred onto a glass filter funnel and washed with ice-cold water until all CNBr is removed. This washing procedure should be done as quickly as possible so as not to lose reactive groups by hydrolysis. The activated agarose beads should be used immediately for coupling. This procedure gives a coupling capacity for low molecular weight amines of about 10–100 μmol/g drained agarose gel, which is about half the amount that can be obtained by the CNBr titration procedure (40). For proteins the differences are less pronounced. The method has the advantage of being reliable and easy to perform compared with the titration method, which also requires automatic titration equipment.

9.6.1.2.2 Coupling Step

The ligand to be coupled is dissolved in 0.1 M NaHCO₃, pH 8.3, containing 0.5 M NaCl. This is the most commonly used coupling buffer. Other buffers with lower ionic strength in the range 8–10 (such as borate or phosphate buffers) can also be used. In this pH interval the amino groups on the ligand are predominantly in the unprotonated and reactive form. The high salt concentration in the buffer (0.5 M NaCl) serves to minimize ionic protein–protein adsorption.

The amount of ligand added to the reaction mixture depends on what ligand density is desired. As a rule, a higher ligand concentration in the reaction medium leads to higher ligand content in the adsorbent. However, as discussed earlier, it might not always be advantageous to obtain a very high ligand content as this might have adverse effects on the affinity chromatography. For an efficient adsorbent, 1–10 μmol of low molecular weight ligand/mL gel and 5–10 mg protein/mL gel for protein are recommended. These figures can be obtained by adding two to three times excess protein to the reaction mixture. A lower ligand concentration may in fact be more effective in, for example, immunoadsorbents, as it facilitates desorption.

The washed and drained activated gel is suspended in the ligand solution. If necessary, more buffer may be added to make a slurry that can be efficiently mixed. The reaction mixture is then agitated for 2 h at room temperature (22–25°C) or overnight at 4°C. A number of residual active groups may remain on the gel after coupling (this is particularly the case after immobilizing high molecular weight ligands such as proteins). These groups can usually be removed by hydrolysis by leaving the gel for 2 h with Tris-HCl buffer, pH 8, or by adding an excess of a small primary amine (e.g., ethanolamine, glycine, or glutamic acid). The obtained gel product is finally washed and stored as described earlier.

9.6.1.3 The CDAP Method

The agarose gel (e.g., Sepharose 4B) is washed with water, then with acetone:water (3:7), and finally with acetone:water (6:4) (43). The gel is drained for a moment by mild suction (see earlier discussion). Drained gel (10 g) is transferred into a 50-mL glass beaker and 10 mL of acetone:water (6:4) is added. This should
TABLE 9.9 Amounts of CDAP and TEA Employed for the Activation of 10 g Drained Agarose (Sepharose 4B) (see Reference 43)

<table>
<thead>
<tr>
<th>Degree of Activation</th>
<th>Coupling Capacity, μmol/g gel</th>
<th>CDAP in Dry Acetonitrile (0.1 g/mL), mL</th>
<th>0.2 M Aqueous Solution of TEA, mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weak</td>
<td>5</td>
<td>0.25</td>
<td>0.2</td>
</tr>
<tr>
<td>Moderate</td>
<td>15</td>
<td>0.75</td>
<td>0.6</td>
</tr>
<tr>
<td>Strong</td>
<td>30</td>
<td>1.50</td>
<td>1.2</td>
</tr>
</tbody>
</table>

give a dense but easily stirrable slurry (gel suspension). In the original standard technique the gel suspension is cooled at 0°C, the desired volume of CDAP stock solution (see Table 9.9) is first added under vigorous stirring and after 30 sec the corresponding volume of TEA solution is added dropwise over a period of 1–2 min (43).

The entire reaction mixture is then rapidly transferred into 200 mL of ice-cold 50 mM HCl. This is to hydrolyze and remove from the gel the pyridinium isourea moieties, which are formed as a byproduct of the activation reaction (the active group, the cyanate ester, is stable toward dilute mineral acid and will thus not be affected). The gel is allowed to sediment for 15 min, then washed on a glass filter funnel with ice-cold water, and is used for coupling immediately. Coupling of ligand as well as deactivation is performed as described earlier for the CNBr buffer method.

A modified protocol for an improved activation of Sepharose 4B with CDAP has been optimized (98). To the gel suspension precooled to 4°C, CDAP (250 mg) dissolved in 2 mL of acetonitrile/water (6:4 v/v) is added under vigorous stirring at 4°C for 3 min; then, 360 μL of 200 mM TEA solution is added dropwise over 1–2 min. After 3 min the entire reaction mixture is quickly added to 50 mL of ice-cold 50 mM HCl. The recommended incubation period of the activated gel suspension with 50 mM HCl is cut down to 3 min for optimal immobilization yields. Thus, it was shown that after a short (3 min) incubation period with 50 mM HCl, the CDAP-activated agarose formed was able to immobilize almost eightfold more concanavalin A than after a longer (15 min) incubation period. The high density Con A-Sepharose adsorbents obtained were successfully applied for the purification of horseradish peroxidase (HRP) from crude roots extract (99). With the optimized CDAP technique, much higher degrees of activation can be obtained and thus higher amount of ligands, such as lectin, can be coupled to the agarose beads.

For the activation of soluble polysaccharides, special CDAP activation procedures have been optimized, thus allowing the preparation of glycoconjugates constituted by the S. pneumoniae serotype 14 capsular polysaccharide and bovine serum albumin (100).

9.6.1.4 The DSC Method This method is essentially as that described by Wilchek and Miron (47). Beaded agarose (10 g of wet gel) is dehydrated (washed) and mixed slowly under agitation with 1.5 mmol of DSC (which can be obtained from Sigma Chemical Co.). A 1.5- to twofold molar excess (with respect to DSC) of base catalyst (either 0.38 mL TEA in 10 mL pyridine or 325 mg DAP in 10 mL acetone) is added to this suspension slowly under agitation.

After agitating the suspension for 30–60 min at room temperature, the gel is washed successively with solutions of acetone, 5% acetic acid in acetone, methanol, and 1 mM HCl (4°C). If the gel is to be used within a few hours, it can be stored in 1 mM HCl. As a suspension in acetone at 4°C, the activated gel is stable for several weeks. Proper washing of the gel can be checked by diluting an aliquot of the methanol washings with 0.25 M NH₄OH. Using this treatment, the remaining reagent will turn into N-hydroxysuccinimide, which can be detected photometrically at 260 nm (described earlier).

With the previously described conditions, an activated gel containing 20–40 μmol hydroxysuccinimide carbonate groups/g gel is obtained. The degree of substitution depends, among other things, on the excess DSC used in the reaction.

Coupling of amino-containing ligands and proteins is performed at pH 6–9 by mixing the activated gel, after filtering off the 1 mM HCl or acetone, with the ligand dissolved in either 0.1–0.2 M phosphate buffer, pH 7.5, or fresh solutions of 0.1–0.2 M NaHCO₃, pH 8.3, for 4–16 h at 4°C. A ligand density of 37 μmol/mL gel was obtained when aminocaproic acid was added to the activated gel suspension to give a final concentration of 0.5 M, and 2.5 mg of protein A/mL gel was bound as a result of adding 3.0 mg of protein/mL gel. A higher degree of substitution is obtained by adding more protein to the activated gel. Deactivation is not necessary, as the N-hydroxysuccinimide carbonate groups are rapidly hydrolyzed at the conditions of coupling.

9.6.1.5 The Tresylchloride Method This method is essentially as described by Nilsson and Mosbach (46). A 10-mL gel aliquot is washed with 3–4 volumes of water, then sequentially with acetone:water mixtures (30:70, 60:40, 80:20), and finally with pure acetone: 10–20 mL per washing and three to five washings for each mixture.

The drained gel is suspended in 5 mL of dry acetone. Pyridine is added to twice the volume of the tresylchloride. Under agitation, 0.05–0.2 mL of tresylchloride is added dropwise (total addition time, 1 min). The reaction mixture is then agitated for another 10–15 min. Wash twice with 10 volumes of acetone and then wash sequentially with mixtures of acetone and 1 mM HCl (in water) (70:30, 50:50, 20:80), and finally with 3 volumes of 1 mM HCl.

CDAP is commercially available (e.g., Sigma Chemical Company, Missouri, USA).
The coupling is performed by suspending the gel in an equal volume of suitable buffer of pH 7.5–9.5 (e.g., 0.1–0.2 M carbonate or phosphate) containing the ligand. The reaction mixture is then agitated overnight at 4°C or 25°C, depending on the stability of ligand. The gel is washed with the coupling buffer and is then resuspended in an equal volume of 0.1 M buffered ethanalamine, pH 7.5–8.5, and agitated for an additional 3–5 h at 4°C or room temperature. The obtained gel derivative is washed and stored as described earlier.

9.6.1.6 Immobilization with Water-Soluble Carboxdiimides This method should be described essentially as described in Reference 10 (see also References 50–53). An agarose derivative with carboxyl or amino groups substituted directly on the polysaccharide backbone or via a spacer is purchased or prepared. The gel should contain carboxyl groups if the ligand is an amine and amino groups if the ligand is to be coupled through a carboxyl group. The selected drained agarose derivative (10 mL) is washed with 3–4 volumes of 0.5 M NaCl and then distilled water. The gel is then transferred to a reaction vessel containing 5 mL of 0.04–0.1 M ligand dissolved in water or organic solvent (dioxane, ethyleneglycol, ethanol, methanol, or acetone). The recommended concentration range will result in a molar excess, relative to groups on the gel, of low molecular weight ligands for which the method is recommended. Protein ligands are preferably immobilized by the technique based on activated matrix carboxyl groups, which is described later. The coupling is usually performed in unbuffered medium. In all cases, avoid using buffers containing amine, carboxyl, or phosphate groups.

Adjust the pH to 4.7 with 0.1 M HCl or 0.1 M NaOH. Add 5.2 mL of 0.1 M EDCL [1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide hydrochloride] or CMCL [1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide-metho-p-toluenesulfonate] in water or mixed solvent. Up to 50% of organic solvents can be used when the ligand is poorly water soluble. Maintain the pH at 4.5–5 for 1 h (pH usually decreases under this time) by adding dilute NaOH. The reaction mixture is agitated overnight at room temperature.

A blocking reaction is not usually necessary when an excess of ligand is used, but can be carried out with ethanolamine or glucosamine in the case of carboxyl-containing agarose and with acetic acid when the matrix is an amino agarose derivative.

The gel is washed and stored as described earlier.

9.6.1.7 Immobilization to Activated Carboxyl Agarose This is a modification of the procedure described by Cuatrecasas and Parikh (55). Carboxyl-containing gel (10 mL) is washed with deionized water and successively with 10 × 50 mL dioxane to remove all traces of water from the gel. The gel is then suspended in 15 mL of dioxane. N-Hydroxysuccinimide is added (240 mg) and agitation is performed until this compound is completely dissolved. Cyclohexyl carbodiimide is then added (400 mg) and agitation is continued for another 2 h. The gel is then washed with 4–10 volumes of dioxane and four times with 1 volume of pure methanol (to eliminate the poorly soluble N,N'-dialkyurea derivative produced during activation). After washing the gel another three times with dioxane (or isopropanol) it can be stored as a suspension in a well sealed vessel in the dark at 4°C until used. This procedure usually gives a degree of activation of ~12 μmol of ester groups/mL gel. The degree of substitution can be determined photometrically after the release of N-hydroxysuccinimide as described in Section 9.4.1.4.

Before coupling of the ligand, excess solvent is removed. The gel is then suspended (either directly or after being washed with 1 mM HCl) in 100 mL of the chosen buffer at pH 5–9 (e.g., 0.1 M NaHCO₃, pH 8.3) containing the ligand solution (if protein is 2–20 mg protein/mL gel). The use of a lower pH has the advantage that hydrolysis of the active ester is minimized. Preferential reaction of α-amino groups, as opposed to ε-amino groups, can be obtained by coupling at low pH, due to the lower pKₐ of α-amino groups. The reaction mixture is agitated for 10 min to 6 h at room temperature or at 4°C (coupling reaction is normally very rapid).

Excess activated groups usually hydrolyze at pH > 7, but blocking can also be performed by reacting the gel with, for example, 0.1 M ethanolamine buffered to pH 7.5–8.5 for 1 h at 4°C.

The gel is washed and stored as described earlier.

9.6.1.8 Immobilization with Bisepoxirane and Epichlorhydrin This method is performed essentially as described by Sundberg and Porath (35) and Porath and Fornstedt (45). A 10 mL gel aliquot is washed with 3–4 volumes of deionized water and suspended in 5–10 mL of deionized water. One milliliter of the reagent (bisepoxirane, e.g., 1,4-bis-butanediol-diglycidylether, or epichlorhydrin) and 3 mL of 2 M NaOH containing 20 mg of sodium borohydride are added under agitation. The activation is carried out during agitation at room temperature for 2 h. The activated gel is then carefully washed with deionized water until the reagents are completely removed.

The activated gel is suspended in an equal volume of a buffer of pH 9–13 (carbonate, borate, or phosphate buffers can be used; higher pH for carbohydrate ligands) in which 0.5–1 mmol/mL of small ligand and 5–10 mg/mL gel of macromolecular ligand are dissolved. If necessary, up to 50% organic solvent (e.g., dioxane, DMF) may be used to dissolve the ligand. The mixture is agitated for 15–48 h at 20–45°C.

Oxirane groups not utilized for coupling of ligand are usually hydrolyzed at high pH. When lower pH (<10)
has been used, it may be necessary to block the remaining oxirane groups with ethanolamine or β-mercaptoethanol, as described earlier. The gel is then washed and stored as described earlier.

The degree of activation obtained on 4% agarose beads with a bisepoxirane such as 1,4-bis-butanediol-diglycidyl-ether is 10–20 μmol of oxirane groups/mL gel, but with epichlorohydrin higher degrees are usually achieved. Scoble and Scopes (101) have discussed in some detail the conditions for epichlorohydrin activation of Sepharose 4B; they reported amounts up to 31 μmol/mL active groups, when using 0.2 mL of epichlorohydrin per gram of drained gel. Higher levels of reagent did not cause much increase in the activation, with only 34 μmol/mL active groups obtained from 0.4 mL of epichlorohydrin per gram of Sepharose. However, Franco Ftraguas and colleagues (102) have shown that higher activation degrees were possible when using 0.55 mL of epichlorohydrin per gram of drained Sepharose 4B and the incubation period was shortened to 1.5 h at room temperature. Under these experimental conditions they were able to reach levels of 45 μmol of oxirane groups per mL of packed gel, which was equivalent to 75% of the oxirane groups present in the gel after 19 h of incubation at room temperature.

Because in most cases a rather small percentage of these groups can be utilized for ligand immobilization, protocols yielding high activation degrees are recommended when high ligand densities are required for an efficient performance of the affinity adsorbent (102).

9.6.1.9 Immobilization to Polyacrylamide with Glutaraldehyde Gel (10 mL) is washed with 3–4 volumes of distilled water and then 3–4 volumes of 0.5 M potassium phosphate buffer, pH 7.6 (other buffers with pH between 6.9 and 8.5 can also be used) (4). The drained gel is transferred to a flask containing 100 mL of 25% aqueous glutaraldehyde, a treatment that also sterilizes the gel. Adjust the pH to 7.4. The suspension is agitated for 18 h at 37°C. The gel is then washed with 15–20 volumes of distilled water or with 0.5 M phosphate buffer, pH 7.7, and is then transferred to a flask containing 10 mL of 0.5 M potassium phosphate buffer, pH 7.6, in which the ligand (e.g., a protein) is dissolved. Buffers with pH from 6.9 to 8.5 containing 5–10 mg of protein per mL of gel should be used. After the removal of free glutaraldehyde, the activated polyacrylamide beads can also be stored at pH 7.7 and 4°C for several days.

To remove remaining reactive aldehyde groups on the gel, treat the gel with one volume of 0.1 M buffered ethanolamine at pH 7.5–8.5 for 3 h at 4°C (amino acids can also be used). Alternatively, the free aldehyde groups can be blocked by treatment with one volume of 0.1 M borate buffer, pH 8.5–9, containing 500 mg NaBH₄ for 15–20 min (this treatment should not be used when the immobilized ligand contains disulfide bonds). The absolute amount of protein coupled to the polyacrylamide beads depends on the nature of the protein ligand and the excess of protein used in the reaction; 0.4–2 mg/mL gel is typically obtained when 1–2 mg protein is added per milliliter of activated gel, that is, a yield of 20–100%. The gel is then washed and stored as described earlier.

9.6.1.10 Immobilization to γ-Glycidoxypropylsilica (Epoxy-Silica) Silica (10 g), LiChrospher Si 1000, is washed briefly with 20% HNO₃, distilled water, 0.5 M NaCl, distilled water, acetone, and ether and put into a 500-mL three-neck flask where it is dried for 4 h at 150°C under vacuum (28). The reaction flask is then cooled, and sodium-dried toluene (150 mL.) is sucked into the flask. γ-Glycidoxypropyl trimethoxy silane (2.5 mL, Dow Corning Z6040) and trimethylamine (0.05 mL) are added, and the reaction mixture is agitated by an overhead stirrer and refluxed for 16 h; a slow stream of dry nitrogen gas will ensure anhydrous conditions. The formed epoxy-silica is then washed on a glass filter with toluene, acetone, and ether and dried under vacuum. The procedure gives an epoxy (oxirane) group content of ~50 μmol/g as determined with the method described earlier for bisoxirane- and epichlorohydrine-activated agarose derivatives. Amino- and thiol-containing ligands can be coupled directly to the obtained silica derivative according to the procedure described earlier (28). A pH higher than 8 should not be used. This means that the coupling, at least for amino ligands, will be rather slow and should be speeded up by using high concentrations of ligand and, if possible, an elevated temperature. The coupling is finished by converting excess epoxy groups to more hydrophilic entities. The preferred way is acid hydrolysis, provided that the ligand is stable under these conditions, which will result in a diol structure.

Heating of γ-glycidoxypropyl-silica to 50°C at pH 2 for 3 h is enough for complete hydrolysis of the epoxide groups. Excess oxirane groups can also be converted into more hydrophilic structures by treating the silica derivative with 1 M mercaptoethanol at pH 8 at room temperature for 2 h. This method is more suitable after immobilization of proteins, provided these do not contain disulfides.

Other ligand coupling methods described earlier, such as the DSC and tetryl chloride methods, can also be used if the epoxy-silica is first converted to diol-silica by acid hydrolysis. This is performed by mild agitation of 10 g of epoxy-silica in 1 L of 0.01 M HCl at 90°C for 1 h. Diol-silica can also be obtained commercially (e.g., Li Chrospher DIOL, E. Merck AG).

9.6.2 Affinity Chromatography

9.6.2.1 Purification of Kinases and Dehydrogenases on Blue Sepharose CL-6B (103) Affinity gels with group specificity have the potential advantage of being useful for
the isolation of many compounds belonging to a particular group. This technique was nicely demonstrated by Easterday and colleagues (103), who purified enzymes from crude yeast extract on the dye column Blue Sepharose CL-6B and eluted them by competitive elution with enzyme-specific cofactors at different pH.

Dried bakers yeast was extracted in 1 M Na₂HPO₄ for 3 h at 37°C and then centrifuged at 13,700 g for 1 h. After filtration, the supernatant was precipitated with (NH₄)₂SO₄ (75% saturation) at 4°C. After centrifugation, 220 mg of the precipitate was dissolved in 10 mL of starting buffer (0.02 M Tris-HCl, pH 6.4, containing 5 mM MgCl₂, 0.4 mM EDTA, and 2 μM mercaptoethanol). Blue Sepharose CL-6B was packed in a column with dimensions 1.6 × 5 cm (bed volume 10 mL) and equilibrated with starting buffer before application of the sample. A peak of inactive material was eluted with starting buffer (Fig. 9.4); 5 mM NAD⁺ and 20 mM NADP⁺ dissolved in starting buffer were used to elute alcohol dehydrogenase (ADH) and glucose-6-phosphate dehydrogenase (Glu-6-PO₄-DH), respectively. Hexokinase (HK) was eluted when the pH of the eluent was raised to 8.6 and glyceraldehyde-3-phosphate dehydrogenase (Gly-3-PO₄-DH) was eluted with 10 mM NAD⁺ at the same pH.

9.6.2.2 Purification of a Lectin on N-Acetyl-D-galactosamine Sepharose 6B Grosund Falcata Japonica seeds (20 g) were suspended in 200 mL of 0.01 M PBS (pH 7.4), stirred overnight at 4°C, and then centrifuged at 2300 g for 15 min (83). The precipitate was extracted with a half-volume of starting PBS. Pooled supernatants were ultracentrifuged to remove the lipid materials. The crude extract (200 mL) was applied to a column of N-acetyl-D-galactosamine (GalNAc) coupled to epoxi-activated Sepharose 6B (1.6 × 5.0 cm, 10-mL bed volume). The column was washed with 1 mM PBS (pH 7.0) until the absorbance of the eluate at 280 nm was less than 0.05. The bound lectin was desorbed by a specific competitive elution adding 25 mM GalNAc to the starting buffer (Fig. 9.5). A similar result was obtained by nonspecific elution by decreasing the pH to 2.2 with glycine-HCl buffer. A 1000-fold purification was achieved in one step.

9.6.2.3 Binding of Immunoglobulins to Protein A Sepharose Protein A is a group-specific ligand with affinity for many different immunoglobulins (104, 105). The subclasses can be eluted separately according to binding strength by a stepwise decrease of pH.

All subclasses of mouse IgG can be purified on Protein A Sepharose (105, 106). Even IgG₁, which is known to have low affinity for Protein A (107–109), binds efficiently under certain buffer conditions (104). The two parameters normally changed to affect the binding of proteins to affinity adsorbents are pH and ionic strength. Biewenga and colleagues (110) studied the influence of these two parameters during the binding of human myeloma IgA and human polyclonal IgG. They found that binding of IgA decreased with decreasing pH. At salt concentrations up to 2 M NaCl, IgA binding decreased, whereas IgG binding was constant (Fig. 9.6).

Fredriksson and colleagues (105) studied the effect of ionic strength on the binding of mouse monoclonal antibodies of different IgG subclasses to Protein A Sepharose at high pH. They found that a high ionic strength (3 M NaCl) is necessary for efficient binding of IgG. The binding buffer (1.5 M glycine, 3 M NaCl adjusted to pH 8.9 with 5 M NaOH) is used (Fig. 9.6) to adsorb polyclonal immunoglobulins from mouse serum to the Protein A gel.

Figure 9.4 Purification of kinases and dehydrogenases from crude yeast extract by affinity chromatography on Blue Sepharose CL-6B. Reproduced from Reference 103, with permission.
Protein A Sepharose CL-4B (1 mL) was packed in a column with bed dimensions $1 \times 1.2$ cm and run at a flow rate of 0.8 mL/min. After the column was equilibrated with binding buffer, 5 mL of mouse serum was diluted with 5 mL of binding buffer and applied to the column. Elution was then performed using a series of buffers (0.1 M citric acid adjusted to pH 6.0, 5.0, 4.0, and 3.0). These pH values can also be used to elute the different IgG subclasses when purifying monoclonal mouse antibodies, thus eliminating acid conditions.

9.6.2.4 Purification of Catechol-O-Methyltransferase

Catechol-O-methyltransferase (COMT) is a very labile protein. Previous methods for purification of this enzyme were often laborious and time-consuming. Veser and May (111) described a rapid and specific purification method that combines ion-exchange chromatography and affinity chromatography. A low molecular weight ligand, S-adenosyl-L-homocysteine (AdoHcy) immobilized to a spacer-arm-containing preactivated gel, AH-Sepharose 4B, was used as the affinity adsorbent.

COMT was prepared from rat liver and partially purified by ion-exchange chromatography (111). AdoHcy-linked AH-Sepharose was packed into a column ($1 \times 10$ cm, bed volume 8 mL) and equilibrated with 4 mM phosphate buffer, pH 6.4, containing 0.02% sodium azide, 0.2 mM magnesium chloride, 1 mM mercaptoethanol, 0.3 mM dithiothreitol, and 2% glycerol. The COMT-containing fractions from the ion-exchange chromatography were pooled and dialyzed against the equilibration buffer mentioned earlier and applied to the affinity column at a flow rate of 0.75 mL/min. The enzyme was eluted with equilibration buffer containing 0.1 mM $S$-adenosyl-L-homocysteine (AdoAdoHcy) (Fig. 9.7). The enzyme activity could also be eluted by a small increase in pH from 6.4 to 7.4. The column was regenerated and non-specific bound material eluted with 0.1 M NaCl in the

**Figure 9.5** Nonspecific elution of GalNAc-binding lectin from GalNAc-Sepharose 6B with glycine-HCl buffer pH 2.2. Similar results were achieved by specific elution with 25 mM GalNAc. Reproduced from Reference 83, with permission.

**Figure 9.6** Elution of different IgG subclasses in mouse serum from a column of protein A Sepharose CL-4B by a stepwise reduction in pH. Reproduced from Reference 104, with permission.

**Figure 9.7** Affinity chromatography of catechol-O-methyltransferase on a column of $S$-adenosyl-L-homocysteine immobilized to AH Sepharose 4B. Reproduced from Reference 111, with permission.
equilibration buffer. The recovery of the enzyme after the affinity chromatography step was 95%.

9.6.2.5 Purification of the Angiotensin-Converting Enzyme from Human Heart Using an Immobilized Inhibitor (112) The angiotensin-converting enzyme from human heart was isolated by a procedure including two chromatographic steps. After extraction, the enzyme was partially purified by a batch adsorption to DEAE-cellulose followed by affinity chromatography on \(N\)-[1-(S)-carboxy-5-aminopentyl]-Gly-Gly-linked to Sepharose 6B via a spacer arm. The affinity adsorbent (0.9 \(\times\) 8.5 cm, bed volume 5 mL) was equilibrated with 20 mM MES, pH 6.0, containing 0.5 M NaCl, 0.1 mM zinc acetate, and 0.1% Nonidet P-40 at a flow rate of 8 mL/h. The enzyme was eluted by a buffer change to 50 mM sodium borate, pH 8.9 (Fig. 9.8).

9.6.2.6 Simultaneous Detection of HSA and IgG in Serum by HPLAC (113) This application demonstrates the use of HPLAC in an analytical mode for the simultaneous detection of human serum albumin (HSA) and IgG in a single sample of serum by a dual-column system (Fig. 9.9). Reference 113 also presents a general scheme for the design and optimization of such a multianalyte affinity system. The ligands chosen, Protein A and anti-HSA antibodies, were immobilized to activated diol-bonded LiChrospher Si-4000 and Si-500. (For a detailed description of the activation see References 28 and 114, and for the immobilization see Reference 113.) Quantitation of serum samples was performed on two minicolumns (6.35 mm length \(\times\) 4.1 mm i.d.) connected in series and a 10-\(\mu\)L injection loop. The application buffer was 0.05 M phosphate and 0.05 M citrate buffer, pH 7. The elution buffer was 0.05 M phosphate and 0.05 M citrate, pH 3. The serum sample was diluted 1:5 with application buffer prior to injection. The anti-HSA column was placed before the Protein A column to avoid nonspecific adsorption of albumin to the Protein A adsorbent (the details of this optimization experiment are given in Reference 105). Therefore, after the nonretained peak had been eluted from both columns, the Protein A column was switched off-line and the anti-HSA column was eluted with pH 3 buffer. After the HSA had been eluted, the protein A column was switched on line to elute the IgG. Standards with HSA and IgG were analyzed. This method gave results in good agreement with commercially available methods, while requiring only 2 \(\mu\)L of serum and 6 min per cycle.

9.6.2.7 Use of the Avidin–Biotin System for Immunosorption (114) This application describes how the avidin–biotin system can be used to prepare immunosorbsents on glass beads with directed immobilization of the antibodies. The methods described by Babashak and Phillips (115) apply both conventional (IAC) and high performance immunoaffinity chromatography (HPIAC). Monoclonal antibodies (Mabs) were biotinylated with biotin hydrazine. This reagent couples the biotin to the carbohydrate moieties of the antibodies. The carbohydrate part of most antibodies is present in the FC region, and attachment of biotin thus ensures correct orientation of the antibody. For details of the biotynilation procedure, see Reference 115. For silanization and derivatization of the glass beads, see References 115 and 116. The

![Figure 9.8](image.png)  
**Figure 9.8** Affinity chromatography of human heart angiotensin-converting enzyme on immobilized N1-(S)-carboxy-5-aminopentyl]-Gly-Gly-Sepharose 6B. Elution by increase of pH to 8.9. Reproduced from Reference 112, with permission.

![Figure 9.9](image.png)  
**Figure 9.9** Chromatograms obtained after injections of (a) HSA, (b) IgG, (c) HSA plus IgG, and (d) normal serum into the dual column system. Event sequence: 0.00 min, switch from pH 3 to pH 7 buffer; 0.50 min, sample injection; 2.25 min, protein A column switched off-line, switch to pH 3 buffer; 4.00 min, protein A column switched on-line. Reproduced from Reference 113, with permission.
streptavidin form of avidin was immobilized to the activated glass beads by incubation at pH 9 for 18 h at 4°C. The biotinylated Mabs were then attached to the streptavidin-coated beads by incubation for 1 h at 4°C in PBS on an overhead mixer. The beads were washed (PBS, pH 7) and slurry packed into 10 cm × 4.6 mm i.d. stainless-steel columns at 250 psi. The immunosorbent was used to isolate the B 27 human leucocyte antigen component from detergent-solubilized human leucocyte membranes. The B 27 antigen is retained on the column, while most of the membrane components are washed through it (Fig. 9.10). The bound antigen is eluted by decreased pH or by chaotropic ions. The binding between streptavidin–biotin is so strong that no elution of biotinylated Mabs occurs in these conditions. More than 20 batches of streptavidin-coated glass beads were produced and used in HPIAC. The derivatized beads bound between 1.5 and 1.85 mg of streptavidin per 2 g batch of beads, and the streptavidin beads bound between 195 and 245 µg of hydrazine biotinylated antibodies. Running the chromatograms at 4°C is preferable to room temperature (both for peak performance and bead lifetime). Also, chaotropic elution is preferable to acid elution, as the bead lifetime increases twofold.

9.6.2.8 Preparation and Evaluation of Ricinus communis Agglutinin Affinity Adsorbents Using Polymeric Supports

The lectin (hemagglutinin) of *Ricinus communis* (RCA) has special interest because of its high affinity and particular specificity for glycoconjugates containing terminal β-d-galactosyl residues (117). RCA shows binding and agglutination of polysaccharides and glycoproteins and furthermore of liposomes and micelles containing glycolipids with galactosyl residues.

An efficient and reproducible protocol for preparation of RCA adsorbents using tresylated polymeric supports has been reported (117). Immobilization was performed in 0.5 M phosphate buffer, pH 8.0, containing a 50-fold molar excess of lactose (coupling buffer) for protection of the RCA binding sites during the process. For immobilization of RCA two different polymer-based supports, Toyopearl and TSKgel (TosoHaas), were used, which are known to be suitable for low pressure and HPLAC applications, respectively. For preparation of the HPLAC adsorbent, RCA was coupled directly onto the preactivated support TSKgel Tresyl 5PW, prepacked in a stainless-steel column (7.5 × 75 mm). After equilibration of the column with water and coupling buffer, a solution of 20 mg RCA in 6 mL of coupling buffer was recirculated through the column at 20°C at a flow rate of 0.2 mL/min. The immobilization process was stopped after 11 h, and the unreacted tresyl groups were blocked by reaction with 0.2 M Tris-HCl, pH 8.0, for 2 h. The prepared RCA-TSKgel column was washed with 0.01 M phosphate buffer pH 7.2, 0.15 M NaCl, and stored at 4°C in the same buffer containing sodium azide.

RCA has been successfully immobilized onto the above-mentioned supports with amounts of coupled ligand between 15 and 23 mg/g dry support and corresponding coupling yields of 69–93% (w/w). The prepared affinity adsorbents were evaluated for capacity, ligand accessibility, and long term stability. They displayed a capacity of 1.7–2.5 mg/mL of adsorbent for the glycoprotein asialofetuin (ASF) and accessibility of 80% of RCA-binding sites. Regarding long term stability, no change in capacity or performance could be noticed for a period of at least 10 months.

RCA-TSKgel was successfully applied for HPLAC of glycoproteins (asialofetuin, fetuin) and nitrophenyl-derivatized carbohydrates. To perform the affinity separation, 400 µg of glycoprotein sample in 0.01 M phosphate buffer pH 7.2, 0.15 M NaCl, was pumped onto 1 mL of adsorbent at 1.0 mL/min and, after thorough washing, elution was

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**Figure 9.10** Chromatograms of an HPLAC isolation of the B 27 antigen isolated from detergent-solubilized membranes. The 100-µL sample was applied in 0.9% sodium chloride, 0.1 M sodium acetate buffer at pH 6.5, and nonadsorbed material was washed off the column before elution started. (a) The antigen was desorbed by a pH gradient from pH 6.5 to 1.0 by the addition of 0.1 M hydrochloric acid to the initial running buffer. (b) The antigen was eluted by a chaotropic ion gradient of 0 to 2.5 M sodium thiocyanate. Reproduced from Reference 115, with permission.
performed with a step gradient of 0.1 M lactose. The RCA-TSKgel column proved to be suitable for selective adsorption of glycoproteins and carbohydrates with respect to β-d-galactosyl residues. This column is suited for analytical separations and also allows preparative purification of glycoproteins in the range of a few milligrams per cycle (the capacity for ASF is 5.5 mg per column cycle).

9.6.2.9 Affinity High-Performance Monolith Disk Chromatography The quantitative characteristics of the interaction between three genetically designed forms of protein G (monofunctional IgG-binding protein G, monofunctional SA-binding protein G, and bifunctional IgG/SA-binding protein G) and the target proteins have been evaluated with the use of high performance monolithic disk chromatography (HPMDC) (118). Additionally, the unique possibility of simultaneous isolation of IgG and SA from human blood plasma and rabbits sera by affinity HPMDC was demonstrated.

All three forms of recombinant protein G were bound directly to the CIM support by means of the reaction between the epoxy groups of polymeric material and protein amino groups. The amount of ligands coupled to the disks seemed to be similar and ranged from 0.8 to 0.9 mg per disk. The behavior of the immobilized ligands with respect to IgG and SA binding has been quantitatively investigated using solutions of standard plasma proteins. Affinity-bound proteins (IgG, SA) were eluted with 0.01 M HCl (pH 2.0). To maintain the activity of the isolated proteins, the pH of the solutions obtained after the desorption step was immediately adjusted to 7.5 with 1 M NaOH. The immobilized monofunctional IgG-binding protein G demonstrated high specific capacity for IgG (0.9 mg of IgG per disk), whereas the capacity of monofunctional SA-binding protein G disk was found to be seven times lower (~0.15 mg of SA adsorbed).

In agreement with these results, when immobilized bifunctional protein G was used as affinity ligand for both IgG and SA, the average disk capacity for IgG was 1.3 mg, whereas for SA it was about one order of magnitude lower (0.13 mg).

The special construction of the cartridge used for the installation of CIM disks allows the use of several disks of different functionalities stacked into a continuous monolith unit and, therefore, a simultaneous fractionation of target molecules from crude biological fluids. In these experiments, such a multifunctional fractionation approach was applied to recover IgG and SA from mammalian plasma. The procedure has been developed with the use of a simple scheme:

1. IgG-binding protein G disk and SA-binding protein G disk are installed consecutively into the same cartridge.
2. Serum is passed through the stacked disks.
3. The SA-binding disk is removed from the housing and the desorption of IgG from single IgG-binding disk is accomplished.
4. The SA-binding protein G disk is reinstalled into the chromatographic line, and the affinity-bound SA is eluted. In this way, the whole processing operation requires ~15 min at a flow rate of 2.5 mL/min.

The most significant result was that the same dynamic capacities for the IgG and SA isolated from human plasma or rabbit sera were achieved as those found for IgG and SA used in model experiments. Thus, ~1 mg of immunoglobulin and 0.13 mg of serum albumin were isolated from blood media within a quarter of an hour.

Scaling up of the purification process for IgG by using several stacked disks or a monolithic tube with immobilized recombinant protein G was also successfully performed (118).

9.7 REFERENCES

9.7 REFERENCES


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10

AFFINITY LIGANDS FROM CHEMICAL COMBINATORIAL LIBRARIES

ENRIQUE CARREDANO AND HERBERT BAUMANN
GE Healthcare Bio-Sciences AB, SE-751 84 Uppsala, Sweden

10.1 INTRODUCTION

Natural molecules such as proteins and carbohydrates, which by their nature bind to target proteins with high specificity, are plausible candidates to function as affinity “ligands” for those target proteins. However, such ligands are often less likely to be stable under the acidic and basic conditions often used in cleaning-in-place (CIP) protocols in industry. Protein ligands are often susceptible to several different types of degradation (proteolysis, deamidation, isomerization, denaturation, etc.), and these problems usually become aggravated under conditions of extreme pH. Another drawback with these kinds of macromolecular ligands is that they are relatively expensive to produce. These concerns have triggered the search for stable organic molecules, including relatively small oligopeptides, as affinity ligands. A number of publications on this subject have already appeared in the literature. Two recent general reviews cover different approaches to the design and selection of such affinity ligands and illustrate, with examples, some of the techniques available (1, 2). The different classes of compounds include natural binders (such as substrates and inhibitors) plus their derivatives and so-called mimetics of such ligands, as well as other small organic molecule ligands obtained by screening various types of compound libraries). Small-molecule libraries may also be designed and synthesized around a particular type of scaffold, such as the triazine scaffold found in several dyes.

The methods of finding ligands can be ordered on a scale from empiric to strictly knowledge-based. The dynamic aspects of molecular interactions and the difficulty to predict how ligand immobilization (e.g., on a chromatographic support) may affect target binding suggests that a large amount
of empirical screening is needed in all cases, but an appropriate use of the available knowledge about a target may allow a reduction in the size of the ligand test set.

Regardless of the amount of information available, the first crucial step is primary screening of a set of molecules for binding to the target protein. At least two strategies can be identified. One approach is to screen first in solution and then immobilize the most interesting ligands to a suitable solid phase for further tests. The other approach is to make use of a solid-phase screening method.

10.2 PRINCIPLES OF LIGAND DESIGN

10.2.1 Four Different Scenarios and Corresponding Approaches

Depending on the type of target structure information available, it is possible to identify four different scenarios, for which four corresponding approaches to obtaining small ligands are most appropriate (Fig. 10.1). This classification is commonly used within drug discovery research. The corresponding approaches are as follows:

- the trial-and-error or random approach
- the ligand-based approach
- the de novo design approach
- the complex structure-based approach.

This listing does not exclude the possibility of using the various methods for scenarios with higher levels of information. The random approach is, for instance, not restricted to the orphan ligand scenario, but can be used in all scenarios. On the other hand, all the approaches can be applied (and the complex structure-based method exclusively) to the information-rich, known site–ligand scenario. Furthermore, it is common through experimentation to be able to move from one approach to more sophisticated approaches as structures and interactions become better understood.

10.2.2 Random Approach

In the case that there is neither a known ligand nor a known useful structure it may be possible to identify affinity ligands by a purely trial-and-error approach, which may be combinatorial in nature. This has been shown for synthetic peptide ligands. For example, a peptide with the sequence Tyr–Tyr–Tyr–Trp–Leu–His–His was discovered in this way and verified as an effective affinity ligand for staphylococcal enterotoxin (3). The peptide Ala–Pro–Ala–Arg (APAR) was selected from a library composed of 132,000 tetrapeptides and used to purify an anti-granulocyte macrophage colony stimulating factor monoclonal antibody (4). In general, because of their nature, having well established and straightforward chemistries, plus the possibility to use different amino acids as building blocks, small synthetic peptides are an attractive alternative to the orphan ligand scenario (5). A more recent variant of this approach is the high throughput development of affinity resins, in which the ligands are synthesized on encoded beads (6). For other small organic molecules there is, in principle, the possibility to order a combinatorial library from a commercial synthetic chemistry company involved in, for instance, lead generation for drug discovery. The synthesized compounds have to be screened for binding to the target protein. Some synthetic chemistry companies may also be able to provide screening of the library. A comprehensive list can be found, for instance, at the website of Wendy Warr & Associates at http://www.warr.com/.

Two further tools that can be mentioned as examples of the trial-and-error approach, and that may prove useful in the orphan ligand scenario are (1) screening for low affinity of small fragments and (2) screening of small libraries of multimodal ligands.

10.2.2.1 Low Affinity Fragment Based Approach (www.grafinity.com) Libraries of thousand of small fragments (<300 Da) are synthesized with nanoscale solid-phase organic synthesis and immobilized onto the surfaces of chemical microarrays via a spacer molecule. About 10,000 compounds are displayed on top of so-called self-assembled monolayers, allowing for high throughput label-free low affinity fragment screenings based on a surface plasmon resonance (SPR) platform requiring a thin gold metal film on the array’s glass carrier. Changes of refractive index at the gold/liquid interface induce a wavelength shift corresponding to the increase of mass concentration obtained when the protein in solution binds one of the immobilized ligands. The technology allows processing of ~10,000 measuring points simultaneously. Positive hits can then be optimized
10.2.2.2 Small Libraries of Multimodal Ligands (7, 8)
Multimodal ligands for chromatography purification are immobilized ligands providing the possibility of multiple interactions, including hydrophobic, electrostatic, and hydrogen-bond donor or acceptor and specific van der Waals interactions. When compared to traditional ion-exchange and Hydrophobic Interaction Chromatography (HIC) ligands, multimodal ligands are complex and their behavior more difficult to predict. The combination of the long range of the electrostatic interaction and the other shorter range interactions contribute to the selective and often unique behavior of multimodal ligands. Compared to traditional affinity ligands, multimodal ligands are usually of lower specificity (more general), and their success in protein purification applications relies in the possibility to tailor selectivity by varying binding and elution conditions in terms of pH and salt concentration. Small libraries of both multimodal anionic and multimodal anionic mixed-mode ligands are available, for example, in a 96-well filter plate format for quick evaluation (www.gelifesciences.com). Using a multimodal ligand one may no longer be able to differentiate between an “affinity like” one-site interaction between the protein and the ligand and a nonspecific multisite interaction; it can on the other hand be possible to optimize binding and elution conditions considerably using different types of screening conditions.

10.2.3 Ligand-Based Approach
If there is information in the form of a known ligand or ligand profile, this can be used to positively bias the nature of the compound library. The most obvious approach for enzymes and other bioactive proteins is to take a natural substrate and immobilize it directly or after chemical derivatization. Two examples are the derivatization and immobilization of adenosyl sulfur compounds for the purification of a transferase (9) and the immobilization of an iminobiotin to purify avidin from a mixture with Bovine Serum Albumin (BSA) (10).

The so-called mimetic approach, where the binding interface of the ligand is used as a template, can also be seen as a ligand-based approach. The long known textile dyes belong to this category, as many resemble natural ligands such as coenzymes, nucleotides, nucleic acids, ATP, and so on (11–13). Reactive dyes have been of interest since the 1970s as a complement to the more traditional ligands used for HIC and ion exchange. Some ligands, such as Cibachrome Blue F3G-A and Procion Red, have been used extensively. Two disadvantages often mentioned in the context of dyes are their moderate selectivity and potential product contamination by leaked dyes. It should also be noted that some dyes may be carcinogenic. To improve selectivity, derivatives have been constructed by merging a dye and a natural substrate or cofactor of the protein of interest. These chimeric molecules, often referred to as biomimetic dyes, combine the binding capacity of the dye with the specificity of the substrate or cofactor, and have proven useful as affinity ligands (14, 15). More recent examples are biomimetic glutathionyl derivatives targeting glutathione-recognizing enzymes (16) and a galactosyl–biomimetic dye ligand used for the purification of Dactylolium dendroides galactose oxidase (17).

10.2.4 De Novo Design
In cases where the structure of a binding site or at least a putative binding site is known, it can be translated into a complementary structure of a supposed ligand, and so-called de novo design can be an alternative to random screening. Two examples of this approach are the design of a ligand to the insulin precursor to complement a hydrophobic patch on its surface (18) and the design of affinity ligands towards Fab fragments of κ type based on knowledge of the structure of a putative binding cavity in combination with virtual screening techniques (19).

10.2.5 Complex Structure-Based Approach
This approach can only be applied to the most generous scenario where the structure is available for the complex of the target protein with a known ligand. The complex describes in detail the essential features of the interaction, and this information can then be used to attempt to design a new molecule that retains those features. One example of this approach is the design of a protein A mimetic in which two of the side chains of protein A involved in binding to Fc are used as decorations of a triazine scaffold (20). A combinatorial strategy was applied in subsequent studies, where a directed library around the structure of the first protein A mimetic was synthesized and screened for binding towards IgG followed by chromatographic evaluation of a few selected candidates (21, 22). Another example is the modeling of a biomimetic chimeric ligand for the purification of bovine heart lactate dehydrogenase (LDH) (23) based on the available structure of porcine heart LDH–lactate–NAD\(^+\) complex.

The structure-based approach with in silico or virtual screening of the active site of porcine pancreas α-amylase (PPA) has been applied, with the knowledge of how the known ligand acarbose binds to the protein being formulated in a three-dimensional query to screen a large data base: the Available Chemicals Directory (ACD). One group consisting of 84 hits containing saccharide residues in accordance with the carbohydrate-binding nature of the enzyme generated good ideas for the structure-based design, synthesis, and screening of a glucuronic acid scaffold library of 23 compounds (24) (Fig. 10.2). The analysis of another group of about 50 compounds comprising (hydroxy)imine-, sulfonic
acid-, phenolic-, and anilinic derivatives led to the discovery of a new affinity ligand based on a coumarin structure (25) (Fig. 10.3).

Yet another example of the complex-structure-based approach is one where information from the interactions between Protein L and human Fab were used as guide in the combinatorial synthesis of a 169-membered triazine-derived agarose-bound library, which was evaluated for IgG and Fab fragments (26, 27).

10.2.6 Comparison of the Different Approaches

Based on the number of reports, the ligand-based approach is by far the most successful method for the identification of potential affinity chromatography ligands. Most reports concern mimetic dyes. Not surprisingly, the orphan ligand scenario is the most challenging, and existing trial-and-error reports concern small peptides, which in some cases may suffer from the known disadvantages of protein ligands. Finally, there are a few examples illustrating the use of a known target structure or a complex. However the affinity of the designed ligands is relatively low in these cases and a nonspecific component in the binding cannot be ruled out.

10.3 SCREENING METHODOLOGIES

There are, in principle, two ways to screen for affinity chromatography ligands. One approach is to first screen in solution and then immobilize the most interesting hits to a suitable matrix. This approach may require an additional synthetic step at the risk of altering affinity. However, it has the advantage of allowing the exploration of a large chemical space during the initial screening, as the molecules are not restricted to having an attachment point for immobilization to the solid phase. The other approach is to make use of a solid-phase screening method. The compounds can be synthesized directly on gel beads using solid-phase synthesis or they can be synthesized in solution and then immobilized to a gel before screening. In the first case a combinatorial chemistry approach can be used to expand the diversity. However, the solid-phase screening approach is limited by the requirement for an attachment point on the molecules to be tested.

10.3.1 Solution Screening Approach

A flow chart of solution-based screening is shown in Figure 10.4. After the first round of screening, compounds that already have attachment points or can be modified with such functionality are selected for the second round of screening. In general it is not desirable to introduce artifactual interactions that could lead to false positives. For instance, when a carboxylic acid group is used as an attachment point, it should be modified to a methyl ester before solution screening to avoid ionic interactions not available after attachment to the gel.

Useful solution-based screening methods are, for example, those based on SPR, where the protein is immobilized to a chip and the compounds are injected in solution, and a range of nuclear magnetic resonance (NMR)-based screening methods.

Figure 10.2 Reference and STD NMR spectra of selected compounds from a glucuronic scaffold library. The spectra have been expanded between 5 and 8.5 ppm. (a) Reference one-dimensional NMR spectrum. (b) Corresponding STD NMR spectrum collected using 25 μM protein. (c) Corresponding STD NMR spectrum collected using 8 μM protein. (d) Corresponding STD NMR spectrum collected using 2 μM protein. Reproduced from Reference 24, with permission.

Figure 10.3 Sensogram of the selected Coumarin affinity ligand. Reproduced from Reference 25, with permission.
10.3.2 SPR Screening in Solution

In the case of solution-phase screening, the protein is covalently immobilized onto a sensor chip by, for example, N-hydroxysuccinimide (NHS)-activation of surface carboxymethylated dextran. A flow cell with an unmodified surface is used as the reference surface. The response from the reference surface is then subtracted from the target surface. The observed response can be compared with the theoretical \( R_{max} \), calculated as the ratio between the molecular weights of the ligand and the protein multiplied by the immobilization level, assuming a one-to-one interaction. Using solution-based SPR screening, the theoretical \( R_{max} \) is often very low and this simple check reveals if binding stoichiometries are higher than one (28). In many cases it is advantageous to improve the solubility of the putative ligands by introducing small amounts of Dimethyl sulfoxide (DMSO) in the buffer. With the introduction of DMSO it is important to match the sample and running buffer (29).

The surface is regenerated after each run. However, it is important to check the surface regularly with a known binder to ensure proper function.

A new development in Biacore™ (www.gelifesciences.com) hardware allows for parallel screening or characterization of the interaction between small molecules and a panel of protein targets, while improved software enables fast analysis of interaction data, with, for example, automatic DMSO solvent correction.

10.3.3 NMR Screening

There are many methods available to perform NMR-based solution screening of affinity ligands [see for instance a comprehensive review by Lepre and colleagues (30)]. For affinity ligands it is often useful to have an affinity constant of \( 10 - 100 \mu M \) (18). Within this affinity window the one-dimensional saturation transfer difference method (STD NMR) is very useful as a screening assay (31). One further advantage with this method is the small amount of protein needed for screening. Ligands may be tested one by one or in mixtures containing up to 10 compounds. A one-dimensional \( ^1H \) spectrum is obtained first, as reference, and subsequently a saturation transfer difference (STD) spectrum is acquired. A positive result is obtained if signals from the ligand are observed in the STD spectrum. An advantage of this method is that there is no need for a specific assay to be developed for each target protein because the result is seen directly in the resulting NMR spectrum of the ligand. Thus new proteins are screened quickly and efficiently.

The STD method is also advantageous in that the detection limits can be tuned for binding strength. At higher protein concentrations, binders with weak to medium affinity will be detected, but at lower protein concentrations the weaker binders will not be detected (32).

10.3.4 Immobilization of Ligand after Solution Screening

When good candidates are identified by solution screening they may have to be modified with a handle before immobilization to the gel. Examples of suitable functionalities on the ligand for attachment to a gel are aldehydes, amines, carboxylic acids, thiols, and hydroxyl groups. Ligand candidates not containing any of these groups may require more extensive synthetic conversions or alternative synthetic routes in order to introduce suitable functionality.

10.3.5 Solid-Phase Screening

The strategy of solid-phase screening is to test an already immobilized ligand with a structure similar or identical to the desired affinity medium. One advantage with this
TABLE 10.1 Structure, Target, and Reference for Small Organic Affinity Ligands Taken from the Literature. The Symbol X in the Figures Denotes the Attachment Point, where Known

<table>
<thead>
<tr>
<th>No.</th>
<th>Affinity Ligand</th>
<th>Target Protein/Scenario</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ala–Pro–Ala–Arg (APAR)</td>
<td>GM–CSF Mab</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Val–Phe–ψ[PO₂–CH₂]–Leu–His–NH₂</td>
<td>Human BHMT</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>Tyr–Tyr–Trp–Leu–His–His</td>
<td>Staphylococcal enterotoxin B</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>S-adenosylmethionine-dependent 3-amino-3carboxypropyl transferase</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>Arachidonic acid</td>
<td>Avidin</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>Imidobiotin</td>
<td>Human insulin precursor</td>
<td>18</td>
</tr>
<tr>
<td>7</td>
<td>Human Fab fragments of κ-type</td>
<td></td>
<td>19</td>
</tr>
<tr>
<td>8</td>
<td>Human Fc, IgG</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>Human IgG</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td>10</td>
<td>Bovine heart l-lactate dehydrogenase</td>
<td></td>
<td>23</td>
</tr>
</tbody>
</table>

(Continued)
approach is that conditions for optimal binding and elution can be evaluated in a straightforward manner. Two different procedures can be used to create a solid-phase library, either by building up ligand diversity through solid-phase synthesis or synthesizing a library in solution and then immobilizing the ligands to a solid phase. One example of the former approach is a strategy reported for the purification of a recombinant human insulin precursor from a crude fermentation broth of *Saccharomyces cerevisiae* (18). In an example of the latter approach, 13 ligands of a combinatorial library were individually immobilized on agarose beads and gold SPR devices, respectively (33). Because the response

<table>
<thead>
<tr>
<th>No.</th>
<th>Affinity Ligand</th>
<th>Target Protein/Scenario</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td><img src="image1" alt="BM1" /></td>
<td>Glutathione-recognizing enzymes NAD$^+$-dependent formaldehyde dehydrogenase from <em>Candida boidinii</em>. NAD(P)$^+$-dependent glutathione reductase form <em>S. cerevisiae</em>, Recombinant maize glutathione S-transferase I</td>
<td>16</td>
</tr>
<tr>
<td>12</td>
<td><img src="image2" alt="BM1" /></td>
<td><em>Dactylis glomerata</em> galactose oxidase</td>
<td>17</td>
</tr>
<tr>
<td>13</td>
<td><img src="image3" alt="G5-A33" /></td>
<td>Porcine pancreas α-amylase</td>
<td>24</td>
</tr>
<tr>
<td>14</td>
<td><img src="image4" alt="Cpd 5" /></td>
<td>Porcine pancreas α-amylase</td>
<td>25</td>
</tr>
<tr>
<td>15</td>
<td><img src="image5" alt="8/7" /></td>
<td>Fab fragments</td>
<td>26, 27</td>
</tr>
</tbody>
</table>
of an SPR system is largely determined by the ratio between the molecular weight of the molecule in solution and the immobilized target molecule, this approach gives a rather large signal compared to the solution screening approach where the protein is immobilized on a chip. Another example of the latter approach is a method for rapid screening of textile dyes based on the determination of the adsorption isotherms of 24 individual dyes coupled to Sepharose™ CL-4B (34). After coupling, the media were transferred to Eppendorf tubes and evaluated by batchwise adsorption/desorption.

In a more recent approach, a 96-well filter device was used in a high throughput fashion (35). Batch binding followed by sequential washing and elution of each well was used to mimic the behavior of a chromatographic column.

10.4 SUMMARY

The above discussion provides examples of how to utilize the possibilities arising from different scenarios, related to the level of information available, to identify low molecular weight organic molecule affinity ligands to target proteins. In Table 10.1 the different published results are summarized in terms of the structure of the ligand, the target protein, and a reference to the relevant publication.

Common to all reported cases of small molecule affinity ligands is a considerably lower selectivity and affinity compared to natural protein ligands. This lower affinity has to be compensated with more thorough work in the optimization of binding and elution conditions to obtain significant recoveries and purification factors.

10.5 ACKNOWLEDGMENTS

The authors would like to acknowledge Dr. Ib Johannsen for a useful discussion and Dr. James Van Alstine for language corrections.

10.6 REFERENCES

11

AFFINITY LIGANDS FROM BIOLOGICAL COMBINATORIAL LIBRARIES

PER-ÅKE NYGREN
Division of Molecular Biotechnology, School of Biotechnology, Royal Institute of Technology (KTH), SE-106 91 Stockholm, Sweden

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11.1 INTRODUCTION

Advances in methodologies for the construction and handling of protein libraries have opened up for new routes to obtain highly selective protein ligands for use in bioseparation applications. From pools (denoted “libraries”) containing very large numbers (typically 10^7–10^9 or more) of peptides or related variants of a given protein, rare molecular species capable of selective and reversible interaction with a given target molecule can be enriched and identified by in vitro selection routes. Such libraries, if properly designed, can serve as general and broad sources of ligands to many different target molecules. Thus, traditional routes involving immunization of laboratory animals to obtain either polyclonal or monoclonal antibodies towards the target molecule can be bypassed using genetic engineering methods, which also allows for the development of non-immunoglobulin ligands of different classes ranging from polypeptides to larger and folded proteins (1–3).

The overall workflow for these approaches can be described to include three main steps: (i) design and construction of the library, including choice of starting protein or peptide framework, as well as the selection system to be used; (ii) expression of the library and selection of variants capable of selective and reversible interaction with the target molecule; (iii) production and characterization of identified variants for evaluation of their performance as ligands in affinity chromatography.

11.2 DESIGN OF LIBRARY

11.2.1 Combinatorial Design Principle

To construct the large pools of variants constituting the library from which novel target-specific ligands are to be selected, combinatorial protein engineering principles are used. This involves the recruitment of a number of either consecutive or sequentially distributed amino-acid positions within a peptide or protein, which are genetically randomized such that any of the 20 natural amino acids, or a specified subset thereof, are allowed at those positions at the protein level. For example, if the library design involves four such
positions within a protein, a library comprising up to \(20^4\) \((20 \times 20 \times 20 \times 20)\) variants of that protein can be constructed. The gene pool encoding such a library is typically assembled by standard gene technology methods (polymerase chain reaction (PCR), endonuclease restriction, ligation, etc.) via the use of mixtures of synthetic oligonucleotides containing so-called degenerate codons at the positions corresponding to the positions to be randomized in the protein. Based on the genetic code comprising in total 61 trinucleotide codons for the 20 amino acids (some amino acids are encoded by more than one codon) and the three stop codons, different degenerate codons can be designed to include codons for all 20 or different subsets of the amino acids that are encoded by more than one codon and the three stop codons, different degenerate codons can be designed to include codons for all 20 or different subsets of the amino acids to be used for combinatorial library design. An example of a degenerate codon is (A:G:C:T)/(A:G:C:T)/(G:T) or (NNK), which includes 32 possible codons covering all 20 amino acids and one of the stop codons (TAG). Similarly, examining the genetic code matrix, other degenerate codons can be designed that cover more narrow subsets of the 20 amino acids. In a few examples, preformed trinucleotides have been used as building blocks for oligonucleotide synthesis (4–7). Such trinucleotides, each corresponding to a full amino-acid codon, can be freely mixed in any proportions during the stepwise synthesis of the oligonucleotide mixture, allowing any desired combination of codons to be included for a specific position in the sequence. The resulting oligonucleotide mixtures can subsequently be used for library construction, either directly after DNA polymerase-mediated synthesis of the complementary strands, or as building blocks for the assembly of larger gene fragments, and transferred into a gene expression context (e.g., plasmid/phagemid/phage vector or PCR product), specified by the selection system to be used (see Section 11.3).

The number of codons included in the combinatorial design, and their distribution within a peptide or protein-encoding sequence, can vary significantly, and should be considered in each case by taking into account several factors including the scope of the work, the characteristics of the protein used as the scaffold (see below), the necessity and probability of complete sampling of all possible variants, and the likelihood of successfully addressing a wide variety of target molecules with a particular library diversity.

11.2.2 Ligand classes

The different ligands developed by combinatorial protein engineering approaches can essentially be divided into three categories: peptides, antibody fragments, and scaffold proteins. The least complex approaches involve the construction of linear peptide libraries, in which a number of consecutive positions of a polypeptide are combinatorially varied (8, 9). Typically, such peptides contain up to 20 randomized positions, often flanked on one or both sides by a few non-varied residues. In so-called cyclic peptide libraries, each such flanking sequence comprises a cysteine residue, which together form a disulfide bridge providing an increased structural constraint on the randomized segment forced into a loop structure. Libraries of antibody fragments (e.g., Fv, scFv, VH, or VL), for example constructed from Ig cDNA of naive or immunized laboratory animals or by synthetic gene assembly, have been extensively and successfully investigated as sources of novel affinity proteins (10). However, in contrast to what could be expected from the established field of immunoaffinity chromatography involving antibody ligands derived from natural sources or hybridomas, the use of engineered recombinant antibody fragments as affinity ligands in chromatography has been limited (11, 12). A different category of ligands is based on combinatorial re-engineering of the surface of an existing protein, recruited as a supporting framework (scaffold). Here, various non-immunoglobulin proteins of different sizes and architectures have been used for library constructions aimed at the isolation of variants binding a desired target molecule. These approaches have involved substitutions or insertions in single loops, multiple loops, or in more rigid secondary structures (β-sheets and α-helices) of proteins showing high tolerance to engineering (13, 14). Examples of the scaffold proteins used include the antibody VH-domain-like fibronectin 10 Fn3 domain (10 kDa) (15), the β-barrel protein class of lipocalins (20 kDa) (16) and Z (6 kDa), a three-helix-bundle, single-domain staphylococcal protein A analog (17, 18). These proteins all have native binding activities, and, typically, the residues involved in those interactions have been subject to randomization during the library constructions in order to develop variants capable of binding to a desired target molecule.

11.3 SELECTION METHODS

Intimately coupled to the library construction step is the choice of selection system, that is, the choice of strategy used for expression of the library members while maintaining a physical link between them and their respective encoding nucleic acids. Such a linkage is crucial for later identification of selected library members, performed via DNA sequencing. The working horse in most laboratories is phage display technology (reviewed in Reference 19), which involves the periplasmic expression of library members as genetically fused (phage or phagemid vector) to the gene encoding an Escherichia coli filamentous phage coat protein (typically protein III or protein VIII of Fd or M13 phage) for display (multi- or single-copy) on phage particles, which inside their coats harbor the vector from which the library member was expressed, thus establishing the desired genotype–phenotype coupling (Fig. 11.1). Typically, subjecting a mixture of phage particles, each displaying a unique library member, allows for enrichment of variants capable of
interacting with a labeled or surface-immobilized target molecule. Re-infection of bacteria with the subsequently eluted phage population results in bacterial clones corresponding to enriched target-binding variants, which can be further propagated for an additional round of phage selection (often four to five rounds are performed) or analyzed directly or after transfer to an appropriate expression system (depending on phage system, or alternatively chemically synthesized for smaller polypeptides) at the clonal level (binding affinity/selectivity, DNA sequencing, performance as ligands in affinity chromatography). Other phage classes have also been utilized for library work, including the lytic E. coli phage T7, involving cytosolic expression of library members for assembly of hybrid phage particles (19).

In addition to phage display, many other selection systems have been described, including ribosomal display, mRNA–peptide fusion, bacterial or yeast cell surface display (allowing flow cytometric screening of the library), and plasmid display (reviewed in Reference 20). Ribosomal display and mRNA–peptide fusion technologies comprise the so-called in vitro methods, which are characterized by transcription and translation of the library in vitro, using a suitable cell extract to which the library (at the DNA level) is added by pipetting. This circumvents one of the bottlenecks in

Figure 11.1 Basic principles of phage display technology for affinity ligand development (E. coli filamentous phage). Mixtures of related oligonucleotides containing degenerated codons, each oligonucleotide encoding a section of, or the whole, individual library member peptide/protein are used for the construction of pools of phage or phagemid vectors, in which the library member genes are genetically fused to a gene encoding a phage coat protein for leader peptide (L)-mediated periplasmic production of the fusion construct. These vectors also contain a suitable promoter (P) for expression cassette transcription, an antibiotic resistance marker gene, phage DNA packaging signals, and an E. coli origin of replication (ori). After transformation of E. coli with the vector pool and subsequent phage production (w/o the use of helper phage particles) the resulting phage particle library is subjected to selection, during which phage particles displaying target molecule-interacting library members are specifically enriched. Isolated clones capable of reversible interaction with the target molecule under the specified conditions by virtue of their particular amino acid sequences can subsequently be identified by DNA sequencing via the phage particle-packed vector DNA, establishing the desired phenotype-genotype coupling during the procedure.
achieving large library sizes, namely the transformation of cells with DNA. Whereas phage and cell libraries typically have $10^7$–$10^9$ members, in vitro selection systems allow the construction of libraries with more than $10^{13}$ members (21, 22). The increased likelihood of finding high affinity binding proteins for a given target molecule using a larger library has been shown in several studies, either directly (comparing two differently complex aliquots from the same library) or indirectly (through affinity maturation of a first-generation ligand) (23, 24).

The choice of selection system for a specific library depends on several factors, including first, the desired experimental complexity. Phage display is relatively straightforward to implement if cloning competence is available, whereas ribosomal display and mRNA–peptide fusion are somewhat more complex systems. The characteristics of the ligand class to be investigated comprise the second factor. The described selection systems differ significantly in how library members are expressed (in vitro/in vivo, periplasm/cytosol, non-fused/N- or C-terminal gene fusion, etc.). For example, in standard phage display (using filamentous phage), library members need to be compatible with secreted (periplasmic) production in E. coli as fused in their C-terminal to a phage coat protein. A good tip is to first investigate a candidate selection system with a representative protein and to perform selections from model libraries to address the suitability of the system for real library work.

The third factor is the intended selection conditions. The entities of the library (phage particles, ribosome–nucleic acid complexes, cells, etc.) must be compatible with the chemical and biophysical conditions used during selection (including binding, washing, elution). These conditions can differ significantly (see below) for different scopes and need to be considered before constructing the library.

The methodology used in the selection step for identification of variants in a library capable of a reversible interaction with a desired target molecule under specified conditions is crucial for success (Fig. 11.2). Conditions during selection should mimic as closely as possible the conditions expected in a final application of the binding protein as ligand in affinity chromatography. The selection phase involves a number of parameters, each of which must be considered in detail, including (i) target molecule concentration and presentation [free in solution, coupled to a solid phase (directed or randomly), labeled with biotin/fluorophore, etc.], (ii) binding conditions (preselection step, buffer system, temperature, presence of competitor, time, etc.), (iii) elution principle (chemical/biophysical/
Candidate ligands are further characterized for relevant applications and to facilitate the identification of relevant clones for further characterization.

A standard first-round selection setup using phage display typically involves the use of a concentrated phage library preparation ($10^{12}$ to $10^{13}$ cfu/mL) and presentation of a biotinylated target protein (typically at a final concentration of 500 nM) on streptavidin-coated paramagnetic microbeads in a physiological buffer containing detergent (e.g., Tween 20) and blocking agent (gelatin/dry milk proteins) to reduce non-specific adsorption of non-relevant phage particles. Pre-incubation of the phage solution with naked beads, or switching to a different immobilization chemistry in the following cycles, can further reduce the selection of irrelevant clones, including streptavidin-binding variants. After end-over-end incubation for a couple of hours, beads are washed and phage particles eluted at low pH (glycine–HCl buffer, pH 3), followed by neutralization and infection of a log-phase culture of pili-bearing E. coli for phage production, as input for a subsequent selection cycle. These conditions can obviously be altered significantly depending on the prerequisites set out for a specific case (25). For example, if the target protein is labile to extreme pH values, a range of milder elution conditions (e.g., 50% ethylene glycol) might be preferred during selection to identify ligands with desired binding characteristics. An optimized elution condition (i.e., complete desorption of the captured target molecule with as mild conditions as possible) is obviously also of relevance for the longevity (i.e., multicycle use) of the ligand when eventually used immobilized onto a chromatographic resin.

Ideally, after an appropriate number of selection cycles in which the selection stringency (or pressure) is typically gradually increased (e.g., more washes, lower target molecule concentration, etc.), the initial library diversity should have narrowed to include target molecule binding variants in the majority (Fig. 11.2). Usually, although DNA sequencing of candidate clones can be performed directly after selection to reveal homologies or identities between enriched variants, an enzyme-linked immunosorbent assay (ELISA) screening step (based on a phage or soluble protein format) is usually first performed on a larger number of clones (preferably from different selection cycles) to verify a successful selection and to facilitate the identification of relevant clones for further characterization.

### 11.4 Characterization and Further Engineering of Ligands

Candidate ligands are further characterized for relevant application-dependent parameters, such as productivity, solubility, affinity, specificity, selectivity, and stability, to identify ligands passing the set criteria. This obviously first requires the production of ligands, either by recombinant means (often after subcloning of the gene from the selection format into an adequate expression system) or by chemical synthesis (option for peptides and smaller scaffold-derived ligands). Even at this stage, differences between variants in terms of productivity, solubility, and stability can generally be seen. Affinity measurements can be performed using a number of methods, including isothermal titration calorimetry (26), affinity chromatography (27), fluorescence anisotropy (28), ELISA (29), and biosensor technology (30). As discussed in Chapter 10, the practical “affinity window” ($K_D$, dissociation constant) for affinity chromatography ligands is in the range $10^{-4}$ to $10^{-9}$ M. Affinity determinations using real-time biosensor instruments (based on surface plasmon resonance or quartz crystal microbalance principles) can, in addition to the overall affinity constant, give information on the separate association and dissociation rate constants. Recovery tests for the target from complex mixtures (e.g., a cellular lysate or serum spiked with the target molecule) performed on a small scale using the ligand candidate immobilized onto a chromatographic resin allows for overall performance analyses, including an evaluation of the selectivity. If an industrial use under cGMP is planned, conditions for column sanitation (cleaning-in-place) could be addressed at this level.

Advice regarding coupling chemistries for attaching the ligand either by random multipoint attachment or by directed single-point attachment to the resin follows the general considerations for proteinaceous ligands described in Chapter 10. To facilitate a directed coupling, generally considered to result in increased capacities, the ligand can be engineered post-selection by recombinant or synthetic means to contain a suitable end group (cysteine, biotin, lysine). Some classes of ligands have been described as producible in a multimeric format, that is, with genetic linking of individual monomeric ligand moieties in a head-to-tail fashion. Depending on the situation, this could potentially be associated with some advantages in affinity chromatography, such as increasing the number of target binding sites on the resin per attachment point, resulting in increased binding capacity, advantageous avidity effects upon binding to di- or multimeric target molecules (chelate effect), or providing a spacer function leading to more efficient presentation of the outermost ligand moiety(ies).

In addition to the use of a developed binding protein as resin-immobilized ligand in affinity chromatography, the recruitment of the binding protein as affinity gene fusion partner for the production and affinity recovery of recombinant proteins has also been described (31, 32). Here, selection is performed using a target molecule intended to be used as the immobilized ligand, thus resulting in a generally applicable system.
11.5 APPLICATIONS

11.5.1 Development of a Peptide Ligand for Affinity Chromatography Purification of Recombinant Human Factor VIII

Using three differently designed constrained (cyclic) peptide phage libraries (1.1 × 10⁹–1.7 × 10⁹ diversity) as input during selections towards a recombinant human factor VIII (BDDrFVIII) target protein, Kelley and coworkers (7, 33) described the identification and characterization of a peptide variant denoted TN8.2 binding to BDDrFVIII with an affinity \( (K_D) \) of \( \approx 0.7 \) μM, which has the potential to replace a mAb ligand presently used in a cGMP process. The three peptide phage libraries were constructed for multivalent display (whole-phage vector rather than phagemid) of library members on M13 phage as fused to protein III, and differed regarding the number of variegated residues between (five, six, or seven amino acids) or outside (one or two amino acids) the two flanking cysteines. In addition, diversity was restricted by the use of tailor-made oligonucleotides for library construction, allowing cysteine (involved in unwanted disulfide formations), methionine (oxidation prone), and lysine (complicating later resin immobilization) codons to be avoided at all positions, for example. The three libraries were each used for two parallel three-round selections against biotinylated BDDrFVIII, involving different elution conditions (50% ethylene glycol or pH 2.0). After phage-ELISA screening and DNA sequencing of enriched variants, nine peptide sequences were chosen for chemical synthesis and column performance tests. Here, the peptides were equipped with a C-terminal nonamer extension ending with a unique lysine for directed coupling to \( \text{N}-\text{hydroxysuccinimide-activated agarose.} \) From these tests, the TN8.2 peptide-resin (AEGTGDHRCSLHPCLAEPEGGGGK;

![Figure 11.3](image_url)

**Figure 11.3** Recombinant factor VIII-binding cyclic peptide selected by phage display technology. (a) Schematic representation of the recombinant factor VIII-binding peptide TN8.2 described by Kelley and co-workers (7), developed by phage display technology. The 27-amino-acid peptide contains ten residues (grey) at positions that were randomized during library constructions, six of which are located within the constrained loop structure formed by the disulfide bridge between two invariant cysteine residues. During synthesis of the peptide for affinity chromatography studies, the N-terminus was acetylated to facilitate a directed amine coupling to activated Sepharose via a unique lysine introduced at the C-terminus. (b) SDS-PAGE analysis (8%, silver staining, reducing conditions) of load and peak fractions from an evaluation of the TN8.2 peptide ligand. Reference material for the 90 + 80 heterodimeric recombinant factor VIII protein is compared with both a column-applied complex sample and material eluted from the TN8.2 column after washing. (Reproduced from Reference 33 with permission from Wiley Periodicals, Inc.)
underlined residues represent variegated positions in the library) showed efficient capture of BDDrFVIII, and could be efficiently eluted with the preferred 50% ethylene glycol solution. Ligand density was found to be critical to column performance and intermediate values (2.25–3.5 mg/mL resin) were found optimal (lower values resulted in poor capture and higher values in poor elution efficiencies), at which ~13 mg BDDrFVIII could be bound per mL resin. For a possible implementation of the ligand into a CHO host cell-based cGMP process, removal of host cell protein and DNA (4.2 log 10 and 4.4 log 10 reduction, respectively) as well as model viruses was successfully evaluated, together with conditions for column sanitization (6 M Gdn-HCl at low pH) and removal of leached ligand. A chromatographic evaluation was carried out with a single peak elution of captured material, the composition of which was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (silver staining) and shown to be virtually identical to reference material recovered from the mAb column (Fig. 11.3).

11.5.2 Combinatorial Engineering of Staphylococcal Protein A for Development of Affibody Binding Proteins for Affinity Chromatography Applications

Staphylococcal protein A (SPA) is widely used as a ligand for affinity recovery of antibodies from serum or cell cultures, both in laboratory- and large-scale settings, by virtue of its binding activity towards Fc or Fab regions of antibodies of different species origin. Native SPA has shown several characteristics beneficial for industrial process conditions, including high selectivity and capacity in binding, high stability to feed stream components, low pH elution conditions, and sodium hydroxide-based column sanitization (34–36). In addition, through engineering of the SPA framework, new variants have been developed conferring milder elution conditions (37) or improved alkaline stability (38). In addition, SPA (or derivatives thereof) has also been frequently used as an affinity gene fusion partner for the production, purification, and immobilization of recombinant proteins (39).

Figure 11.4 Recombinant factor VIII-binding affibody binding protein selected by phage display technology. (a) Schematic representation of the recombinant factor VIII-binding affibody binding protein ZfvIII.3 described by Nord and co-workers (24), developed by combinatorial engineering of a single Staphylococcal protein A domain analog denoted Z. The 58-amino-acid non-cystein scaffold used for library constructions contains three anti-parallel α-helices, forming a three-helix bundle structure (left). Thirteen surface-located positions in two helices involved in the Fc-binding activity of the Z domain were randomized during library constructions, and selections using a recombinant factor VIII protein as target molecule resulted in the identification of several positive variants, of which the composition at the 13 variegated positions for a variant denoted ZfvIII.3 is shown here. (b) SDS-PAGE analysis (4–15% gradient gel, Coomassie Brilliant Blue staining, non-reducing conditions) of load and peak fractions from an evaluation of the ZfvIII.3 affibody ligand. Reference material of the 90 + 80 kDa heterodimeric recombinant factor VIII protein purified with immunaffinity chromatography (monoclonal antibody) (lane 4) is compared with both a column-applied complex sample (lane 1), column flow-through (lane 2), and material eluted from the ZfvIII.3 affibody column after washing (lane 3). (Reproduced with permission from Blackwell Publishing, Inc.)
In recent years, a variant (denoted the Z domain) of one of the five highly homologous, 6 kDa, non-cystein, three-helix bundle domains making up the Ig-binding region of full-length SPA has been recruited as a scaffold for library constructions. With the aim of obtaining novel ligands (denoted affibody binding proteins) with new binding specificities for use in affinity biotechnology applications (including affinity chromatography), while retaining the advantageous characteristics of their SPA domain ancestor, the positions at the molecular surface involved in Ig-binding have been combinatorially variegated to construct libraries for use in selections towards desired target molecules.

Using a phagemid-based approach for monovalent display on filamentous phage particles, Nord and co-workers described the construction of libraries of affibody binding proteins based on the recruitment of 13 surface-located positions in the Z domain scaffold for variegation using different degenerate codons (17). From such libraries of different complexities, affibody binding proteins have been selected for a number of different target proteins for use in affinity biotechnology applications, in several cases involving affinity chromatography. In one study (using a library with a diversity of $4 \times 10^7$), affibody binding proteins of micromolar affinities were selected towards biotinylated Taq DNA polymerase and recombinant human apolipoprotein A-I target proteins, immobilized onto paramagnetic streptavidin-coated microbeads, using low pH for phage elution (40). Subsequently, these affibody binding proteins were evaluated for use as ligands in affinity chromatography, where the binding selectivity and stability towards alkaline column sanitization was investigated. For these studies, two identical affibody moieties were genetically linked in a head-to-tail format to result in divalent ligands, which were produced recombinantly in E. coli fused to an affinity fusion partner. Ligands were coupled to N-hydroxysuccinimide-activated Sepharose recruiting primary amines in the ligands. Repeated purification trials (involving 30 consecutive cycles of lyase loading, column washing, and low pH elution) using E. coli cell lysates containing the respective targets showed selective capture and high ligand stability. Interestingly, column sanitization could repeatedly be performed with five column volumes of either 0.1 or 0.5 M sodium hydroxide solutions without loss of binding capacity or selectivity, and elution with 50% ethylene glycol could be used as an alternative to low pH.

In subsequent studies, affibody binding proteins directed towards human IgA (41), a recombinant factor VIII (24) (the same BDDFVIII as described in Section 11.5.1; see Fig. 11.4), the ancestral SPA or other affibody binding proteins (resulting in so called anti-idiotypic affibody pairs) (32, 42) have also been used for affinity chromatography applications. The small size of the affibody scaffold (58 amino acids) and its ability to spontaneously fold into the three-helix structure allows for the production of selected affibody binding proteins by chemical synthesis routes, in analogy with smaller peptide ligands. This has been demonstrated to be useful in the incorporation of end groups (cysteine or biotin), which can be specifically addressed to achieve a directed coupling (18, 24, 43).

### 11.5.3 Further Examples

Additional illustrative examples on the use of phage display library technology for the development of affinity chromatography ligands can be seen in References 6, 8, 44 and 45, which describe successful selections using Fc fragments, von Willebrand factor, serum albumin, and urokinase-type plasminogen activator as targets. Using a synthetic $3 \times 10^7$ peptide library, synthesized directly onto 65-µm-sized Toyopearls, Kaufmann and co-workers exemplified an alternative methodology for constructing and screening a peptide library for fibrinogen-binding peptides (46).

### 11.6 REFERENCES


PART III

OTHER SEPARATION METHODS AND RELATED TECHNIQUES
MEMBRANE SEPARATIONS

JOACHIM K. WALTER
InnoBiologics Sdn Bhd, Lot 1, Persiaran Negeri BBN, 71800 Nilai, Malaysia

ZUWEI JIN
GE Healthcare Life Sciences, Building 1, No 1 Huatuo Road, Zhangjiang Hi-Tech Park, Pudong New Area, Shanghai 201203, China

MAIK W. JORNITZ
Sartorius Stedim North America Inc., 5 Orville Drive, Bohemia, New York 11716, USA

UWE GOTTSCHALK
Sartorius Stedim Biotech GmbH, August-Spindler-Straße 11, D-37079 Göttingen, Germany

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12.1 INTRODUCTION

Protein purification procedures normally encompass different membrane-based separation steps. These include, but are not limited to, various kinds of filtration processes where the product is filtered through the three-dimensional structure of a membrane or depth filter. In particular, sterilizing membrane filtration is an essential part of bioprocesses, because “cold sterilization” is required due to the degradative effects of heat on any biological solution. Filtration is defined either as the mechanical separation of particles or molecules based on their apparent size in relation to the pore size of the respective filter membrane, or the adsorptive capture of contaminants to the membrane or depth filter structure. Most commonly, retention by membrane or depth filters is a combination of both, sieve and adsorptive retention. Surface-modified membranes are used to adsorptively capture target molecules by interaction with the ligand attached to the membrane. These so-called membrane adsorbers are comparable with conventional chromatographic beads, although there are significant differences in their structures and morphologies, which can influence the key applications for either membranes or beads.

This chapter will provide a general overview of the technologies, equipment, procedures, and processes related to membranes for the separation of contaminants and molecules. However, the main focus will be membrane separation applications in the biopharmaceutical industry. Accordingly, it is the sequence of unit operations for the purification of biological molecules and elimination of undesired contaminants—downstream processing—which is of major interest in the use and application of membranes as separation tools.

12.1.1 Filtration Principles

Currently, the use of membranes for filtration processes is the dominant application and is performed throughout the entire course of the purification process. It covers a vast range of filtration methodologies, including various membrane chemistries, filter designs, and pore sizes or retention ratings.

Membrane filtration is a size-based physical and/or charge-based physicochemical separation process (Fig. 12.1). Some, such as ultrafiltration, nanofiltration, and reverse osmosis, can separate dissolved molecules, which is distinct from traditional filtration processes that separate rigid contaminants. In the context of protein purification procedures, membrane or depth filter separation is used to remove host cells such as bacteria, yeast, and animal cells, to remove particles such as cell debris or viruses, to separate and/or concentrate protein from liquid streams, and to purify water.

The membrane material, filter construction, and design are critical in making the above listed processes possible. Although size exclusion is the dominant mechanism, other factors such as the hydrophobic and electrostatic interactions commonly also play a role in membrane separation.

Various modes of filtration are applicable to the mechanical separation of a target product, and, preferably, the mode of filtration is selected in order to provide the best separation efficiency under acceptable operational conditions. There are two basic types of flow path configuration: static filtration (SF) and tangential flow filtration (TFF) (Table 12.1). Static filtration, also called dead-end filtration (DEF) or normal flow filtration (NFF), refers to a process in which the retentate (contaminants like particulates or microorganisms) accumulates on the surface of the membrane and eventually blocks the membrane. Tangential flow filtration, also known as
cross-flow filtration (CFF), provides a mechanism to reduce this accumulation, due to a swiping flow of the feed stream over the membrane and a partial penetration of the fluid through the membrane. As will be further explained in the text, the key to improving filter performance, flow rate, or throughput is to provide a mechanism for reducing fouling and contaminant accumulation on the membrane. Manipulating the flow pattern or, alternatively, moving the membrane itself can be two approaches to designing efficient solutions to avoiding membrane blockage. The approach of “moving membranes” resembles the third configuration, termed “special.” In some cases this leads to the opportunity to design very efficient filter devices. Other, more common, membrane filter protection methods include the use of prefiltration devices to reduce the contaminant load in the feed stream. Both approaches, flow path configuration or prefiltration combinations, are used to improve filtration capacity.

Filtration capacity or total throughput is defined as the amount of solution that passes through the filter or volume through the membrane area (1). At the laboratory scale, flat filter disks, syringe filters, and stirred cell devices are very popular for their simplicity, ease of operation, and disposability. Stirred cells represent an early attempt at overcoming debris accumulation on membrane surfaces by forced mixing on top of the membrane. Stirred cell and disk filters are a small scale concept and do not scale up well. Trial work with such filters can be used as a performance indication, but have to be followed up by verification trials with larger scale devices. A TFF device provides a well regulated flow pattern to clean/lift contaminant accumulation on a membrane surface and is widely accepted for both small scale and large scale designs.

In the downstream processing of biopharmaceutical proteins, SF operations are typically used for particle clearance, sterile filtration, or virus filtration, usually dealing with a clear process fluid, but require diligent validation. In some examples, however, SF is also used for media or cell harvest filtration, and requires specific high total throughput filters to be able to filter the required volume. TFF operations, applied either as microfiltration or ultrafiltration and diafiltration (UF/DF), are the critical filtration processes in the downstream process and form individual unit operations. Because of its importance, TFF and its principles and applications are highlighted in this chapter on membrane separation.

12.1.2 Evaluation Criteria

A filtration device is usually evaluated by the following criteria:

- flow rate
- total throughput or filtration capacity
- retention/rejection capabilities
- unspecific adsorption.

Depending on the application, different criteria become more pronounced and have greater importance. For example, high flow rates are required for fast equipment turn-around and gain emphasis in buffer filtration or any solutions of low contamination load. High total throughputs are needed in media and final product filtration to be able to filter the entire volume and to reduce the hold-up volume. Low unspecific adsorption filter polymers are needed to avoid yield losses in the final product filtration. Specific retention capabilities must be met in any case. The retention of the filter device is essential in order to avoid any microbial or particulate contamination reaching the final filled product dose.

Specific tests are commonly performed to determine the performance of individual filtration systems and criteria (1–3). Trial work can be performed on a small scale to obtain an indication of the performance sought (indicator trial), but is commonly followed by larger scale verification trials for scaling purposes.

12.2 Membrane Technology

Membranes can be manufactured using various substrates, including cellulose, polymers, metal, and ceramic. The porous nature of membranes has made them ideal materials for different types of filtration processes. In the area of protein purification applications, membrane pore size distribution and porosity are keys for flux (liquid flow per unit membrane surface area) and retention of wanted or unwanted matter. Flux may also be influenced by membrane pleat densities, effective filtration area, pre- or support fleeces, and the overall construction, in avoiding any flow path restrictions. Chemical
compatibility may also play a key role in membrane performance, because subtle incompatibility could cause membrane swelling and therefore a reduction in flow rate. The individual polymeric membrane materials have an influence on unspecific adsorption, and could cause yield losses or premature fouling. Total throughput is also influenced by the abovementioned operating and design parameters. Membrane polymers are a key element, requiring test work to determine the optimal membrane for a particular application, as there is no perfect filter for all applications.

12.2.1 Membrane Chemistries

There are many different kinds of polymers available for membrane materials. However, for use with proteins, only a few basic polymers are currently relevant (Table 12.2). Table 12.3 lists the general properties of commercial membranes. The chemical and micro-structures of a membrane are the basis with which a membrane performs a separation task. Retention and protein binding are of critical importance, because they are the functional indicator of the filtration. Chemical compatibility and thermostability provide critical environmental information, which usually includes solvent resistance, pH tolerance, and steam sterilizability. In other words, retention, cleanability, and physical compatibility are the basic concerns regarding membrane properties and performances. Wettability and protein binding are more a result of the hydrophobicity and electrical charge of the membrane surface treatment and/or its wetting agent. Protein binding to the membrane polymer is most important for membrane filtration performance in protein purification applications. An inappropriate choice of the membrane polymer might result in premature fouling of the membrane or protein losses to the membrane (4).

Hydrophilicity means “affinity for water”; that is, a hydrophilic membrane is applicable for filtration of aqueous solutions. Hydrophobicity indicates the repulsion of water, so a hydrophobic membrane is applicable for air/gas/solvent filtration.

Most membrane polymers are naturally hydrophobic and gain hydrophilicity due to chemical surface treatment. Surface treatment is essential and requires diligent monitoring, as it can cause elevated leachable levels or spotty removal due to cleaning processes, which could result in false failed integrity tests or increased protein adsorption due to a hydrophobic interaction. Even when naturally hydrophilic membrane polymers are used, membrane/target protein interactions have to be evaluated, as the electrical charges of the membrane polymer can change with the parameters of the solution. The pH can shift the isoelectric point and might result in the undesirable effect of binding. The ionic strength of the solution can create the release of captured contaminants into the filtrate. Additional factors affecting retention are summarized in Table 12.4.

### Table 12.2 Basic Membrane Polymers

<table>
<thead>
<tr>
<th>Type</th>
<th>Polymers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose-Based</td>
<td>Cellulose acetate (CA)</td>
</tr>
<tr>
<td></td>
<td>Regenerated cellulose (RC)</td>
</tr>
<tr>
<td></td>
<td>Cellulose nitrate (CN)</td>
</tr>
<tr>
<td></td>
<td>Hydrated cellulose</td>
</tr>
<tr>
<td>Synthetic</td>
<td>Polysulfone (PS)</td>
</tr>
<tr>
<td></td>
<td>Polylether sulfone (PES)</td>
</tr>
<tr>
<td></td>
<td>Polyvinylidene difluoride (PVDF)</td>
</tr>
<tr>
<td></td>
<td>Polypropylene (PP)</td>
</tr>
<tr>
<td></td>
<td>Polyamide (PA)</td>
</tr>
<tr>
<td></td>
<td>Polyacrylonitrile (PAN)</td>
</tr>
<tr>
<td></td>
<td>Polycarbonate (PC)</td>
</tr>
<tr>
<td></td>
<td>Polytetrafluoroethylene (PTFE)</td>
</tr>
<tr>
<td>Inorganic</td>
<td>Ceramic (sintered clay)</td>
</tr>
<tr>
<td></td>
<td>Stainless steel</td>
</tr>
</tbody>
</table>

### Table 12.3 General Membrane Properties

<table>
<thead>
<tr>
<th>Property</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical structure</td>
</tr>
<tr>
<td>Microstructure</td>
</tr>
<tr>
<td>Hydrophobicity</td>
</tr>
<tr>
<td>Wettability</td>
</tr>
<tr>
<td>Charge/surface treatment</td>
</tr>
<tr>
<td>Nonspecific binding</td>
</tr>
<tr>
<td>Thermal stability/mechanical strength</td>
</tr>
<tr>
<td>Performance</td>
</tr>
<tr>
<td>Flow rate</td>
</tr>
<tr>
<td>Throughput</td>
</tr>
<tr>
<td>Chemical compatibility</td>
</tr>
<tr>
<td>Retentivity</td>
</tr>
<tr>
<td>Extractables/leachables</td>
</tr>
</tbody>
</table>

### Table 12.4 Factors Affecting Retention

<table>
<thead>
<tr>
<th>Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane pore size (nominal molecular weight cut-off, NMWC)</td>
</tr>
<tr>
<td>Molecular weight/size of the target</td>
</tr>
<tr>
<td>Membrane wettabily</td>
</tr>
<tr>
<td>Polymer surface chemistry</td>
</tr>
<tr>
<td>Degree of hydrophobicity</td>
</tr>
<tr>
<td>Wetting agents</td>
</tr>
<tr>
<td>Shape of the molecules</td>
</tr>
<tr>
<td>Presence of other solutes</td>
</tr>
<tr>
<td>Operating conditions</td>
</tr>
<tr>
<td>Membrane structure or thickness</td>
</tr>
<tr>
<td>Fluid properties</td>
</tr>
</tbody>
</table>
Many commercial membranes are made from cellulose derivatives, such as regenerated cellulose (RC) and cellulose acetate (CA). RC and CA are hydrophilic, which gives them a significant advantage over other polymers (Fig. 12.2). The chemical process for CA manufacturing is cellulose acetylation, and because it is an evaporation casting process it is considered a slow and complex production process. Owing to its hydrophilicity, CA is widely used for aqueous solutions, water, buffer reagents, media, and protein solutions, as the protein binding capacity of a membrane can be critical for filtering protein solutions. CA has the lowest protein binding capacity on the market. In biopharmaceutical processing, CA is the preferred membrane material for sterile filtration.

12.2.1.2 Synthetic Membranes Most synthetic membranes are cast by quenching or precipitation processes. The casting process is faster and easier to control than evaporation casting. Polysulfone (PS) and its derivative polyethersulfone (PES) are widely used in both microfiltration and ultrafiltration (Fig. 12.3). They are characterized by their diphenylene sulfone repeating units. Their molecular structure provides excellent chemical stability, wide pH compatibility, strength, and rigidity. Polyethersulfone and polysulfone have high thermal and oxidative stability. The membrane structures of PS and PES are often designed to be asymmetric, which allows higher throughputs due to fractionate retention.

Owing to their hydrophobic structure, PS and PES surfaces need to be modified with wetting agents (e.g., PVP) or surface treatments to process aqueous solutions or water. The hydrophobic character increases the tendency to fouling when compared to the hydrophilic cellulose polymers.

Polyvinylidene fluoride (PVDF) is another widespread membrane polymer. It shows excellent low unspecific adsorption, a good pH range and chemical compatibility. PVDF is naturally hydrophobic and requires surface modification. It is important to check specific applications for potentially harmful cleaning cycles, which could wash off the surface treatment and reveal hydrophobic spots.

Polyamide (PA) is also a common membrane polymer, an example of which is nylon. Nylon is dry autoclavable, thermally and mechanically stable, easily wettable, but may show significant protein/nucleic acid binding. Nylon is ideal when processing aggressive solutes such as hyaluronic acid or solvents, which would degrade most other membranes.
Nylo, however, should not be used in oxidative applications, like hot water, as it would degrade rapidly.

### 12.2.2 Membrane Structures

The pore sizes of microfiltration membranes range from 0.04 to 8 μm and are typical for sterile filtration and particle removal. Microfiltration membranes with pore sizes of 0.65 μm, 0.45 μm, 0.2 μm, and 0.1 μm are widely used for the separation of mammalian cells. Microfiltration, as well as using the already described membrane filter materials, also uses the most conventional filter media such as paper, glass fiber, ceramic, and so on. The nominal retention rating for these filters ranges from 0.65 to 150 μm. The most widely used microfiltration step is sterilizing-grade membrane filtration. It is an essential and critical step in aseptic processing of biologics.

Ultrafiltration membranes are characterized by their molecular weight cut-offs, ranging from 1 kDa to 1000 kDa NMWC (nominal molecular weight cutoff). They are commonly used for protein and biological separations or buffer exchange (diafiltration). Reverse osmosis (RO) is a special variety of ultrafiltration. RO membranes are significantly tighter in pore size than UF membranes. Although UF is intended to separate larger molecules such as proteins, RO retains salts and ions, so it is typically applied to fresh water preparation (e.g., desalting of sea water). Accordingly, both filtrations feature significant differences in practical impact: due to the higher molecular weight species used in UF the osmotic pressure differences are much smaller compared to RO, and simultaneously the liquid diffusivity of these molecules is much lower. As a consequence, membrane fouling and concentration polarization are dramatically more pronounced and significant in UF.

Microstructures for MF and UF membranes differ. MF typically has a uniform microporous structure (there are exceptions when MF membranes have an asymmetric structure) with or without a skin layer, whereas UF has an asymmetric structure, a skin layer for separation, and is usually cast on a support material. MF membranes can be made from a variety of polymers and with many different configurations. Pore size can be indicated by the bubble point test, when correlated to the bacteria challenge test (5). The most significant commercial use of MF is the retention of microorganisms and particulate matter. General characteristics of microporous membranes are given in Table 12.5.

There are two principal types of microporous structures: the nodular structure and open cell foam (Fig. 12.4). In the nodular structure, the continuous phase is lumen (nodular structure is more likely for pore sizes of <0.8 μm); in the open cell foam structure the membrane material is the continuous phase.

Ultrafiltration membranes are asymmetrical in their microstructure, and can again be differentiated into two basic structures, macrovoid and macrovoid–free (Fig. 12.5). Both types of membranes have a delicate skin that determines the separation cut-off. Although macrovoid membranes have finger-void structures, macrovoid-free membranes have a dense supportive layer that significantly increases the discriminative membrane layer. Macrovoid-free membranes are considered to be state of the art.

Ultrafiltration membranes are used throughout modern protein purification processes. The applications include concentration and/or diafiltration of product containing cell free culture fluid, diafiltration before a chromatography step for the adjustment of physical conditions, concentration and/or diafiltration after a chromatography step to adjust product concentration, and buffer exchange for the subsequent step in the purification process. Finally, there is the process of concentration and/or diafiltration to transfer the protein product into the designated formulation buffer or (optional) concentration of the protein product with buffer exchange for formulation by gel permeation chromatography.

#### 12.2.2.1 Pore Size and Testing

Membranes for MF and UF are characterized by different methods to indicate their pore sizes and structure according to their physical features (Table 12.6). The bubble point test is a quantitative measure widely used as an indicator for the pore sizes of MF membranes, when correlated to the bacteria challenge test (5).

<table>
<thead>
<tr>
<th>Microporous structure</th>
<th>Pore size, 0.04–8 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sponge-like structures/thickness, 150 μm</td>
<td></td>
</tr>
<tr>
<td>Highly porous</td>
<td></td>
</tr>
<tr>
<td>Narrow pore size distribution</td>
<td></td>
</tr>
<tr>
<td>Passes all dissolved products quantitatively</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 12.4 Microstructures of membranes.**
The test is based on capillary action and is influenced by surface tension. When the applied air pressure exceeds the water capillary force in the membrane pores, water is pushed out of the largest pore, bulk air starts to pass through the freed pore, and air bubbles can be observed (Fig. 12.6). This effect is defined as the bubble point of the tested membrane (Fig. 12.7). Membrane bubble point can be closely linked to the largest pore diameters of the tested membrane. Because the test is based on upstream pressure applied to the membrane, the bubble point test is typically only used for MF membranes and requires a pressure of 1–4 bar (14.5–58 psi). The pressure required to perform a bubble test with ultrafiltration membranes is excessively higher and would lead to an irreversible destruction of the UF membrane. The bubble point test is different from pressure hold and air diffusion tests, which are other commonly used membrane/ system integrity tests. The pressure hold and diffusive flow test are routinely performed at a test pressure specified as 80% of the bubble point pressure. Air bubble or flow will be possibly observed from the filtrate/permeate side even during air diffusion or pressure hold tests. When the bubble point is reached, the air is passing through the membrane pores as obvious bulk flow. During the air diffusion or pressure hold test, air on the upstream side has higher pressure and will dissolve into the wetting liquid within the membrane pores, which will in turn diffuse through the membrane pores to the permeate side and emerge as air bubbles in the permeate. The physical principle of an air diffusion test and pressure hold test is comparable. The difference is that the air diffusion test measures the air flow escaping from the permeate/downstream side while the pressure hold test measures the pressure drop on

<table>
<thead>
<tr>
<th>TABLE 12.6 Membrane Characterization</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ultrafiltration (UF)</strong></td>
</tr>
<tr>
<td>Clean water flux</td>
</tr>
<tr>
<td>Marker retention</td>
</tr>
<tr>
<td><strong>Microfiltration (MF)</strong></td>
</tr>
<tr>
<td>Clean water flux</td>
</tr>
<tr>
<td>Bacteria retention</td>
</tr>
<tr>
<td>Bubble point</td>
</tr>
</tbody>
</table>

**Figure 12.5** Microstructure of UF membranes.

**Figure 12.6** Bubble point test set-up.
The rationale of air diffusion/pressure hold test for checking integrity is that the air flux (air flow divided by membrane area) or the pressure drop rate should be less than the filter manufacturer’s designated specifications. If there is a flaw within the pore structure of the filter, the wetting agent within this region will thin, which influences the volume diffusing through the wetting liquid layer.

Bubble point, pressure hold, and diffusive flow testing are used for particular filter sizes, as the effective filtration area influences the test sensitivity. Bubble point integrity testing is commonly used for small scale filtration devices, because the diffusive flow through these devices would be minimal. Diffusive flow and pressure hold would be used for larger scale devices. In some cases it is advisable to use both test methods, bubble point and diffusive flow, as they each have their advantages and disadvantages (6).

The preset conditions for integrity tests are constant temperature, pressure supply, and wetting agent conditions. Because it is more difficult to determine the retention rating for UF membrane via integrity testing, markers are used to challenge the membrane. These markers are typically molecules that are relevant for the intended separation, such as proteins. Two markers at least should be applied and tested for high rejection and high passage.

There are no fixed industrial standards for rating the pore sizes of ultrafiltration membranes. Hence, the comparability of various membranes from different suppliers cannot be based on the published cut-off rating, but requires a thorough investigation by the users applying their distinctive target solutes (Table 12.7). Ultrafiltration membranes are typically to be quality control tested by markers such as PVP, dextran, proteins with one marker for high rejection and another for high passage. Dextran rejection curves are greatly affected by process conditions. Protein should not have that much variation. Dextran with different molecular weights is used to challenge ultrafiltration membranes in order to characterize membrane pore size and distribution. Dextran rejection curves will be greatly affected by the types of dextran used and process conditions, so such curves are more suitable for marketing than real applications.

### TABLE 12.7 Membrane Cassette Characteristics

#### Flat Sheet Cassettes

<table>
<thead>
<tr>
<th>Membrane</th>
<th>PVP Passage</th>
<th>Protein Passage</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 kDa</td>
<td>C15 = 70–90%</td>
<td>BSA &lt; 2%</td>
</tr>
<tr>
<td>30 kDa</td>
<td>K30 = 35–50%</td>
<td>BSA &lt; 5%</td>
</tr>
<tr>
<td>50 kDa</td>
<td>K30 = 55–80%</td>
<td>BSA &lt; 15%</td>
</tr>
<tr>
<td>100 kDa</td>
<td>n.a.</td>
<td>BSA &gt; 85%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IgG &lt; 10%</td>
</tr>
</tbody>
</table>

#### Hollow Fibers

<table>
<thead>
<tr>
<th>Hollow Fiber</th>
<th>MgSO₄ Rejection</th>
<th>PVP-K15 Rejection (≏10 kDa)</th>
<th>PVP-K30 Rejection (≏40 kDa)</th>
<th>PVP-K90 Rejection (≏630 kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 kDa</td>
<td>6%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 kDa</td>
<td>1%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 kDa</td>
<td></td>
<td>80%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 kDa</td>
<td></td>
<td>75%</td>
<td>90%</td>
<td></td>
</tr>
<tr>
<td>30 kDa</td>
<td></td>
<td></td>
<td>70%</td>
<td></td>
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<tr>
<td>100 kDa</td>
<td></td>
<td></td>
<td></td>
<td>95%</td>
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<tr>
<td>300 kDa</td>
<td></td>
<td></td>
<td></td>
<td>90%</td>
</tr>
<tr>
<td>500 kDa</td>
<td></td>
<td></td>
<td></td>
<td>80%</td>
</tr>
<tr>
<td>750 kDa</td>
<td></td>
<td></td>
<td></td>
<td>60%</td>
</tr>
</tbody>
</table>
12.2.2.2 Pore Distribution  
Filtration retention effectiveness is mainly a consequence of pore size, which is determined by the three-dimensional matrix of the polymer membrane and the casting conditions. Applying UF membranes, separation occurs at the discriminating skin layer. MF membranes may also have a skin layer, but the separation of particulate matter happens not only on the surface of the membrane, but also within the depth, especially when asymmetric membrane designs are used. If the retention relies on adsorptive sequestration, the thickness of the membrane and the length of the tortuous path through the membrane are of importance to the retention efficiency. The thicker the membrane, the higher the likelihood that the contaminant is captured.

MF filters have a narrower pore distribution than a traditional depth filter (0.65–50 μm in traditional depth filtration). Uniform pore size occurs only in track-etched membranes and certain crystallization-formed membranes. Although such membranes give ideal, sharp cut-off retention characteristics, the filtration capacity of such membranes is usually so low that often an accumulated contaminant layer rapidly blocks the membrane. In pre- or depth filtration an accumulation of particulates can form a secondary retention layer, which eventually shifts the retention characteristics of the filter. Pre- or depth filters lacking distinguished pore sizes and retaining contaminants within the depth of the filter membrane or fleece are classified by “nominal retention” values (Fig. 12.8). These values create an indication for the end-user as to what to expect regarding the retentivity of the filter. Nominal retention ratings are measured by particle challenge tests and commonly represent 95–99% capture of the labeled size.

Because of membrane pore distribution, molecules of similar sizes will not be able to be separated by membrane filtration. In normal protein separation applications, a significant molecular weight difference may be needed for efficient separation by typical membranes. For retention, the NMWC of the membrane should usually be 3–5 times smaller than the molecular weight of the target molecule. For passage, the NMWC of the membrane should usually be 5 to 10 times larger than the molecular weight of the target molecule.

In practice, the NMWC of an UF membrane is considered only an approximation: the chemistry of the solute/membrane interaction is an important factor for separation as is also the effective radius of the protein molecules (Fig. 12.9). The base polymer surface chemistry is selected for maximum hydrophilicity to increase flux and reduce fouling in aqueous buffer solutions. Proteins may vary in their effective size due to their physicochemical properties and the particular chemical microenvironment. This “true” physical size is characterized by Stoke’s radius, which also includes the steric effect of glycosylation. Proteins may also vary significantly in shape. They are not ideally globular. Despite an “identical” molecular weight, same-weight proteins will significantly differ in their effective three-dimensional size (Fig. 12.10).

Filtration performance is significantly influenced both by the solutes to be filtered and by the operating conditions. It is predominantly the interaction with particles (MF) and macromolecules (UF) that will lead to the formation of a gel layer. Such a gel layer consists of retained solutes, resulting in a dynamic, secondary membrane. This effect is known as gel-layer formation or membrane fouling, and can cause a significant rejection of the target products as well as flux reduction (see Section 12.4.2).

12.2.2.3 Flow Rate  
Flow rate becomes a major factor when the fluid to be filtered has a limited amount of contaminants or fouling components. Often a particular fluid volume must be filtered in the fastest time possible. Differential
pressure sensitivity, due to possible fouling and restriction in the flow, does not play a role in this application. Filter flow rate dictates some of the down time of the equipment and therefore the capacity available within a production facility. High flow rates are most commonly required in the filtration of buffers or large volume parenterals. Membrane filters are optimized to serve these high flow applications. To obtain optimal flow rates from membrane filters, there are only limited parameters that can be controlled within the filtration process. Flow rate depends on the entire filter cartridge design and not solely on the membrane’s porosity, thickness, and construction. If a membrane with an exceptional flow rate cannot be pleated, it is useless within a filter cartridge construction. The optimization of filtration processes requires tests using comparable filter elements, commonly 100 filter cartridges used in the production process. A side-by-side trial can be performed using only such comparable elements, and the test would take into account the entire design of the filter, the membrane design, as well as the effective filtration area, flow distribution due to pleat densities, and fleece thickness. The test would be performed under the required or specified process conditions, commonly using a set inlet

![Figure 12.9 Typical retention characteristics of ultrafiltration membranes.](image)

![Figure 12.10 Effect of the shape of molecules.](image)
12.2.2.4 Retention The key function of filtration is the removal of contaminants, including particulates, microbes, viruses, colloids, or gels. When a filtration system is designed, the first question is what retention grade is required. The answer to this question is the basis for any step and decision that follows. For every application and removal purpose, filter types and designs are selected to achieve an optimal result. Separation mechanisms for the various contaminants differ; filter construction can be affected by the contamination type, and the performance of a filter is determined by the form and load of contaminant. For example, does the separation take place on the surface of the membrane or in the depth of a filter? What are the particular contaminants and which solids are required to go through the filter? As an example, colloidal contaminants, haze, or lipids are best retained by adsorptive filter forms, such as depth filter pads, as found in lenticular filters. Microbial retentive filters, especially sterilizing grade filters, should rather be sieve retentive to assure organism removal and filtrate sterility. Pore size choice depends on the contaminant removal purpose, as a filter that is too tight could result in performance losses or oversized systems. Most of the time, the purpose of filtration is to remove the contaminant, but pass the drug target through the filter. Again, a membrane that is sized too tight could jeopardize the yield outcome. Removal need must be well defined and should fit the filtrate quality requirement without losing filtration performance (4).

The design or construction of the filter is determined by the removal requirement and contamination load. In case the load is high and the particulate matter size distribution wide, the filter should be designed to increase fractionate retention, so that larger particles are retained first and smaller particles gradually within the depth of the filter. Such a filter would have a multilayer construction to cope with the load and spectrum. If the contaminant is well defined, a sharper retentivity can be used and it may well be that just a single-layer membrane will be sufficient to separate the target contaminant. The design of a filter element and/or a filter combination depends upon the contamination form and load.

12.2.3 Membrane Properties

Membrane properties can, in the broadest sense, mean everything from chemical composition to physical porous structures to the functional outcome (e.g., retentivity). Hydrophobicity or wettability, chemical compatibility, and thermostability are the important physical properties of a membrane.

12.2.3.1 Hydrophobicity and Hydrophilicity Hydrophobicity represents the tendency of the material to repel water molecules, and hydrophilicity represents the ability of the material to be wetted by water. This tendency can be physically characterized by the liquid–solid contact angle. Wettability is a function of the wetting angle. The contact angle is the quantitative measure of the wetting of a solid by a liquid, with the angle formed by the liquid at the three-phase boundary. The smaller the contact angle, the better the wettability. A wettable material for a liquid (water) has a contact angle of less than \( \pi/2 \).

Non-specific binding on a membrane surface and in membrane pores is usually caused by hydrophobic and/or ionic interactions between the membrane and the solutes (7–9). Hydrophobicity is significant in membrane–solute interactions and plays a critical role in membrane separation performance. Applying surface treatments is a common approach to modifying the hydrophobicity of synthetic membranes. A PS/PES membrane would bind only 2–10 \( \mu \)g protein/cm\(^2\) with surface treatment, rather than up to 100 \( \mu \)g/cm\(^2\) without such treatment. However, wetting agents or surface treatments could also introduce undesired additional interactions to the membrane, as illustrated in Table 12.8, where one of the wetting agents obviously had highly negatively charged molecules. Both hydrophobic and ionic interactions can be influenced by conditioning the solutes in different levels of pH and ionic strengths. These effects can sometimes be manipulated to change rejection characteristics or avoid fouling on the membrane.

Besides non-specific protein binding, membrane wettability and hydrophobicity also affect the filtration flux and rejection characteristics. Better wettability improves filtration flux through the membrane. Poor wettability leads to additional rejection and membrane fouling because of the interaction between the membrane and the solutes.

In biopharmaceutical applications, minimum non-specific protein binding should be pursued and the membrane manufacturers choose the membrane material and the wetting agents for minimum hydrophobicity. CA and RC are the two most hydrophilic membrane materials on the market.

<table>
<thead>
<tr>
<th>Membrane Type</th>
<th>BSA, ( \mu )g/cm(^2)</th>
<th>Lysozyme, ( \mu )g/cm(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuf. A PES</td>
<td>4.5</td>
<td>2.8</td>
</tr>
<tr>
<td>Manuf. B PES</td>
<td>2.0</td>
<td>28.7</td>
</tr>
<tr>
<td>Manuf. C PES</td>
<td>3.0</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*Binding of protein to polyethersulfone (PES) membranes with different wetting agents: BSA (negatively charged) and lysozyme (positively charged).*
and should always be chosen when nonspecific binding is a concern. Despite their hydrophobic properties, PS and PES are also widely used in the biopharmaceutical industry for their wider range of pH and thermostability, thus providing robustness to manufacturing processes.

12.2.3.2 Chemical Compatibility and Thermostability
Chemical compatibility and thermal stability are important practically when choosing a membrane. Cellulose-based membranes are largely resistant to organic solvents but are susceptible to acid and alkaline pH, whereas most synthetic membranes are resistant to acid and alkaline pH, but susceptible to organic solvents. In ultrafiltration, CA is sensitive to temperature and shows only a limited pH tolerance (up to 30°C and pH 2–8). RC can resist temperatures up to 75°C. CA is sensitive to aggressive cleaning and does not resist chlorine well, which is a common ion in process and cleaning buffers; in contrast, RC is more stable. Both polymers are biodegradable. PS can tolerate temperatures up to 75°C, while PES can be used at temperatures up to 125°C and thus can be sterilized by autoclaving. Both polymers have a wide range of pH tolerance (pH 1–14) and show good resistance to solvents including alcohols and aliphatic hydrocarbons. PS and PES do not resist esters, ethers, ketones, and aromatic hydrocarbons. PVDF and PTFE are able to tolerate high temperatures (up to 260°C, but not the supporting material, so the temperature limit remains at ~140°C), a wide range of pH conditions, and many different kinds of solvents such as aliphatic hydrocarbons, aromatic hydrocarbons, ketones, and esters. In static microfiltration all described membranes are used and are steam sterilized at temperatures up to 140°C. It is, however, important to always monitor pressure conditions. The filter manufacturers list specific temperature/pressure conditions that have to be met to ensure the integrity of the filter devices. Steam sterilization qualification is another essential part of the validation process, as the influence on the filter needs to be determined as well as the robustness of the sterilization cycle. Chemical compatibility is tested by many different mechanisms, mainly extractable/leachable and/or integrity tests (1). When choosing membrane materials, the decision in fact relates to all the materials involved in the filter module. The pH and temperature limitations posed by the manufacturers are usually the limiting factor arising from an overall consideration of the materials (including supporting, potting, sealing, and housing materials).

12.3 STATIC FILTRATION (SF)

Static filtration (also known as dead-end filtration, DEF, or normal flow filtration, NFF) features the most simple arrangement of a membrane and the fluid path to achieve filtration (Fig. 12.11). In general, SF devices have a lower filtration capacity than cross-flow devices because of their lack of mechanism for reducing solute accumulation on the membrane surface. However, the need to protect columns or UF/DF steps and their large design versatility make these devices still very popular for many routine protein purification procedures.

Pleated filter modules belong to the group of static filters. In the protein purification context they are excellent, and are suitable for sterile filtration of media prior to use in a bioreactor or for sterile filtration of buffers. Other important applications are viral removal or as a “police” filter, in line before chromatographic columns or filter housings to protect these from any burden of particulate matter that could be loaded onto the chromatographic matrix or the filter. Such protection is highly recommended, at least for any larger scale application, as the cost for the filter device is marginal compared to the effort and expense linked to repacking of a column or re-installing membranes in a holder. Stacked disks are layers of depth filters and can be combined with diatomaceous earth for removal of high particulate feed streams. Such adsorptive filtration is applied for the clearance of hazy fluids, and can be supportive for the partial removal of lipids and lipoid substances in the feed stream. In particular, lipids and lipoid substances in the product solution can complicate and impact the performance of an ultrafiltration process by promoting gel-layer formation on the membrane surface and finally clogging the membrane. It may result in an extension of process time, and—under negative circumstances—necessitate an intermediate purification of the membranes.

12.3.1 Devices and Systems

Filters and filtration systems have designs and constructions that are driven mainly by the purpose of the filtration. The most common filter devices are flat disks, pleated devices such as filter cartridges, stack devices like lenticular filters, single-use filters such as the syringe filter, and small scale centrifugal devices for concentration or clarification purposes (Fig. 12.12). Disk filters are available in the above-listed polymer compositions in diameters ranging from 13 to 293 mm. The most common use for disk filters is microbiological tests to determine organism species and level.
These membranes are mainly gridded. Syringe end filters are used to clarify analytical samples, injected into systems like HPLC, or for sterilization of small scale water samples. The application range of these filters is as wide as the available pore sizes and combinations. These single-use devices are also used for small scale vent filtration. The diameters available range from 4 to 50 mm. The next larger single-use devices would be encapsulated pleated filters or so-called filter capsules. These capsules are available in many different membrane or prefiter polymer combinations with a variety of pore sizes or nominal retention ratings. The filtration area range of these filters is 0.01–2.5 m². Encapsulated filter devices have the benefit of avoiding cleaning requirements, and protect the end user from product contact, which is necessary when cytotoxic fluids are filtered. Most often, encapsulated filters are used for liquid applications, often connected to disposable bag systems to store the filtrate appropriately. When connected to a bag assembly, the filter is gamma-irradiated with the bag assembly. It is of importance to understand that these filters require materials of construction that are gamma radiation stable. The pleated filters, built into encapsulated devices, are also available as pleated filters for use in filter housings. The sizes of these filters range from 5” to 40”, and the filtration area from 0.01 to 36 m². Stacked or lenticular filters are mainly of a cellulosic/diatomaceous combination and comprise a thick pad. These filters are highly adsorptive, separate in the depth of the filter pad, and have a very high dirt load capacity. They are excellent for use in cell harvest or prefiltration applications. The common diameters are 12” or 16”, with different stack sizes. However, because trial work needs to be performed with these filters, manufacturers make small scale devices available to either clarify small volume test samples or even production material. Centrifugal devices are used as the syringe end filters, although the centrifugal modus creates a higher throughput option and the fluid is not pushed through the filter by the pressure differential, but by the centrifugal force. These devices are used mainly for sample preparation, concentration, and clarification purposes. Often these devices form the beginnings of the use of ultrafiltration membranes, except that these membranes are expanded to cassette or hollow-fiber format at larger scales (see Section 12.4).

12.3.2 Operation and Optimization

Because of its unidirectional flow through the membrane, conventional static filtration leads to the deposition and accumulation of retained material on the membrane surface (Fig. 12.13). The contaminants are separated by sieve retention or size exclusion and are held as a filter cake on the surface of the membrane. The particles are larger than the largest pore and accumulate on the membrane. In microfiltration applications, such as cell separation after fermentation, another mechanism of fouling can be observed: particles with sizes comparable to the membrane pore sizes may become entrapped within the pores, resulting in mechanical clogging of the membrane. Adsorptive separation is commonly used

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Figure 12.12 Static filtration filter formats.

Figure 12.13 Static filtration system set-up.
in filtration and results in another contaminant collection or blocking process, being known as membrane fouling.

Membrane fouling is caused by the interaction between fluid components and the membrane surface. Physical adsorption is a typical effect when filtering protein solutions derived from biological sources. The deposition of protein or contaminants from the process fluid onto the membrane surface cannot be precisely predicted, as it is a fairly complex cascade of interactions. Fouling is influenced by the chemical nature of a membrane (hydrophilic–hydrophobic), the physicochemical properties of the solutes (hydrophilic/hydrophobic proteins, lipids, and lipidoid substances) and finally the properties of the carrier solution (buffer), for example, pH, conductivity, ionic strength, and temperature.

Fouling may even be enhanced in filtration processes for concentrating the product. The solubility of the retained material will become decisive with regard to fouling effects, due to accumulation of precipitate on the membrane surface. Fouling is time-dependent and may occur in a few minutes or up to hours of processing. As a result, the flux is reduced. Membrane blockage builds up on top of the membrane surface, fouling also within the membrane, and is usually irreversible, so blocking or fouling cannot be eliminated or reversed by any modification of the operating parameters (flow rates, pressures).

The degree of blockage or fouling can be evaluated by measuring the water permeability after filtration; the reduction in water permeability is due to the contaminants on top of the membrane surface or contamination bridging or layering within the membrane.

Flux decay can also be caused by another effect during filtration; the concentration of retained material builds up at the membrane surface and leads to a concentration/gel polarization. The effect is a filtrate flux that is inversely proportional to the concentration of the retained solute. This is different than membrane fouling—concentration/gel polarization is time-independent and reversible. Concentration/gel polarization leads to smaller incremental increases in flux as pressure is increased, to a point in time when a gel layer is formed. At this point the flux no longer increases with pressure. This flux is called the limiting flux.

12.3.3 Depth Filtration

The most efficient way to separate a product solution from cells or cell debris is static filtration, as it offers low capital cost and can make use of a wide range of filter products. However, static filtration is sensitive to capacity and early filter blockage may occur. Static filtration may not even be applicable for some feed streams, such as bacterial lysates with high viscosity. Thus, in order to process particle loaded feed streams, a cascade of filters of different pore sizes is usually required for processing, which makes the handling less elegant and creates a high hold-up volume. Such a filter cascade is typically led by depth filters for clarification of feed streams, and is followed by various kinds of microfilters.

In general, two kinds of depth filter are mainly used, which technically differ in their separating principle, adsorptive depth filters, and noncharged depth filters. A good example of adsorptive filters are filters showing a zeta potential. These filters include cellulose-based depth filtration media designed to retain contaminants by both mechanical entrapment and electrokinetic adsorption. Molecules carrying a positive charge are chemically bonded to the filter matrix, permanently forming an interconnected filtration structure with positively charged electrokinetic capture sites. Particles as small as 0.005 \(\mu\)m are retained. A depth filter with no designated adsorption effect might be a string-wound or nonwoven pleated filter device. These filters mainly use polypropylene or polyamide as the filter materials and are available in a wide range of nominal retention ratings. The appropriate prefilter for a particular application or function has to be determined by the use of filterability trials. These trials create an indication of the performance to be expected at the process scale. The resulting filter combination needs to be optimized regarding filter size, once the process parameters and volumes are known.

12.3.4 Sterile Filtration

Sterile filtration is a widely used application in any biopharmaceutical process, including both gas and fluid handling. It starts early on in cell culture and fermentation, with filtration of cell culture media and media components, and sterile filters are used for aeration and venting of culture flasks, bioreactors, and fermentors. In downstream processing the respective process buffers are usually sterile filtered. As downstream processing is typically performed in a classified but not sterile environment, sterile filtration is typically performed between the individual unit operations in order to keep the bioburden of process fluids low and avoid potential elevated endotoxin levels. Sterile filters are also frequently used as guard filters at the ports of chromatographic columns and the TFF filter holder to protect the surface of chromatographic matrices as well as membranes from particle burden. Such preventive measures are crucial for the performance and quality of these unit operations in the environment of biopharmaceutical manufacturing. The typical pore size for sterile filters is 0.2 \(\mu\)m. An ultrafiltration process of crude feedstock from cell separation after fermentation can be facilitated and its performance improved by replacing the 0.2-\(\mu\)m filtration with a 0.1-\(\mu\)m filtration. A typical range of operation would be feed pressures of up to 5 bar/75 psi at 20°C, at a differential pressure of up to 4 bar/58 psi for cartridges, and 2–3 bar/43.5 psi for capsules at 20°C. Manufacturer’s specified values have to be considered for individual filter products.
The membranes used for sterile filtration of gas are made of hydrophobic polymers such as polytetrafluoroethylene, and so on. Filters for fluid filtration are based on hydrophilic materials as some applications in the biopharmaceutical industry require low adsorption capabilities. The dominant base material for very low nonspecific binding of proteins is cellulose acetate. Such membranes provide excellent protein yield and are therefore ideally suited to processing high value biopharmaceutical solutions such as dilute protein solutions. In addition to cellulose-based modified polymer filter materials such as hydrophilic polyvinylidene fluoride (PVDF) or hydrophilic polyethersulfone (PES) as sterilizing-grade 0.2- and 0.1-μm Mycoplasma retentive membranes are well accepted for their low protein binding, high flow rates and throughputs. PVDF and PES show broad chemical compatibility, low extractables, and nonfiber-releasing properties.

In order to increase throughput and provide high flow rates at low differential pressure for gentle product treatment, many sterile filters are designed as composite filters providing a built-in prefiltration, for example, by applying a 0.45-μm membrane as protective cover for the 0.2-μm membrane.

Sterile filters are available in traditional cartridge formats and disposable capsules ranging from cm² to m² for simple linear scale-up and process flexibility (10). Certified sterile filters of reputable manufacturers have the common link that the filter cartridges are fully validated as sterilizing grade filter elements according to regulatory guidelines. As a substantial measure of quality control, each individual element is integrity tested by diffusion and bubble point tests before release, ensuring absolute reliability. In order to select particular filters in compliance with regulatory requirements for pharmaceutical products, validation and extractables guides should be requested from the supplier.

12.3.5 Applications

Static filtration performed mainly with pleated filter cartridges or lenticular depth filters is used in a multitude of different applications, some critical (e.g., sterilizing grade filtration). Such filtration systems have often been regarded as straightforward or just a commodity item, both observations being incorrect, as the performance criteria for such filters are highly critical. The list of applications includes the following:

- sterilizing filtration of monoclonal antibodies, recombinant proteins, LVP and SVP, vaccines, water, buffer, cell culture media, and so on
- bioburden reduction, such as column protection filters or prefilters for sterilizing grade filters
- prefilters or particulate filters, which are utilized to clarify solutions or protect membrane filters
- depth filters, mainly lenticular filters, commonly used for cell harvesting or lipid removal in blood fractionation applications
- air and gas membrane filters for venting, compressed service gas or fermentation air intake filtration.

This list is most certainly incomplete, as one finds pleated, static filters in other fields, such as the semiconductor or food and beverage industries. It is important to understand that every application has specific requirements and performance criteria, which must be met by the filter device utilized. A random, unspecific choice of filter design, polymer, or construction could be detrimental if not properly chosen and tested. Initial investments might look attractive, if low, but can inevitably rise to a painful level when running costs or yield losses accumulate later in routine production. For example, single-use filtration devices might look more costly than a pleated filter utilized in a stainless steel housing. However, such a filter-holding device needs to be cleaned and sterilized, as well as set up, all of which are energy-, material-, and labor-intensive, leading to a higher cost than the single-use costs in the first place. As another example, in some cases a 0.45-μm-rated filter suffices to obtain a sterile effluent, but in other applications 0.1-μm-rated filters are needed, such as in cell culture media filtration to remove possible Mycoplasma contaminants. The EMEA–CPMP Guideline of 1996 (11) allows only a presterilizing grade filter bioburden level of 10 cfu/100 mL; that is, 0.45-μm-rated filters could accomplish this task.

Application- and process-driven filter choice, validation, and utilization are required to enable an optimal filtration process. Every application has its distinct needs and specification, which should preferably be investigated together with the filter device manufacturer to make use of their wealth of experience and data bases.

12.4 TANGENTIAL FLOW FILTRATION (TFF)

TFF (also known as cross-flow filtration, CFF) is a configuration of filtration technology in which the process fluid is directed tangentially across the membrane surface, thus “sweeping along” retained particles or molecules rather than allowing them to build up on the membrane surface (Fig. 12.14). The perpendicular flow reduces the contact time between the process fluid and membrane surface, resulting in greater filtrate flux and improved product recovery, even if there are large amounts of retentate. TFF is therefore popular for clarification and size fractionation operations, where normal filters would rapidly become clogged with retentate (12).

TFF is a highly dynamic process and operations are therefore technically demanding. The process parameters must be carefully controlled, but even then there may be significant
variations in performance between one process run and the next. Regular cleaning of the entire TFF system (and replacement of disposable filter modules, if appropriate) is necessary to maintain performance and prevent the system’s physical limitations (including boundary layer formation, intrusion of particulate matter into the membrane skin, and fouling) from interfering with the overall process. TFF modules are available in a wide range of sizes so that an optimal membrane area can be achieved for different processes and scales. Standard equipment is manufactured with capacities of up to several hundred liters for large scale processes, and customized modules can be developed where necessary.

One of the key advantages of TFF is its general insensitivity to process volume and solids concentration, which makes it suitable for separation tasks where the shear sensitivity of a product or producer cell is low or negligible. Such applications typically include separation processes for microorganisms and mammalian cells (microfiltration) and for molecules (ultrafiltration), in which there is a long history of operational success (13).

TFF is also more versatile than SF. Whereas SF is used primarily for filtration or concentration of a product that is larger in size than the membrane’s pore size or molecular weight cut-off. In the latter case, the product stays in the retentate and is concentrated in the TFF module or transferred into another carrier fluid (diafiltration). This mode of operation is a typical application of ultrafiltration membranes during the processing of proteins, where recovery of the retained product is increased. Where the product is smaller than the membrane’s pore size or molecular weight cut-off, it passes through the membrane in the filtrate or permeate. This mode of operation is applied when the aim is cell separation using microporous membranes, virus removal by ultrafiltration or nanofiltration, or buffer depyrogenation.

The flow and pressure profiles for SF and TFF differ considerably (Fig. 12.15). Two major operating variables determine TFF performance: transmembrane pressure (TMP) and cross-flow velocity (CF). The TMP is the driving force for the flux through the membrane: the permeate flux. TMP is the pressure difference between the two sides of the membrane, and is defined as

$$\text{TMP} = \left(\frac{P_F + P_R}{2}\right) - P_P,$$

where $P_F$ is the inlet or feed pressure, $P_R$ is the outlet or retentate pressure, and $P_P$ is the permeate pressure.

The cross-flow velocity is equivalent to the retentate flow rate. Accordingly, CF can be determined easily and is proportional to the difference between the feed pressure and retentate pressure:

$$CF \approx P_F - P_R.$$

As well as the TMP and CF, TFF performance is affected by feed concentration, viscosity, temperature, pH and ionic strength, and device geometry, but TMP and CF are the major factors, especially in terms of membrane productivity. They are closely associated with a mechanism called concentration/gel polarization (discussed below). Indeed, concentration/gel polarization is considered the single most significant factor in membrane productivity. The goal of TFF is to achieve optimal permeate flux, with TMP as the driving force.

It is important to evaluate the filtration behavior of a membrane before its first use and compare this to its operational performance (6, 14). One important parameter is the normalized water permeability (NWP), which decreases with declining membrane performance and is the decisive indicator used for performance evaluation. NWP is a simple and elegant method to determine the efficacy of a membrane regeneration procedure by comparing the NWP prior to use and after cleaning, with full recovery indicated when the two values
are identical. In practice, however, the NWP decreases slightly and continuously depending on the feedstock and its tendency to foul the membrane.

The permeability of a membrane is defined as the filtrate flux divided by the applied pressure. In TFF systems the filtrate flux is a function of two parameters, TMP and feed temperature. The temperature affects the viscosity of the fluid, so the filtrate flux (at constant TMP) will increase with increasing temperature. To compare the water permeability after each process, the water permeability is normalized to a standard temperature:

\[
\text{NWP} = \frac{\text{Water flux}}{\text{TMP}} \times \text{TCF}
\]

The water flux is defined as permeate flow rate/membrane area/time (typical units L/m²/h). The temperature correction factor (TCF), which must be determined for individual fluids, is only required if the temperature varies from process to process.

12.4.1 Devices and Systems

TFF modules come in many designs, differing in terms of channel spacing, packing density, cost, pumping energy requirements, plugging tendency, and ease of cleaning, so the design must be chosen on a case-by-case basis for each bioprocess, depending on the implications of the above criteria in the context of the overall process train (15). Investment costs have to be taken into account in the design of TFF systems. Two principle membrane design formats are available for the separation of cells: hollow-fiber and open-channel flatsheet (Figs. 12.16 and 12.17).

The flat-sheet format, as the name suggests, involves flat membranes that are often presented in the form of a cassette module to increase the surface area available for filtration without increasing the footprint (Fig. 12.16). Hollow fibers are self-supporting tubular membranes that have unique physical properties due to their favorable high precision geometrical shape, that is, the accuracy of membrane casting and assembly (Fig. 12.17). Although hollow-fiber membranes offer high membrane areas in a small footprint, this comes...
at the expense of relatively high dead volumes that are not acceptable in applications where a high product concentration has to be maintained.

When it comes to the operation of a TFF system, the cost calculation for processing should include the costs of the consumable parts, the filter cartridges, and cassettes. Although manufactured from inert materials, these have a limited lifetime because filtration performance declines over time, despite rigorous cleaning. Membranes also need to be replaced if a filtration skid or a facility is used to manufacture different products to avoid the risk of product carryover as a result of insufficient cleaning. A typical system set-up for a TFF process is outlined in Figure 12.18. This presents all relevant operational components, independent of scale and membrane design.

Tangential flow microfiltration operations often require strict permeate flux control, because the pore size and the porosity of typical microfiltration membranes could allow a high permeate flux. Microfiltration for cell separation is usually performed by applying only moderate TMP (e.g., 0.1 – 0.8 bar). This could be achieved with a slower pump speed, but the CF would then be insufficient and result in rapid membrane fouling. The permeate flux can be reduced either by throttling the permeate valve or by placing an additional pump in the permeate line in order to control the flux (Figs. 12.19 and 12.20).

### 12.4.2 Transfer Mechanism and Operation Dynamics in TFF Processes

Even if the basic flow/membrane configuration is correct, the key to successful TFF is understanding the transfer mechanism that determines its performance and operational dynamics (16). For a pure water feed, flux increases linearly with transmembrane pressure because resistance and
viscosity remain constant. During a typical filtration process, however, the flux tends to decline over time because both viscosity and resistance increase (Figs. 12.21 and 12.22). During cross-flow filtration, flux declines rapidly at first, then the decline slows down, and eventually a steady state is achieved. This reflects two simultaneous phenomena—gel-layer formation and fouling—both of which are taken into account by modern membrane development and suitable operational strategies (17). In tangential flow microfiltration the gel layer (also known as the filter cake) builds up when retentate particles accumulate on the feed side and achieve a packing density that causes them to form a defined layer on top of the membrane (18). The shear forces created by the tangential flow across the membrane surface support the removal of such attached particles and molecules, but the removal is not complete and cannot be complete for physical reasons, for example, because of stagnant fluid due to friction in molecular proximity to the membrane surface. This has two consequences. First, the gel layer resists the flow of permeate by effectively acting as an additional filter bed, increasing the thickness of the filter membrane, and leading to increased resistance. Second, the local viscosity of the feed is increased because the concentration of particles near the filter surface is higher than in the bulk feed, resulting in reduced flux. In ultrafiltration, the equivalent phenomenon is known as concentration polarization, where the retentate remains in solution but builds up on the feed side, increasing in concentration to such an extent that the osmotic pressure of the retained solute opposes the force driving the permeate across the membrane. At very high concentrations, the retentate can approach its solubility limit and form a gel layer on the membrane surface that also obstructs the flow of permeate (19).
Under certain conditions these layers can either aid in the filtration retention process or act as a barrier by plugging the membrane. Practically, gel polarization is either unlikely or minimal with purified proteins at concentrations \(<0.1\%\). Polarization will likely become a problem at protein concentrations \(\geq1\%\), and becomes obvious during ultrafiltration processes when permeate flux rates decline as concentration increases. Concentration polarization and gel layer formation govern how process parameters such as flux, TMP, and CF interact with each other, as discussed in more detail below.

The transfer of molecules through a membrane is resisted by up to three components: membrane resistance \((R_m)\), concentration boundary resistance \((R_c)\), and gel layer resistance \((R_g)\) (19) (Fig. 12.23). Accordingly, the three representative situations are: membrane-controlled filtration, for example, pure water or buffer, showing no concentration boundary \((R_m)\) (Fig. 12.24), membrane and concentration boundary control, that is, process fluid with concentration polarization but no gel polarization \((R_m + R_c)\) (Fig. 12.25), and process fluid with membrane resistance, gel layer, and concentration polarization \((R_m + R_c + R_g)\) (Fig. 12.26). Transfer through a membrane is also influenced by the additional environmental parameters listed in Table 12.9.

Concentration affects filtration performance by influencing the viscosity, diffusivity, and concentration gradient of the liquid inside the boundary. Greater viscosity caused by higher feed concentration generates a thicker concentration boundary with increased viscous resistance. Higher feed concentration results in lower diffusivity and higher \(C_w\) therefore increasing the likelihood of gel polarization. The pH and ionic strength of the feed affects performance by influencing solute solubility \((C_G)\) and diffusivity \((D_s)\). The effect is eventually caused by \(C_G\) and \(D_s\) through concentration/gel polarization.

Temperature also has a significant impact on feed viscosity, diffusivity, and solute solubility, which affects TFF performance by influencing concentration/gel polarization. Higher temperatures increase \(C_G\) (thus reducing the likelihood of gel polarization), increase diffusivity (thus increasing filtrate flux), and lower viscosity (thus reducing the thickness of the concentration boundary layer).

The geometric characteristics of the flow channels also affect the performance of a TFF module by influencing laminar and/or turbulent flow, the efficiency with which retentate is swept from the membrane surface, and thus the concentration boundary thickness. The flow pattern is described by a dimensionless number, the Reynolds number \((N_{Re})\). Empirically, laminar flow in straight pipe occurs where \(N_{Re}\leq1800\) and becomes turbulent flow when \(N_{Re}/C^2\geq4000\), the intervening range being described as transitional (Fig. 12.27).

Theoretically, turbulence could be introduced by two factors: inertia and flow path geometry. \(N_{Re}\), as defined in pipe...
flow, does not tell the whole story, and would need to be redefined for different geometries, which therefore have lower thresholds for turbulence.

Shear forces also evolve from the cross-flow along the membrane, that is, moving molecules or particles passing a fixed surface at a given velocity, where the velocity is the quotient of flow rate and cross-sectional area. Shear rate is always greatest on the membrane wall and is zero in the center of the channel, and can be calculated using \( \tau_{\text{wall}} = \Delta P r^2 / L \) for a simple geometry such as a hollow fiber. It is proportional to feed/retentate pressure drop, and therefore also to cross-flow velocity.

Shear rate in its physical sense is the velocity gradient as defined in Newton’s law. Based on the shear balance above, shear rate = \( \tau_{\text{wall}} / \mu = \Delta P r^2 / L / \mu \), which takes the form of \( 4q / \pi r^3 \) in a laminar flow situation (\( q \) is the flow rate going through one pipe). Viscosity is resistance to shear force, and can be defined as Newtonian fluid viscosity (in which shear is proportional to stress) and non-Newtonian fluid viscosity (where shear and stress have a nonlinear relationship). The effect of shear forces on process fluid therefore depends on the nature of the solute.

In microfiltration applications related to cell separation, cell damage may occur if the feed stream flow rate is excessive. Typical shear rates are \( \sim 8000 - 16,000 \) per second for the harvest of \( E. \text{coli} \) cells, and \( 4000 \) per second for shear-sensitive mammalian cells. In protein ultrafiltration, excessive shear may cause agglomeration, and the aeration of proteins may lead to irreversible denaturation. The ultrafiltration concentrate return line is therefore submerged and sealed to prevent this.

Figure 12.26 illustrates the opposite effect caused by the applied transmembrane pressure (TMP) which enforces the build up of a gel layer formation and the cross flow (CF) which decisively contributes to reduce the concentration polarization close to the membrane surface. Figure 12.29

### Table 12.9 Factors Affecting Concentration/Gel Polarization

<table>
<thead>
<tr>
<th>Factor</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (CB)</td>
<td>( C_{\text{G}} / \text{viscosity (( \mu ))} / \text{flow type (( \delta ))} / \text{diffusivity (( D_s ))} )</td>
</tr>
<tr>
<td>pH/Ionic strength</td>
<td>( \text{Solubility (( C_G ))} / \text{diffusivity (( D_s ))} )</td>
</tr>
<tr>
<td>Temperature</td>
<td>( \text{Viscosity (( \mu )) / diffusivity (( D_s )) / solubility (( C_G ))} )</td>
</tr>
<tr>
<td>Flow path geometry</td>
<td>( \text{Flow types and concentration boundary thickness (( \delta ))} )</td>
</tr>
<tr>
<td>Retained solutes or particulates other than the target product</td>
<td>( \text{Flow path geometry} )</td>
</tr>
</tbody>
</table>

- Reynolds number in pipe flow
  - \( \text{Laminar: } N_{\text{Re}} < 1800 \)
  - \( \text{Turbulent: } N_{\text{Re}} > 4000 \)

- Effect of geometric characteristics
  - \( \text{Fluid density (\( p \)) / Fluid velocity (\( u \)) / Pipe diameter (\( d \)) / Fluid viscosity (\( \mu \))} \)

**Figure 12.26** Gel layer formation.

**Figure 12.27** Laminar and turbulent flow.
shows how concentration on the membrane interface is built up in response to increased TMP, and how the concentration boundary is formed and defined. Concentration and gel polarization is such a universal phenomenon in filtration operations that understanding the process is as important as understanding the membrane itself, explaining why it is considered the single most significant factor in the success of TFF.

12.4.2.1 System Hardware and TFF Operation Dynamics
Additional causes of induced shear are often related to the system hardware. TFF involves two basic flow streams; the filtrate (or permeate), which crosses the membrane, and the tangential flow (or cross-flow), which sweeps across it. The filtrate/permeate stream is driven by TMP and the cross-flow is usually driven by an external pump. The TMP is an average value for the filtration system and consists of incremental local TMPs across the membrane.

To understand pipe fluid dynamics, there are two key elements: pump operating characteristics and pipe flow pressure, the final flow in a pipe system reflecting their combined effects. A pump that always delivers a set flow rate regardless of opposing pressure is called positive displacement pump, for example, a piston pump (at least until it fails). Peristaltic and rotary lobe pumps may temporarily achieve positive displacement, but would be unable to cope at high pressures. Pressure drop through chambers and pipes reflects the flow rate, especially the linear velocity. Fluid dynamics in pipe flow can be studied by the integrated form of the Navier–Stokes (NS) equation on pipe boundaries. The NS equation is fundamental in fluid dynamics and is highly complex (a time-dependent, nonlinear, second-order, three-dimensional partial differential equation set). A simple, integrated form for pipe flow is the Bernoulli equation, which relates pressure and flow according to the cross-sectional area of the pipes. It can be represented as $0 = \Delta Z + \Delta P/\rho + \Delta V^2/2g$, where $\Delta Z$ is the elevation from one cross-sectional area to the other on the pipe, $\Delta P$ is the pressure difference between the two cross-sectional areas, $\rho$ is the fluid density, $g$ is gravity.
constant, and $\Delta V^2$ is the difference of the square of average flow velocities at the two cross-sectional areas on the pipe. For dense media such as membrane cassettes or hollow fibers, where viscous forces are more relevant than cross-sectional area, Darcy’s law may be more appropriate. This can be represented by the equation $V = k/\mu \cdot \Delta P - \rho g$, where $k$ is the permeability of the dense media, $V$ is the superficial velocity, and $\mu$ the fluid viscosity. Darcy’s law is essentially the same as the Hagen–Poiseuille equation discussed below in relation to membrane permeability.

Without going into detailed theoretical analysis, the relations described by the above equations have the following practical effects in terms of TFF:

• The higher the flow rate going through a pipe or chamber, the greater the pressure drop from inlet to outlet. The amount of pressure drop depends on the flow type (laminar or turbulent), the number of turns and bends, and any sudden changes in diameter as may occur in valves and connectors. However, these effects are dwarfed by the impact of dense media such as membrane cassettes or hollow fibers. Therefore an apparent linear correlation between $\Delta P$ and cross-flow is usually observed.

• Nonpositive displacement pumps do not deliver a set flow rate because the flow rate is a result of the interaction between the pump and the pipe system. A nonpositive displacement pump cannot be calibrated in the flow system because the flow resistance changes during operation. Furthermore, even a positive displacement pump cannot guarantee a constant cross flow because it will only deliver a set inlet flow to the membrane module but not a set retentate flow, which is the inlet flow minus the permeate flow.

• Appropriate shear forces can be exploited as a “cleaning force,” with the cleaning effect in relation to gel polarization. It is the shear rate, not the flow rate, that reduces the concentration boundary thickness.

• Lower channel height or lumen diameter will reduce the concentration boundary and increase membrane filtering capacity (permeate volume/membrane area) at the cost of increasing hydraulic resistance for pumping and increased likelihood of clogging. Spacers, as used in many cassette devices, can induce turbulence and thus help reduce concentration and gel polarization while serving as a mechanical support for membranes and keeping the channel height open. Screen spacers sometimes cause dead spots when the flow is not fully turbulent, and a gel layer accumulates.

Path length is the distance between inlet and outlet represented by an active membrane. As a rule of thumb, the variation of TMP and CF along the membrane path length should not significantly affect flux across the membrane as a whole (this holds for most ultrafiltration operations, where the permeate is a small fraction of CF). Extended path lengths can affect flux significantly, so this is not a recommended method for increasing flux. Also the longer the path, the greater the variation of local TMP, which could lead to local gel polarization (Fig. 12.30).

The direct driving force for permeate is TMP (no matter what pressure the pump adds to the system) (Fig. 12.31).

$$J = \frac{\Delta P_{\text{TMP}}}{R_g + R_m + R_c}$$

Figure 12.30 Overall TMP and local TMP.

Figure 12.31 Permeate flux.
But cross-flow is mostly driven by the pressure lift provided by the pump. This is important for understanding the transfer processes involved in TFF filtration separation. Figure 12.32 shows a control diagram in the form of a logic tree for achieving a specific cross-flow and a specific TMP, as would be required in typical TFF operations.

12.4.3 Cleaning and Storage

Understanding the cleaning methods and chemistry in TFF is important for the reuse of a system when membrane lifetime is a significant concern, as it contributes to operating costs (20). As explained above, TFF performance will decrease over time and runs as the permeate flux decreases, even if the operating conditions remain the same. Changes in the rejection characteristics (additional rejection) may not be a problem for protein concentration, but it would certainly reduce the efficiency of protein clarification, for example, in virus filtration, when additional rejection of the target protein will cause a decrease in yield. A permanent decline in membrane performance (fouling) reflects many different physical mechanisms including irreversible gel formation due to covalent binding of foulants to the membrane (not the same as the reversible phenomenon of gel polarization described above). When this process occurs, changes in operating conditions such as CF and TMP have no effect. The hydrophobicity of a membrane has a significant effect on its fouling tendency, with hydrophilic cellulose acetate membranes relatively less likely to suffer. Rougher surfaces also promote fouling by increasing the spatial accessibility of surface molecules.

Cleaning is precisely defined in any given process and must achieve specific goals that can be verified by assay and form the basis of cleaning validation procedures. In the case of filter membranes, cleaning must achieve physical cleanliness (membranes must be free from visible particulate matter), chemical cleanliness (membranes must be free from foulants and contaminants within acceptable predefined tolerances), and biological cleanliness (membranes must be sanitized or sterile, and pyrogen-free). Effective cleaning is confirmed by reasonable recovery of the initial water flux, which should be tested prior to initial use as a basis for retesting after cleaning. Typical water flux recovery is 65–95% of the original flux, but this does not necessarily imply similar performance in terms of the sample. Temperature and water quality are critical in determining NWP.

Cleaning in place (CIP) usually means chemical decomposition of the materials on the membrane surface that create resistance to permeate flow, whereas sanitization in place (SIP) refers to depyrogenation, where aseptic processing is required. Most existing biopharmaceutical processes achieve cleaning with NaOH, NaOCl, and/or H₃PO₄ at ambient or elevated temperatures (Table 12.10), but the reagent of choice depends on the nature of the fouling agent, which is process-specific and also depends on the specific type of membrane product used. The contact time and the temperature of the cleaning agent have a much greater effect than pressure or flow. For example, leaving a membrane cartridge soaking overnight can sometimes restore a fouled cartridge to functionality. Feed streams containing particulates may benefit from a brief “backwash” (usually <10 psig) to remove gross contamination, but care has to be taken not to destroy the membrane due to reversed flow.

TFF systems may need to be stored between runs. Preferably, membranes should be kept wet for storage after cleaning, and to avoid microbial proliferation, the storage solution should be bacteriostatic (most common storage agents are listed in Table 12.11). Alternatively (and preferably for long-term storage) membranes can be carefully dried. In both cases, the preparation procedures must be defined and validated to ensure that the membrane performance characteristics do not decline in storage.

12.4.4 Applications

12.4.4.1 TFF Microfiltration TFF using hollow fibers was the cell separation technology of choice when large scale mammalian cell fermentation was developed (21). The first ever large scale biopharmaceutical manufacturing process for a recombinant human protein (tissue plasminogen activator) from a recombinant mammalian cell line (CHO)
TABLE 12.10 Typical Cleaning Agents in TFF

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mode of Action</th>
<th>Chemicals</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caustic solution</td>
<td>Hydrolysis, solubilization</td>
<td>NaOH</td>
<td>Organic and microbial foulants</td>
</tr>
<tr>
<td>Oxidants/disinfectants</td>
<td>Oxidation, disinfection</td>
<td>NaOCl, H₂O₂, peroxyacetic acid</td>
<td>Increases hydrophilicity of foulant and so reduces the adhesion of fouling materials to membranes; may cause swelling of membrane</td>
</tr>
<tr>
<td>Acids</td>
<td>Solubilization</td>
<td>Phosphoric acid, citric acid</td>
<td>Removes scales and metal dioxides</td>
</tr>
<tr>
<td>Chelating agents</td>
<td>Chelation</td>
<td>EDTA, citric acid</td>
<td>Removal of organic foulant intensified by divalent cations</td>
</tr>
<tr>
<td>Surfactants</td>
<td>Emulsifying, dispersion</td>
<td>Surfactants, detergents</td>
<td>Forms micelles with fat, oil, and proteins in aqueous solutions, affects fouling dominated by the formation of biofilms</td>
</tr>
</tbody>
</table>

was established jointly by Boehringer Ingelheim Pharma KG, Biberach and Genentech, Inc., San Francisco, between 1984 and 1986, and involved fermentation at the 12,000 L scale, which is still popular today. The manufacturing process was based on batch fermentation and included media exchange and cell separation steps, both based on hollow-fiber TFF. More compact and versatile modular systems for cell separation based on flatsheet membranes have been developed, for example, Prostak (Millipore Corp.), although such devices feature unfavorable meandric fluid pathways and do not perform as well as hollow fibers.

The current status of mammalian cell technology sees high titer processes, with cell productivities in the range of up to 5 g/L, that is, an order of magnitude higher than a decade ago (22). This level of productivity stresses the producer cells and makes them sensitive to shear forces, thus increasing the technical difficulties in cell separation. Membrane design has a limited impact on this phenomenon, even if one considers the hollow fiber as the ideal geometry, because physical forces have the most profound effect on fragile cells (i.e., the pressure drop and cross-flow rate, and related hardware). However, although tangential flow microfiltration can deal with product feed streams featuring a high particle load, often causing pore plugging early on, resulting in a decline in productivity. Smaller pore size membranes tend to provide the highest permeate flux once the system is in a steady state (see Section 12.2.2.2, Fig. 12.7), which in some cases persuades manufacturers to use ultrafiltration membranes for particle separation (e.g., a 500,000 NMWC ultrafiltration membrane in a hollow-fiber design was shown to be highly efficient for harvesting E. coli cells, even though the pore size is significantly smaller than the cell). Empirical data suggest cell harvesting is most efficient with small pore size microfiltration membranes or open pore size ultrafiltration membranes. The cartridge path length is defined by the concentration of particulates (cells, cell debris) in the feed solution, with shorter path lengths more suited to higher particle load. For cell harvesting, a cartridge length of 30 or 60 cm typically works better than a cartridge length of 110 cm, because the longer cartridge requires higher inlet pressure due to increased dynamic friction along the membrane at a given flow rate. The diameter chosen for hollow fibers depends on the nature of the feed stream, with those of 0.75–1.0 mm i.d. best for the typical low to moderate viscosity feed streams, and wider lumens better for more viscous feed streams. In each case, the TMP should be kept below 1.0 bar.

In cell clarification operations the target product is separated from the cells or lysate and recovered. The efficiency of cell recovery is influenced by the cell concentration and the permeate flux, which is in turn affected by the TMP and the temperature. The efficiency of cell recovery is typically around 70–90% for most cell types, but can be as high as 95% for highly efficient retrograde clarifiers. The main disadvantage of TFF is the relatively high energy consumption, which is primarily due to the need for high TMP to achieve the desired permeate flux. TFF is also more expensive than other clarification methods, such as centrifugation, due to the high cost of the membranes and the need for continuous operation.

TABLE 12.11 Filter Storage

<table>
<thead>
<tr>
<th>Agent</th>
<th>Chemicals</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caustic solution</td>
<td>NaOH</td>
<td>Typical range is 0.1–0.5 N</td>
</tr>
<tr>
<td>Alcohol</td>
<td>Ethanol, benzylalcohol</td>
<td>20–25% v/v, may cause swelling of membrane</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1–2% v/v, superior bacteriostatic properties compared to ethanol</td>
</tr>
<tr>
<td>Azide</td>
<td>NaN₃</td>
<td>Toxic</td>
</tr>
</tbody>
</table>
Figure 12.33  Cell clarification: recovery of recombinant IgG from CHO cell culture process design: open channel hollow fiber, shorter path length; microfiltration pore sizes to promote passage of large molecules; permeate flow control to promote protein passage at low inlet pressures. [Cartridge, CFP-4-E-4X2MA, GE Healthcare; system design, single hollow fiber cartridge, QuixStand, GE Healthcare; membrane area, 850 cm² (0.9 ft²); pore size, 0.45-μm microfiltration membrane; flow path, 1.0-mm i.d. fibers, 60-cm path length; process, 20× concentration, cell viability 20%; permeate flow control, 30 Lmh, 50 Lmh.]

<table>
<thead>
<tr>
<th>TABLE 12.12 Selection Guidelines for Tangential Flow Microfiltration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Factor</strong></td>
</tr>
<tr>
<td>Membrane pore size</td>
</tr>
<tr>
<td>Cartridge path length</td>
</tr>
<tr>
<td>Fiber lumen diameter</td>
</tr>
</tbody>
</table>

**Process Conditions**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recirculation flow rate</td>
<td>2000–4000 s⁻¹ for shear-sensitive feed streams, such as mammalian cells and large viruses 6000–8000 s⁻¹ for yeast cultures due to high viscosity 8000–16,000 s⁻¹ for bacterial cells, lysates, and most proteins</td>
</tr>
<tr>
<td>TMP</td>
<td>Low to moderate TMP (&lt;1 bar/15 psi) Low TMP (&lt;0.7 bar/10 psi) Permeate flow control recommended</td>
</tr>
<tr>
<td>Process temperature</td>
<td>20–25°C if process components are stable, otherwise 4–12°C, but flux will be reduced at lower temperatures</td>
</tr>
</tbody>
</table>
of the process therefore depends on the passage and permeation of the target product. Open pore size microfiltration membranes (pore size, 0.2–0.8 μm) are superior for the recovery of recombinant proteins as well as virus vaccines and plasmids. As a general guideline, a membrane pore size at least ten times larger than the target product is recommended. The rationale for selecting cartridge path length and fiber lumen diameter is identical to cell harvest considerations. Owing to the large pore sizes, transmembrane pressure has to be carefully controlled. For optimal control and to keep the TMP low (∼<0.7 bar), the permeate flow should also be limited and controlled, either by pump or valve (see Section 12.4.1, Fig. 12.9). Typical values for permeate flow control are in the range of 30–50 Lmh for clarification of recombinant proteins from mammalian cell culture with intact cells (Fig. 12.33), and ∼10 Lmh for the clarification of enzymes from a bacterial lysate.

As stated above, particles within the feed stream can interfere with product filtration, and fine particles may even coat the membrane surface, creating a secondary rejection layer. To minimize these problems, diafiltration of the feed stream should be incorporated into the operation, optimally while there is still a good permeate flow rate. Table 12.12 summarizes recommendations for cell harvesting and clarification.

### 12.4.4.2 TFF Ultrafiltration

Tangential flow ultrafiltration is best carried out with screen spacer devices or small diameter hollow fibers with a longer flow path (up to 110 cm) (19). Target products in biopharmaceutical ultrafiltration processes include soluble molecules such as recombinant proteins or plasmids, but also 20–200 nm viruses that act like proteins in solution. The depyrogenation of water and buffers is another important application of tangential flow ultrafiltration. The following cut-off ranges provide an approximate guide on which to base the selection of ultrafiltration membranes:

- Viruses retained by 300,000 and 500,000 NMWC UF membranes
- Plasmids retained by 300,000 NMWC UF membranes
- Monoclonal antibodies retained by 50,000 NMWC UF membranes
- Enzymes retained by 10,000 NMWC UF membranes
- Certain plasmids retained by 1000 and 3000 NMWC UF membranes
- Depyrogenation achieved with 1000 and 3000 NMWC UF membranes.

The dominant application of ultrafiltration technology in bioprocessing is the concentration and diafiltration of proteins prior to column chromatography, and diafiltration for formulation of the bulk product. The following example describes the concentration of a monoclonal antibody in a study design to compare different cross-flow geometries and ultrafiltration pore sizes. The comparison includes an evaluation of flux versus TMP to identify the optimal membrane design for rapid antibody concentration. Both cassettes and hollow-fiber modules were investigated, including two different pore sizes for the cassettes.

The data presented in Figure 12.34 show that flat-sheet cassettes offered higher flux rates in this investigation compared with hollow fibers. The screen spacer geometry of the cassette format provided a two- to threefold higher flux, with the 50,000 NMWC membrane performing best overall. After repeated use in antibody concentration, the hollow-fiber membranes were generally at ∼30 Lmh, whereas flat sheet cassettes were generally at 50–60 Lmh.

Another application of ultrafiltration technology is the purification of virus vaccines. As an alternative to sucrose or CsCl gradient centrifugation, ultrafiltration has been established in the purification of influenza vaccines for the removal of contaminating proteins (ovalbumin). Influenza virus is

![Figure 12.34](image-url)
an 80–120-nm enveloped virus, and shear stress is a major concern in the design of a purification process. The need for an aseptic process required a closed system that could be autoclaved prior to operation. The example shown in Figure 12.35 presents an alternative to buoyancy gradient centrifugation techniques using open pore size ultrafiltration membranes (750,000 NMWC). The process design focused on the removal of ovalbumin as the lead contaminating protein from virus propagated in allantoic fluid. With an emphasis on minimizing shear stress, a cartridge with a short path length (30 cm) and a large surface area was used, with very low pressures (0.05 bar TMP) and minimal recirculation flow rates. The productivity was therefore on the lower side (20 Lmh), but provided a quantitative recovery of virus, and complete removal of contaminating protein. This process was successfully scaled up 20-fold (10 L) from 420 cm² to 1 m², maintaining all process variables with the same pressure profile and process description using hollow fibers of identical pore size, path length, and fiber diameter.

12.5 VIRUS FILTRATION

When protein therapeutics are produced in animal cell lines, the risk of contamination with viruses from the cell line or the culture medium (endogeneous and adventitious viruses) needs to be considered and mitigated (24). As a consequence, the manufacturing process of products from mammalian cell culture has to be designed with an in-built capability to remove viral contaminants (25, 26). The ICH Q5A guidelines (27) require at least two dedicated virus clearance steps with orthogonal mechanisms (i.e., clearance steps based on different separative principles), and the inclusion of polishing steps that remove viruses as well as product-related and process impurities is also favored (27–29). Virus clearance steps in an orthogonal safety concept can be of two types: virus inactivation and virus removal. Whereas the goal of virus inactivation is the irreversible loss of viral infectivity, the goal of virus removal is the (mechanical) reduction of the number of virus particles, expressed as a log reduction value (LRV), the log₁₀ ratio of the input virus load and the output virus load (30). The inclusion of four LRVs is considered to be a robust removal step, and the overall reduction factor for a complete manufacturing process is the sum of the log reduction factors of the individual steps.

Low pH inactivation and 20-nm filtration are the most common representatives of the two categories. For products that are unstable at low pH, other inactivation technologies such as solvent–detergent treatment or UV irradiation are alternatively available (31–33).
The primary approach to virus removal is dead-end filtration, involving the size-based removal of virus particles by passage through a membrane with a small pore size. Filters are available in two categories based on their pore size ratings—retroviral (<50 nm) and parvoviral (<20 nm) (34)—and the industry is now favoring the customarily use of parvoviral filters to meet the higher regulatory standards that have been brought into force (35). Parvoviral filters typically require a larger surface area than other filters because the small pore size results in frequent clogging, even if there are only low levels of particulates and aggregates in the process stream. The virus filtration step is therefore typically placed after at least one of the polishing chromatography steps. The choice is usually based on product stream volume considerations as well as the volume that can be feasibly filtered for that process intermediate (36).

**12.5.1 Principles of Virus Removal**

Each virus removal step must be tested with viruses representative of those most likely to be found in the process under development (37, 38). This involves spiking studies that deliberately contaminate a process stream with viruses relevant to that process, or model viruses with similar physical and chemical properties (28). For example, rodent cell lines often contain endogenous retroviral particles, so processes involving rodent cell lines must undergo spiking studies with an accepted model such as xenotropic murine leukemia virus (X-MuLV). Although it is never possible to test for every conceivable contaminant, it is acceptable to use a panel of model viruses with a broad spectrum of properties (different sizes, pI values, enveloped and nonenveloped, resistance to inactivation) so that the process is challenged to clear any virus likely to be found. Using rodent cell lines again as an example, a panel of four diverse viruses is considered adequate for most spiking studies (Table 12.13). Any process that is capable of clearing all four of these viruses stands a good chance of being able to clear any known virus.

The virus removal step must also be characterized for its mechanical performance when challenged with a contaminated feed stream. Filter quality depends on the materials used in manufacture (generally CA, PES, or PVDF membranes), pore distribution, the physical structure, and its ability to maintain pore size during filtration. An important determinant of filter capacity is the flow decay, which is the rate at which the flow rate through the filter declines as a function of time. Ideally, the flow rate should remain unchanged under constant pressure if the feed stream is uncontaminated, but if contaminants such as aggregates or particles are present, flow decay occurs as the particles accumulate on the filter surface, gradually plugging the pores and impeding the movement of the filtrate. The $V_{max}$ value is the maximum volume of feed that will pass through the filter before it becomes completely clogged and the flow rate falls to zero, so $V_{max}$ is inversely proportional to the particle load in the feed stream. In spiking studies, $V_{max}$ can be determined for known viral loads, allowing the viral load in actual process streams to be calculated by interpolation. Recent studies have shown that an additional consequence of flow decay is the tendency for smaller viruses to pass through the filter, particularly at high loads (39, 40). It has been suggested that filter performance could be tested with phages such as PP7 and φX174 acting as models for small mammalian viruses.

The filtrate from the virus clearance step must be tested to determine virus titer, which allows the efficiency of the operation to be calculated. This tends to be done either by a plaque assay or a 50% tissue culture infectious dose (TCID$_{50}$) assay. The plaque assay is a quantiative test in which each virus is identified by its ability to form a clear area (plaque) on a plate of cells. Samples are serially diluted until the point at which adding the sample to the plates results in only 50% becoming infected. Unlike the plaque assay, viruses in the TCID$_{50}$ assay do not need to kill the cells, but only need to have a noticeable effect on cell morphology. Both assays are sensitive to variation (e.g., in culture conditions or properties of the cells) so should be carefully controlled and repeated to allow statistical analysis of the results. Variation also arises because of the high dilution factors used, which means a nonrandom distribution of virus particles in a diluted sample can lead to false negative results. If only a portion of the sample is tested and the test is negative, the amount of virus that would have to be present in the total sample to achieve a positive result with 95% confidence should be calculated and taken into consideration when calculating a reduction factor.

**12.5.2 Modes of Operation**

**12.5.2.1 Dead-End Filtration** Dead-end virus filtration is typically performed using a conventional cartridge design as in microfiltration applications. Filter pore sizes in the range 20–50 nm utilize the classical depth structures that are typical for commercial microfiltration membranes,
although available products differ in the geometry of the membrane as well as number of membrane layers, both of which have a direct influence on LRV. Tighter pore sizes become difficult to develop and use in practice because the membrane back pressure significantly exceeds 0.3 MPa. Furthermore, the filtrate flux declines as the depth structures are blocked by aggregates and small particles. Therefore, virus filtration is preferably carried out using a 0.1-m m prefiltered feed stream. Typical harmful small viruses in the 20-nm range are the most difficult to remove from large protein molecules such as antibodies because the apparent molecular weight and the effective size and shape of the protein molecules may come close to the size of the virus particles. However, size exclusion based 20-nm filtration is nevertheless a robust step for the removal of most viral contamination and it is now required for the production of almost all therapeutic proteins using mammalian cells. The impressive LRVs for four standard model viruses that can be achieved using 20-nm filtration are summarized in Table 12.14 (41).

12.5.2 TFF
TFF ultrafiltration offers an extensive range of different cut-offs for virus removal, limited mainly by the effective size of the target protein because the protein must pass through the membrane quantitatively while the virus is retained. A maximum MWCO of about 300 kDa will allow the passage of most proteins (including antibodies) while virus particles >40 nm in size are retained. However, the removal of a virus <40 nm in size is challenging, because this can be achieved only at MWCO values of ≤200 kDa. Even so, if the ultrafiltration membrane is chosen well, even proteins with a molecular weight larger than the nominal MWCO of the membrane can be purified, because the molecular weight of a protein need not be directly related to its geometry or size (Stoke’s radius), which are influenced by the buffer choice and environmental variables. Therefore, under the appropriate conditions, even antibodies with a molecular weight of 150–180 kDa can be filtered through a nominal 100 kDa membrane. Furthermore, the MWCO of a filter is not an absolute measure; that is, membrane manufacturers may use different tests and test molecules to determine and nominate this value. Accordingly, membranes with the same nominal MWCO from different suppliers may result in significantly different separation results.

Compared to static virus filters, TFF has two disadvantages: there is no state-of-the-art integrity test available and TFF ultrafiltration has to be performed as an individual unit operation requiring dedicated and expensive equipment. Therefore, with the increasing availability of static filters for virus removal, TFF no longer plays a significant role in virus filtration.

12.5.3 Devices and Systems
Only a small number of commercial parvo virus filtration products claim to feature all properties for the robust removal of small, nonenveloped viruses. Planova filters (Asahi-Kasai) are hollow fibers, whereas DV 20 (Pall), Virosolve NF (Millipore), and Virosart CPV (Sartorius Stedim Biotech) are pleated membranes, and all are supplied in a cartridge or capsule that can be placed in-line anywhere in the downstream process, although a position towards the end of the purification train is recommended to optimize the productivity of this expensive step. Another important recommendation is to prefilter the feed stream at 0.1–0.2 μm to prevent fouling.

A comparison of the four commercially available filters described above with 20-nm filters showed that all could remove murine minute virus particles effectively (Table 12.15), although a constant pressure of 30 psi was used for Virosolve and Virosart filters and 14.5 psi or relatively low pressure was used for Planova and DV20 filters. Although no significant flux decay was observed for Planova and DV20, they were designed for a low load capacity and the filtration time was longer, resulting in low productivity. In this comparison Virosolve demonstrated virus break-through at higher flow decay, a phenomenon that was found to be clearly dependent on the quality of both virus spike and product feed stream (30).

### Table 12.14 Virus Removal with NFP 20-nm Filtration

<table>
<thead>
<tr>
<th>Process Capacity</th>
<th>MMV Clearance and Flux Decay</th>
<th>Reo 3 Clearance and Flux Decay</th>
<th>X-MuLV Clearance and Flux Decay</th>
<th>PRV Clearance and Flux Decay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacity, L/m²</td>
<td>LRV %</td>
<td>LRV %</td>
<td>LRV %</td>
<td>LRV %</td>
</tr>
<tr>
<td>420</td>
<td>&gt;5.92 49.3</td>
<td>&gt;4.52 56</td>
<td>≥4.87 79</td>
<td>&gt;4.43 47</td>
</tr>
<tr>
<td>506</td>
<td>5.92 26.1</td>
<td>≥5.22 35</td>
<td>≥4.87 54</td>
<td>≥5.04 60</td>
</tr>
</tbody>
</table>

### Table 12.15 MMV Clearance with 20-nm Filters

<table>
<thead>
<tr>
<th>Filter</th>
<th>Loading, L/m²</th>
<th>Flux Decay, %</th>
<th>Virus Spike, %</th>
<th>LRV</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFP</td>
<td>246.57</td>
<td>93</td>
<td>0.05</td>
<td>3.52</td>
</tr>
<tr>
<td>Virosmart</td>
<td>374.00</td>
<td>95</td>
<td>0.05</td>
<td>7.27</td>
</tr>
<tr>
<td>DV20</td>
<td>82.81</td>
<td>23</td>
<td>0.5</td>
<td>&gt;7.77</td>
</tr>
<tr>
<td>Planova</td>
<td>100.00</td>
<td>22</td>
<td>0.5</td>
<td>6.98</td>
</tr>
</tbody>
</table>
12.6 MEMBRANE ADSORPTION

The separation of proteins using a charged ultrafiltration membrane was first reported by Nakao and colleagues (42). They achieved the separation of myoglobin and cytochrome c by setting the buffer pH near the pI of one of the proteins, allowing it to permeate the membrane, and thereby rejecting by electrostatic repulsion the protein that carried a charge like the membrane surface. The addition of positive or negative charges to a membrane created a new separation technology that spanned the boundary between filtration and ion-exchange chromatography. Molecules were no longer separated solely by their size or molecular weight, but by a combination of these properties and their charge. When the membrane surface contains a net charge, the sieving coefficient of proteins that also have a net charge is altered according to whether the charges are alike or different (43). Protein fractionation is enhanced by using a highly charged membrane, low ionic strength solutions, and a pH that causes one protein to be highly charged compared to another protein. Broadly speaking, the sieving coefficient is greatest when the buffer pH is near the isoelectric point (pI) of the protein, and when the salt concentration is elevated sufficiently to shield the charges on the protein and membrane. When the protein and membrane charges are alike, then electrostatic repulsion reduces the sieving coefficient to a value lower than would be the case in the absence of electrostatic interactions. When the charges are different, then the charged proteins adsorb to the oppositely charged membrane and form a dynamic membrane that has the same charge as the protein molecules in solution. This also causes electrostatic repulsion and a decrease in the sieving coefficient. The effect of these additional factors on the mathematical principles of separation have been comprehensively discussed (13).

Since the first description of charged filtration membranes, the concept of membrane chromatography has evolved (44–49). Membrane adsorbers have been developed with a wide range of surface chemistries that combine filtration not only with charge-based separation but also with other forms of chromatography such as hydrophobic interaction chromatography and affinity chromatography. Several vendors offer disposable modules that cover a wide range of requirements in the downstream processing of protein pharmaceuticals (see next section). Just as the addition of functional groups can enhance the resolving power of membrane adsorbers compared to standard filters, certain properties of membrane adsorbers also provide advantages over packed-bed resins for a number of chromatography applications. Although column chromatography is still preferred for bind-and-elute steps because of the superior dynamic binding capacity of resins, membrane adsorbers are becoming increasingly popular for flow-through operations (50). Anion-exchange chromatography is one such application where membrane adsorbers are beginning to replace resins. Anion-exchange resins are often used in flow-through mode during the polishing of biopharmaceuticals such as antibodies because the resins bind to nucleic acids, many host cell proteins, and most viruses. Viruses and nucleic acids in particular are large molecules that do not readily diffuse into the pores of traditional chromatography resins because the transport of solutes to their binding sites relies mainly on pore diffusion (Fig. 12.36a). This causes mass transfer resistance and lowers the column efficiency, so longer residence times are required for these larger molecules to find binding ligands, which is achieved

![Figure 12.36](image_url)
by using a greater column bed height and/or by reducing the linear flow rate. Therefore, to keep up with process demand, most traditional polishing steps operate at a flow rate between 100 and 150 cm/h and use oversized columns to accommodate the necessary throughput.

In contrast, membrane adsorbers are synthetic micro-porous or macroporous membranes stacked 10–15 layers deep in a comparatively small cartridge, so the footprint of such devices is much smaller than columns with a similar output. The transport of solutes to their binding sites occurs mainly by convection, while pore diffusion is minimal (Fig. 12.36b). Because of these hydrodynamic benefits, membrane adsorbers can operate at much greater flow rates than columns, considerably reducing buffer consumption and shortening the overall process time to ≏1% of the typical length used with columns (51). The use of membrane adsorbers can be viewed as the equivalent of shortening traditional columns to near zero length, allowing large scale processes to run with only a small pressure drop at very high flow rates.

For example, polishing with an anion-exchange membrane can be conducted with a bed height of 4 mm at flow rates of more than 600 cm/h, providing a high frontal surface area to bed height ratio (Fig. 12.37), and small volume disposable membrane chromatography devices can now handle up to 50 L/min/bar/m². Even at these high flow rates, the membrane pores provide adequate binding capacity for large biomolecules such as viruses and DNA, so they can play an important role in the overall viral clearance strategy for antibody purification (52). Traditionally, the performance of strong anion-exchange chromatography has been variable for the removal of viruses with slightly acidic pl values in high salt buffers, but this has been addressed by the development of membrane adsorbers with alternative ligands (agmatine, tris-2-aminoethylamine, polyhexamethylene biguanide, and polyethyleneimine) that can bind viruses such as bacteriophage ΦX174 and achieve LRVs of >5, even in 150 mM NaCl, compared to 0 LRVs for traditional Q ligands.

In conventional chromatography, the problem of mass transfer resistance has been addressed by perfusion chromatography featuring specifically designed beads, where the mobile phase gains access the core of the chromatographic bead by flow-through pores allowing linear flow rates beyond 1000 cm/h. However, the typical pore sizes of adsorber membranes are in the range 0.2–3.0 μm, that is, about two orders of magnitude above those of gel beads. Water values may indicate pore sizes of up to 5 μm, but optically pore sizes even up to 10 μm can be detected. These transport phenomena have been studied in detail using confocal microscopy, showing that a diffusion-controlled process such as packed-bed chromatography requires several hours for the complete penetration of beads by a protein under static conditions, far exceeding the rate of convective transport seen in membranes. The lower dynamic binding capacity of membrane adsorbers has also been addressed more recently, so that they are becoming viable for bind-and-elute as well as flow-through operations. Grafting polymer chains to the membrane surface in order to increase the dynamic binding capacity affects the diffusion rate only marginally. Flow rates can be kept high with no significant loss in resolution, while simultaneously maintaining a constant dynamic binding capacity. Recent data have shown that the protein binding capacity of new membrane chromatographic media have increased to the levels comparable to typical chromatography resins. At a given membrane thickness of ~275 μm (Sartobind membrane), 1 mL volume of the membrane adsorber is calculated to be equivalent to a total surface of 36.4 cm². Even so, large scale membrane adsorbers remain challenging as it is important to realize an even flow distribution for a very large flow area in order to utilize the accessible surface. It is finally the lower surface-to-bed volume ratio that limits the dynamic binding capacities of membranes compared to beads (53, 54).

The use of disposable membrane devices also results in the complete elimination of cleaning and validation, which provides clear operational advantages just as it does in the case of disposable filters. FDA regulations require the cleaning, maintenance, and sanitization of fixed equipment and piping at appropriate intervals to prevent malfunctions and contamination, but this is unnecessary when exhausted modules can be replaced as necessary.

### 12.6.1 Devices and Systems

Membrane adsorbers are manufactured in various formats by several manufacturers, with Sartobind (Sartorius AG) and
Mustang (Pall Corp.) widely used in an industrial context. The typical principle of a membrane adsorber device includes a stack of membranes in a disposable cartridge, optimized for process speed, throughput, binding, and/or resolution. This design has been successfully realized for the Sartobind Adsorber (Sartorius AG), ranging from 15 (4 mm), 30 (8 mm) to 60 layers (16 mm bed height), and the Mustang capsules, containing 16 layers of membrane. Figure 12.38 shows the flow scheme within a membrane cartridge suitable for pilot scale applications.

System requirements for membrane adsorbers differ from those applied to traditional packed-bed columns. The flow rate for a membrane adsorber is 10–100 times higher if measured in column volumes per unit time (CV/h). Membrane adsorbers can also withstand high operating pressures (up to 4 bar) so they are contained within robust and durable polymer casings (single use) or stainless steel housings (multiple use) (Fig. 12.39). Although both formats are available, today the reusable concept plays only a minor role in industrial applications.

### 12.6.2 Process Development and Optimization

Membrane chromatography process development is similar to that applied to traditional chromatography, but it is essential to choose the correct platform for each application. Generally, membrane adsorbers achieve their full potential when the requirement is for rapid and efficient processing of large volumes of feedstock, as in contaminant removal during polishing (scavenger applications), which is now a mainstream application (52). However, given their particular

![Figure 12.38](image1.png)  
**Figure 12.38** Flow scheme in a multilayer design membrane adsorber, operated in a housing.

![Figure 12.39](image2.png)  
**Figure 12.39** Process-scale membrane adsorber cartridges. (a) Membrane adsorber cartridges designed for multiple use. Cartridges (right) and core modules (left) of the Sartobind MultiSep membrane chromatography system (Sartorius Stedim Biotech). (b) Single-use membrane adsorber capsule (5 L) as a ready-to-use stand-alone unit for operating pressures of up to 0.4 MPa (4 bar, 58 psi) (Sartobind, Sartorius Stedim Biotech).
efficiency for binding large molecules and particles (e.g., DNA and viruses), they have been used in bind-and-elute mode for the large-scale purification of oligonucleotides (55), plasmids (56–58), and viruses used as gene therapy vectors and vaccines (59, 60).

12.6.3 Applications

For use in bind-and-elute steps of molecules up to the size of monoclonal antibodies, the dynamic binding capacity of membranes needs to be improved, and this may include research to identify potential new affinity ligands, to find a way to immobilize these ligands on the membrane, to maximize binding and minimize leakage. Main requirements are a minimal bed volume with minimal membrane dispersion, even flow distribution (plug flow characteristics), and uniform ligand capacity for all flow paths. These attributes require appropriately designed membrane morphologies and dedicated surface chemistries, while still providing high flux at low pressure. Alternative matrix formats to provide a large initial surface are based on hollow-fiber technology and monolith structures.

The advantages of membrane adsorbers (high throughput, ability to bind large molecules, and disposable format) combined with their moderate binding capacity makes them ideal for the removal of trace contaminants in the downstream processing of antibodies and other biopharmaceuticals. Anion-exchange membranes in particular are now widely used for large scale biopharmaceutical manufacture in a trend towards the increasing use of disposable options (50). As well as saving on cleaning and validation costs, disposable membranes also save time and provide flexibility, and can form part of an orthogonal strategy for virus removal (49).

Process development is streamlined and expedited because different modules can be tested in various combinations to arrive quickly at the best overall set of process options, and the absence of cleaning and validation requirements can shorten the time required to develop a finalized process by months or years. The ability to replace each module completely also makes it easier to assemble process trains for new products in existing premises without worrying about cross-contamination. The flexibility is most noticeable during scale-up, because the membrane adsorbers are modular and available in a number of different sizes, which show linear scale-up for important parameters such as frontal surface area, bed volume, flow rate, and static binding capacity, while normalized dynamic capacity remains fairly constant at 10% or complete breakthrough (Fig. 12.40). A performance comparison between Sartobind Q (Sartorius AG) and Q Sepharose FF (GE Healthcare) is presented in Table 12.16.

In the initial experiment the DNA was shown to bind to Sartobind Q in physiological buffer (Fig. 12.41). The dynamic binding capacity (dBC) was not influenced over a broad range

![Figure 12.40 Dynamic binding capacities of SingleSep Q membrane chromatography devices represented by breakthrough values as percentage of total load (C/C0) against membrane volume (mL). Individual curves represent selected lots of different sized devices ranging from nano to 300. Data from Sartorius-Stedim Biotech.](image)

**TABLE 12.16 Adsorption of DNA, Process Parameters**

| Membrane adsorber: Sartobind Q 100 (Sartorius AG) | Area: 0.01 m² |
| Beaded matrix: Q Sepharose FF (GE Healthcare) | Volume: 8.6 mL (D = 1 cm, h = 11 cm) |
| Basic buffer: 10 mM NaH₂PO₄ | 10 mM Na₂HPO₄ |
| 20 mM NaCl | pH 7.25, 25 mS/cm |
| DNA (salmon sperm): 50 µg/mL | 25 µg/mL |
| Recombinant protein: M₀ = 80 kDa, IEP = 5.0–5.5 |
of conductivity (0.45–25 mS/cm). An increase in flow rate of 70% (from 30 mL/min to 50 mL/min for 0.01 m²) reduced the dBC by ~10%. The dBC was influenced by the DNA concentration in the load; it dropped by ~25% when doubling the DNA load from 25 to 50 mg/mL. In a comparison with Q Sepharose FF resin, both matrices were challenged using a solution of a purified recombinant protein (80 kDa, IEP 5.0–5.5) of biopharmaceutical quality. The dBC of the membrane adsorber for DNA was only marginally influenced by the protein.

The dBC of the resin was influenced by the flow rate. When increasing the linear flow rate from 45 to 100 cm/h, the dBC dropped by ~20%, from 726 to 581 mg/mL (Table 12.17).

Based on these experimental data and taking a conservative approach, the dBC for DNA in a phosphate buffer at pH 7.25, 25 mS/cm on the membrane adsorber is in the range of 125 mg DNA/cm. As 1 mL of the Sartobind membrane equals a membrane area of 36.4 cm², having a thickness of 275 μm, the respective volumetric dBC is calculated to be 5023 mg DNA/mL of the membrane adsorber. For the beaded matrix at relevant flow rates the dBC is in the range of 500 mg DNA/mL. Accordingly, 1 mL of beaded matrix is approximately equivalent to about 4 cm² of membrane. How does this relate to process design?

The position of anion-exchange chromatography for DNA removal is far downstream, that is, where DNA is a residual trace impurity. The DNA contamination of a crude product derived from mammalian cell culture after cell separation is typically in the range of 20–50 mg/L; that is, at a 1000 L fermentation scale the total contamination is 20–50 g DNA.

![Figure 12.41](image-url)

**Figure 12.41** Membrane adsorber as DNA scavenger. [Filtration of a DNA spiked protein (0.63 mg/mL, DNA concentration 25 μg/mL) in phosphate buffer pH 7.25, c = 25 mS/cm, f = 30 mL/min through a membrane adsorber with anion exchange functionality (Sartobind Q 100, 0.01 m²). The DNA breakthrough at 624 mL refers to a dynamic binding capacity of 156 μg DNA/cm².]

### Table 12.17 Adsorption of DNA to Anion Exchange Matrices

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Phosphate Buffer pH 7.25</th>
<th>DNA, μg/mL</th>
<th>Protein, mg/mL</th>
<th>Dynamic Binding Capacity, μg DNA/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sartobind Q 100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basic experiment</td>
<td>c = 0.45 mS/cm, f = 30 mL/min</td>
<td>50</td>
<td></td>
<td>5023</td>
</tr>
<tr>
<td>Increased conductivity</td>
<td>c = 25 mS/cm, f = 30 mL/min</td>
<td>50</td>
<td></td>
<td>4987</td>
</tr>
<tr>
<td>Decreased DNA concentration</td>
<td>c = 25 mS/cm, f = 30 mL/min</td>
<td>25</td>
<td></td>
<td>6115</td>
</tr>
<tr>
<td>Increased flow rate</td>
<td>c = 25 mS/cm, f = 50 mL/min</td>
<td>50</td>
<td></td>
<td>4550</td>
</tr>
<tr>
<td>Protein load</td>
<td>c = 25 mS/cm, f = 50 mL/min</td>
<td>25</td>
<td>0.63</td>
<td>5678</td>
</tr>
<tr>
<td>Q Sepharose FF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basic experiment</td>
<td>c = 25 mS/cm, f_{lin} = 45 cm/h</td>
<td>50</td>
<td>0.63</td>
<td>726</td>
</tr>
<tr>
<td>Increased flow rate</td>
<td>c = 25 mS/cm, f_{lin} = 100 cm/h</td>
<td>50</td>
<td>0.63</td>
<td>581</td>
</tr>
</tbody>
</table>

All values for the dynamic binding capacities presented in volumetric units. 1 mL of the Sartobind Q 100 equals a membrane area of 36.4 cm², having a thickness of 275 μm.
TABLE 12.18 Application of Membrane Adsorbers and Bead Matrices as DNA Scavengers

| Advantages of Membrane Adsorbers | Cost benefit with standard equipment when a limited matrix volume (area) is required (negative mode of chromatography) | Easy set-up and handling, superior when used as a disposable technology, no risk of matrix collapse, channeling, or air trapping |

Advantages of Bead Matrices

- Cost benefit with standard equipment when a large matrix volume (area) is required (positive mode of chromatography)
- With increasing lifetime, the cost and time savings regarding set-up of equipment become negligible.

In a negative mode of chromatography (flow-through of product) the removal of total DNA would require 40–100 L of Q Sepharose FF or 15–40 m² of Sartobind Q. At the stage of final purification of a protein, that is, after one to three chromatography steps in the downstream process, the residual DNA is typically below 200 pg/mg of protein. Assuming a cell productivity of ~1 g/L, contaminated with ~200 mg of residual host DNA (at an assumed product concentration of 10 mg/mL at the stage of final purification, the processing volume would be about 50 L). Hence, the excessive removal of residual traces of DNA at a 1000 L fermentation scale would require 0.5 L of Q Sepharose FF or 0.16 m² of Sartobind Q to process 50 L of product fluid.

At a membrane flux of 50 mL/min, 0.01 m², the required process time for the membrane adsorber would be ~1 h, for the beaded matrix at 100 cm/h it would be ~10 h. Accordingly, the column volume would need to be adjusted to 5 L to match the membrane area for process time.

Table 12.18 summarizes the advantages both for membrane adsorbers and beaded matrices based on this simple and general calculation. However, respective details have to be elaborated for designated individual process requirements.

12.7 REFERENCES


13

REFOLDING OF INCLUSION BODY PROTEINS FROM E. COLI

ZHIGUO SU
State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100080, China
DIANNAN LU AND ZHENG LIU
Department of Chemical Engineering, Tsinghua University, Beijing 100084, China

13.1 INTRODUCTION
Recombinant DNA technology enables the production of valuable foreign proteins at high expression rates and high concentrations. Escherichia coli is one of the most widely used host organisms, because its genome is well studied and it can easily be cultured on a large scale with high efficiency. However, expression of proteins in E. coli often leads to the formation of dense insoluble particles, known as inclusion bodies (IBs), containing most of the recombinant proteins in incorrect steric structures without the desired biological activities. To obtain their native activity, the IB proteins have to be solubilized and refolded to the native structure. The process involves disruption of the E. coli to release IBs, followed by separation of the IBs through centrifugation on the basis of density differences (1). The purified IBs are then dissolved in strong denaturing solutions such as 8 M urea or 6 M guanidine hydrochloride (2). The final step, the most important one, is to remove the denaturant, either urea or guanidine, away from the protein molecule to enable its self-refolding. It is this step that is termed protein refolding.

In early reports, direct dilution of unfolded proteins into the refolding buffer was often applied, and this remains the most...
widely used method for protein refolding at different scales of operation. However, large volumes of dilute protein solution are cumbersome to handle in the subsequent purification steps. Therefore, efforts have been made to develop methods for folding proteins at high concentrations without significant aggregation, as detailed in recent reviews (2–5).

This treatise starts with a summary of the chemistry and physics of protein folding in vivo, which is helpful for understanding protein refolding in vitro. The molecular simulation of protein refolding in vitro was introduced as an effective tool to probe molecular insight into protein folding and aggregation. Examples are given of chromatographic refolding techniques, a group of newly developed methods with proven effectiveness and convenience from refolding at different scales.

13.2 PROTEIN FOLDING IN VIVO

In vivo folding of a wild-type protein in a cell, when carried out in the correct cellular environment, is usually a highly efficient process in which >95% of the newly synthesized polypeptides eventually attain their native three-dimensional structure. Polypeptide misfolding and aggregation, which are major problems in protein refolding in vitro, rarely occur in in vivo processes, except in the production of mutant proteins or protein production at elevated temperatures (6).

The E. coli cytoplasm contains a wide variety of different biomolecules such as lipids, nucleic acids, cytoskeletal components, and other macromolecules, which are sometimes collectively termed crowding agents. Although many molecular details of the interaction between the protein undergoing folding and the crowding agents remain unknown, entropic stabilization of the folded state in the presence of crowding agents has been confirmed (7–9). It has also been demonstrated that the folding rate increases nonmonotonically in response to crowding agents. Thus, an optimal concentration of crowding agents or confinement size exists at which maximum folding yield is obtained (10).

In nature, many newly synthesized proteins require complex cellular constituents known as molecular chaperones, together with metabolic energy input, to attain their native states (11). These chaperones inhibit the generation of non-native conformations and aggregates. There are two distinct mechanisms by which molecular chaperones assist de novo folding. The first involves chaperones such as trigger factors (TF) and Hsp70s, which stabilize nascent and newly synthesized chains and prevent aggregation by forming reversible complexes until the unfolded chains have folded properly (12–15). The second mechanism involves large cylindrical chaperonin complexes that provide physically defined compartments (confinement) in which a complete protein, or a protein domain, can fold (16). These two classes of chaperones are conserved across all three domains of life, that is, bacteria, archaea, and eukarya (6, 11, 17).

Protein disulfide isomerase (PDI) catalyzes thiol–disulfide interchange reactions and promotes protein disulfide formation, isomerization, and reduction. PDI does not determine the polypeptide’s folding pathway but facilitates the formation of native disulfide bonds by promoting the reshuffling of non-native disulfide pairings (18). Moreover, proteins with peptidyl prolyl cis–trans isomerase (PPIase) activity catalyze the otherwise slow isomerization of amino acid–proline peptide bonds and accelerate the folding of proline-containing polypeptides (19).

A quality control system is essential to protein folding in vivo, to ensure the export of native proteins from the endoplasmic reticulum (ER). This system recognizes incorrectly or partially folded proteins and captures these in the ER for subsequent refolding or degradation by the proteasome. It has been demonstrated that the calnexin/calreticulin cycle and ER-associated degradation are key elements in the quality control system, and these pave the way for incorrectly folded proteins to fold into their native states. Irregularities in the quality control system may lead to the accumulation of incorrectly folded proteins and several types of diseases (20).

In conclusion, the biochemical machinery of protein folding in vivo, which includes the crowding and confinement mechanisms, molecular chaperones and foldases, and the quality control system, has ensured that protein folding in the cell is a highly efficient process. The chemical machinery used for folding in vivo may inspire the design of de novo refolding techniques in vitro, in which the solution environment is tailored for optimal protein folding.

Although either strong or weak interactions may contribute significantly to the stability and folding kinetics (5), the major interactions underlying protein folding are electrostatic forces, hydrogen bonding, van der Waals interactions, the sum of short- and long-ranged forces among connected neighboring residues termed intrinsic propensities, and hydrophobic interactions (21). A predominant feature of globular protein structures is that nonpolar residues are sequestered into a core where they largely avoid contact with water; consequently, hydrophobic interactions are often the dominant force in protein refolding (22). On the other hand, the major force that opposes protein folding and stability arises from the loss of nonlocal conformational entropy due to the steric constraints imposed on the protein once it is in the folded state. Marginal stabilities of proteins arise from the small difference between these large driving and opposing forces (21).

Anfinsen’s classical experiments on the refolding of ribonuclease in vitro demonstrated that all the information required to determine the final conformation of the protein resides in the polypeptide chain itself (23). However, the pathways by which such polypeptides attain their native state remain unclear (24). Many physical models of folding pathways have been proposed such as the nucleation growth model (25, 26), framework model (27, 28), diffusion–collision mechanism model (29, 30), hydrophobic collapse model (31, 32), and jigsaw model (33). The free-energy
funnel model (34) states that in the early stages of folding, a protein possesses a large ensemble of structures. Folding is not to find a single route but to characterize the dynamics of the ensemble through a statistical description of the topography of the free-energy landscape. Folding is easy if the landscape resembles a multidimensional funnel leading through a myriad of pathways to the native structure (34). Onuchic and colleagues first estimated the folding funnel for a fast-folding 60-residue helical protein (34–37). The width and depth of the funnel represent the entropy and energy. This study showed the flow of the molecule through the molten globule, folding bottleneck, or transition-state ensemble and then through a glass transition region where discrete pathways exist. The free-energy folding funnel has been used to illustrate folding in the presence or absence of intermediates (38–41), folding transition states (42–45), folding in confinement (46, 47), folding and aggregation (48–51), and chaperone-assisted folding (52–54). Parallel folding pathways illustrated by the free-energy folding funnel are indicators of the kinetic competition among different folding products.

In summary, protein folding is driven by the free-energy gradient, and folding occurs such that the protein attains its thermodynamically most stable form, as described by Anfinsen (23). In reality, protein folding occurs through a myriad of pathways, as illustrated by the ragged-energy funnel; this yields not only the native conformation but also misfolded states and aggregates (34). Thus, folding is the art of establishing a kinetic partition that drives the protein toward the native conformation.

13.3 MOLECULAR SIMULATION OF PROTEIN REFOLDING AND AGGREGATION

As a tool that is complementary to experimental studies, molecular simulations offer molecular insight into protein refolding and have attracted increasing attention in recent years (55–60). Although state-of-the-art modeling techniques do not suffice for full-atom simulations of multichain protein systems on a practically relevant timescale, coarse-grained protein models are adequate for illustrating the physical events underlying folding and aggregation. Hall and colleagues used dynamic Monte Carlo simulations and two-dimensional lattice proteins to examine the effects of the changing rate of the folding buffer on aggregation in four typical folding processes (61). Lu and Liu simulated the effect of polymers on the refolding of a lattice protein and showed that a polymer of suitable chain length, concentration, and hydrophobicity can form complexes with partially folded proteins, create various intermediates, and provide enriched pathways for the protein to fold into its native conformation (52, 53). As a result, a smoothened “folding energy funnel” is established that results in improved folding yields and kinetics, as shown in Figure 13.1.

Dynamic MC simulations have shown that the polymer chain length should match its hydrophobicity, otherwise long-chain polymers may hinder protein structural transitions and short-chain polymers may hinder the formation of the hydrophobic core of the protein, leading to low refolding yields, as experimentally observed by Wang (62–64), Lin (65, 66), Cui (67, 68), and Lu (69).

Langevin dynamics simulation of an off-lattice coarse-grained β-barrel model showed that the folding of the β-barrel protein in free solution follows a two-step mechanism; that is, the protein first “hydrophobically collapses” into a partially folded state and then “structurally rearranges” into the native state. The presence of a polymer as a folding aid did not alter the abovementioned “hydrophobic collapse—structural rearrangement” mechanism but accelerated the “hydrophobic collapse” required for subsequent folding and also inhibited protein aggregate formation. However, interaction with the polymer hinders the rearrangement. When a
polymer of linearly decreasing hydrophobicity is used in the folding process, an optimized free-energy funnel for folding can be established, as shown in Figure 13.2c. In this case, the two-energy barrier that existed in the absence of the polymer (Fig. 13.2a) disappears; that is, the energy funnel is smoothened, which favors rapid folding of the protein to the native conformation characterized by a global free-energy minimum (70, 71). Experimentally, lysozyme has been refolded using temperature-responsive polymers, such as DGP (72), PNIPAAm (69), and PIPTB (70), at lower temperatures where the polymer is less hydrophobic. This results in improved folding yield and kinetics, as predicted by molecular simulations. In conclusion, optimal folding conditions can be identified when the structural transitions of the protein occur in concert with changes in the polymer’s hydrophobicity.

In their simulation of protein folding using an off-lattice model protein, Blanch and colleagues identified the existence of “hot” sites for aggregation, which are hydrophobic and buried in the native state. Proteins exposed to environments in which partially folded or unfolded states predominate are more likely to aggregate (73, 74). Subtle mutations by sequence engineering can therefore be used to shift the coexistence lines in the phase diagram of a protein solution.
without altering the structure of the native state (74, 75). Cellmer and colleagues performed Langevin dynamics simulation of a β-barrel model protein and showed multiple routes for aggregate formation (74, 76). Lu and Liu showed that the folding of the β-barrel protein at a high protein concentration follows the hydrophobic collapse—structural rearrangement mechanism but yields products that are in various forms, including single proteins in the native, misfolded, and uncollapsed forms as well as protein aggregates (77). Misfolded and uncollapsed proteins form the “nucleus” of aggregates that also encapsulate correctly folded proteins, that is, native proteins, as shown in Figure 13.3a. A new method for folding proteins at high concentrations has been proposed based on the “oscillatory molecular driving force.” In this method, the solution hydrophobicity swings from weak, which triggers the dissociation of protein aggregates, to strong, which promotes protein folding. These kinetics mimic the protein folding quality control system in vivo. The effectiveness of this method in enhancing protein folding and simultaneously reducing aggregation can be illustrated by comparing it with other methods that are based on direct dilution or the application of a denaturant gradient, as shown in Figure 13.3b.

Dissociation of non-native disulfide bonds and promotion of native bond formation is another challenge in protein refolding in vitro. Recently, Lu and Liu proposed a coarse-grained off-lattice model protein containing disulfide bonds and showed that the disulfide bonds located in the hydrophobic core were formed before the “collapse” step, while those located at the protein surface were formed during the “rearrangement” step (78). Although a reductive environment at the initial stage of folding favored the formation of native disulfide bonds in the hydrophobic core, an oxidative environment at a later stage of folding was required for disulfide bond formation at the protein surface, as shown in Figure 13.4a. An oscillatory redox environment, that is, one that changes from reductive to oxidative, intensified disulfide bond shuffling and resulted in improved recovery of the native conformation, as shown in Figure 13.4b. In this case, the swing of the redox driving force reproduces the functions of PDI, which catalyzes the conversion of non-native disulfide bonds to native ones. The abovementioned simulation has been experimentally validated by refolding hen-egg lysozyme, as shown in Figure 13.4c.

13.4 REFOLDING METHODS

There is a host of published methods for in vitro refolding and for purification of refolded proteins, as well as several excellent reviews (2–5), (79–82). Even so, one needs to consider the advantages of soluble and insoluble (inclusion body, IB) expressions. In general, it is easier to work with a soluble starting material than insoluble IBs. Means to increase the proportion of soluble expression are based on expression systems (a different promoter, using a low copy number plasmid or change host) and culture conditions such as changes in pH, minimal media, concentration of inducer (IPTG), induction temperature, and so on.

However, there is a special benefit for IB production because the IB could be easily separated from large amounts of soluble impurities in the culture by wash and centrifugation. The expression level in IB is usually much higher than in a soluble system. Therefore, IB expression is still widely used in biopharmaceuticals production situations. The development of an efficient refolding process is thus very important for increasing productivity.

13.4.1 Release of IBs from E. coli Cells

This is the first step of IB protein separation after fermentation. The E. coli cells are usually separated from the fermentation broth by centrifugation or filtration, washed, and resuspended in a buffer. Inclusion bodies are released from the cells by cell disruption. The method of disruption is to
a large extent governed by the tools available and the scale at which the refolded recombinant protein is produced (see Chapter 1, Figure 1.5, for an overview). On the laboratory scale, sonication (ultrasound producing cavitation) is a common method. Others include French press (83), enzymatic or chemical cleavage of the cells, and freeze–thawing. For larger scale continuous disruption, high pressure homogenization (84) or bead milling (85) are widely used. The combination of cell disruption and subsequent separation has been studied in the literature for soluble (86) and insoluble proteins (87). A chemical extraction was conducted at 37°C in a shaking incubator at 200 rpm overnight (12 h) with 8 M urea, 34.5 mM spermine and 3 mM EDTA in 0.1 M Tris pH 9.0. The extract, containing the denatured and His-tagged target protein, was applied to a Ni²⁺- or Cu²⁺-saturated IMAC expanded bed adsorption column (EBA) followed by refolding of the purified denatured protein (87).

13.4.2 Isolation of IBs

The IBs must be separated from the cell homogenate after cell disruption to get rid of the cell debris and other soluble impurities. The standard method is differential centrifugation, which often means elaborate washing procedures with a variety of buffer compositions in order to free the IBs from the cell debris and soluble contaminants. Such washing, if carried out carefully, will pay off by reducing the amount of trace contaminants in the chromatographic purification steps following dissolution and refolding of the target protein.

An alternative to differential centrifugation is size-exclusion chromatography (SEC) using a gel media with an exclusion limit that allows all cell homogenate components except the IBs to penetrate the gel network. In the crude homogenate the IBs are in the same size range as DNA/RNA, membrane vesicles, cell walls, and so on. So, before

Figure 13.4 “Disulfide bond shuffling” during protein refolding in vitro: (a) formation and breakage of disulfide bonds; (b) simulation prediction; (c) experimental validation. (See color insert.)
application to the SEC column, the homogenate should be treated with DNAse/RNase, detergents, and lysozyme. These depolymerizing and solubilizing agents are preferably added to the cell suspension before homogenization takes place. It is important that the homogenization is thorough, leaving no intact E. coli cells that will otherwise elute together with the IBs in the SEC fractionation. Repeated sonication or several cycles in the French press are required, followed by visual inspection of the homogenate using a phase contrast microscope. As SEC media, agarose gels with 4% dry weight such as Sepharose High Performance (base matrix) or Sepharose 4 FF have been shown to be adequate (88).

A procedure that has proved useful is based on the following disruption buffer: 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 0.1 μM PMSF, DNase 1 (10 μg/g cell paste), 60 μg/mL lysozyme, and 0.1% Triton X-100. In a typical preparation (3–5) g E. coli cell paste containing IBs is resuspended in 40 mL disruption buffer and homogenized in a French press (three cycles). After homogenization the crude homogenate is allowed to incubate at room temperature for 30 min to 1 h to facilitate the enzymatic breakdown of the DNA and peptidoglycan fragments. The incubated homogenate is then directly applied to a Sepharose 4 FF column equilibrated with disruption buffer containing 0.1% of the nonionic, non-UV-absorbing surfactant Berol 185 (Akzo Nobel Surface Chemistry AB, Stenungsund, Sweden) or equivalent. The exclusion limit of this gel is high enough to allow all sample components (proteins, degraded nucleic acids and peptidoglycan and dissolved membrane vesicles, and so on) to penetrate the gel particles except for the IBs, which are restricted to the void space between the particles. The void peak material containing the purified IBs is then collected by centrifugation and used in the refolding experiments. It is advisable to perform this size-exclusion step without unnecessary delay. Storage of the homogenate for longer periods of time, such as overnight, has occasionally resulted in precipitation phenomena to occur in the column.

13.4.3 Solubilization of IBs

There are many standard solubilization procedures published in the literature (see reviews), the vast majority of which rely on strong denaturants such as 6 M guanidine hydrochloride or 8 M urea. If the protein contains disulfide bonds, a reducing agent such as DTT should be added.

In the abovementioned example where the IBs are purified by SEC, the IBs collected by centrifugation are dissolved in a solution of 8 M urea and 0.2 M DTE. The mixture is kept at room temperature for (5–6) h and subjected to centrifugation at 10,000g for 20 min to remove residual insoluble matter. The particle-free solution containing the dissolved and denatured protein is ready for further refolding operation.

13.4.4 Purification before Refolding

Once the IBs have been solubilized, the denatured protein totally reduced, and the solution is free from particulates, initial purification of the random coil target protein is possible using a variety of chromatographic techniques. The methods used must be based on conditions that keep the protein in solution and that do not affect the separation mechanism. Thus, SEC can be applied regardless of denaturant used, while 8 M urea, but not 6 M guanidine hydrochloride, can be used together with ion exchangers. If the target protein is equipped with a His-tag or with a glutathione S-transferase-tag (GST-tag), the purification can be combined with solid-phase (on-column) refolding in one step (see Section 13.4.7.4).

13.4.5 Introduction to Refolding Procedures and Applications

After the IB protein is fully solubilized from the IB or partially purified by chromatography, refolding is then accomplished by removal of the denaturant away from the protein molecule, allowing it to refold by itself. A number of unwanted phenomena are likely to occur when trying to refold a random coil polypeptide by transformation from the denatured, totally unfolded state to the fully native correctly folded and biologically active conformation. The most common problems are aggregation and misfolding. Aggregation can be regarded purely as a statistical problem. Thus, the larger the distance between the individual protein molecules in the refolding buffer, the less risk of molecular collision and possible interaction leading to the formation of aggregates. The solution to this problem is simple and familiar to all workers in the field: reduce the protein concentration in the refolding buffer and you may increase the yield of correctly folded protein. There are many accounts of this in the literature (2, 89–91).

The next and equally important problem is misfolding. It is generally agreed that the refolding process is composed of two stages. The first, mainly based on hydrophobic collapse, is relatively fast and results in the formation of intermediate structures, often called molten globules. These are subsequently, more slowly, reassembled into the final native tertiary structure including reoxidation to form the correct disulfide bridges. Obviously there exist a number of energetically stable folding alternatives, sometimes referred to as kinetic traps and energy barriers. There is a very wide variation in the tendency of misfolding from protein to protein. Unfortunately, there is no generally applicable solution to this problem. Every protein is unique and thus it is necessary to design individual refolding schemes. However, there are certain rules of thumb to follow as a start. Thus, if the native protein contains disulfide bridges, it is mandatory to first see that all these are fully reduced. It is then advisable to supply the refolding buffer with a suitable redox system.
allowing reshuffling of the disulfide bridges, which often facilitates the formation of structurally correct bridges by reoxidation (92). The more disulfide bridges the protein contains, the more difficult the process and the more important it is to find the optimal redox system.

Many authors have described the advantage of including mini-chaperones or folding enhancers with the refolding buffer. The most well known of these is l-arginine (93–98). Thus, the majority of refolding buffers in use today contain 0.5–1.0 M l-Arg-HCl. Other additives and cosolvents include 0.5–1.5 M Gu-HCl (concentration should be optimized), 1–2 M urea, 10% glycerol (concentration should be optimized), Tris-buffer (at least 0.4 M), polyethylene glycol at low molar excess, alkyl ureas, carbonic acid amides (acetamide, propioamide, and so on), and detergents (CHAPS, SDS, and Triton X-100). One should always bear in mind that the effect of these additives varies from protein to protein. It is also advisable to move the pH of the refolding buffer away from the pI of the target protein, because protein solubility is at its lowest at its pI. Slightly acidic pH is preferable because of the risk of premature incorrect disulfide bridge formation at neutral or slightly alkaline pH. It is also advantageous to lower the ionic strength during refolding to avoid aggregation and misfolding due to premature salting out.

The positive effect on the yield of correctly refolded protein of lowering the temperature to around 10°C has been reported by several authors.

The application of high hydrostatic pressure (1–2 kbar) in combination with low concentrations of denaturants has been reported for the simultaneous solubilization and refolding of IB proteins (99–101), as has the use of lipid micelles, or mixtures of detergents and lipid micelles, for enhanced refolding yield, particularly of membrane proteins (102, 103).

### 13.4.6 Refolding by Dilution

Dilution of the solubilized protein directly into the renaturation buffer is the most commonly used method in small scale refolding studies because of its simplicity. However, protein concentration has to be carefully controlled to prevent aggregation (90, 104). Also, dilution is time-consuming and buffer-consuming. Thus, for large scale production, dilution may require subsequent concentration after the refolding. A method called “pulse renaturation” has been reported that has given ~10% higher yield compared with batch dilution (105, 106).

In order to allow a longer time for the denatured polypeptide to regain its natural conformation, the removal rate of the denaturants can be decreased by diafiltration (107), dialysis (108, 109) or by SEC in descending gradients of the denaturating agent (88). An alternative approach to achieving a reduced refolding rate is to perform the dilution in several consecutive steps. Successful applications of dilution refolding are described in Section 13.1.

### 13.4.7 Refolding by Chromatography

Over the last several years, various solid-phase refolding techniques have been developed. Chromatography has been a very successful technique in protein separation and purification. It uses a solid medium, usually a porous microsphere, packed in a cylindrical column. The feed solution is introduced from one end of the column, moving forward with various liquid solutions. Different substances in the feed are separated during the process. The substance having the least interaction with the solid medium exits the column first, while the substance having the strongest interaction with the medium exits last. In this way, different proteins can be separated and purified. As in protein separation and purification, chromatographic refolding uses the solid media in a similar way, but the purpose and the control strategy are different. In chromatographic refolding, the solid medium acts as a kind of chaperone or assistant to help the protein refolding occur in a correct way, which minimizes misfolding and aggregation. Figure 13.5 is a simplified illustration of chromatographic refolding. The feed solution containing denatured protein and denaturant is loaded into the column packed with porous microspheres. Renaturation buffers are introduced to elute the denatured protein to move through the column. During this process, simultaneous refolding and adsorption take place. The solid phase helps the correct folding of the protein. At the column outlet, the protein may exit in the correct form.

Chromatographic refolding is a very complicated process. There is intramolecular folding, intermolecular interaction between the protein and the ligand on the solid phase, and macroscopic process control over the entire column. The protein is several nanometers in size, but the microsphere has dimensions of several tens of micrometers. The length of the column can be more than half a meter. It is presently difficult to describe the mechanism of chromatographic refolding, because information is very limited. Here, we
discuss the molecular action and column control strategy in three types of chromatographic refolding processes.

13.4.7.1 Refolding by SEC  SEC is a successful tool for protein purification. For protein refolding, two key factors need to be considered (110, 111). The first is the loading of the protein to the column in the presence of a denaturant solution. The second is the change in protein size that occurs as it renatures during elution with the refolding buffer. Figure 13.5 is also an illustration of the fundamental principle of SEC refolding. The pores of the SEC media have selectivity regarding the size and shape of the proteins. Unfolded protein has a stretched, long shape that is difficult to insert into pores. The correctly formed product, being compact in shape and size, can access the depths of the pores, and is therefore separated from unfolded and partially folded ones. A new SEC refolding concept has been introduced. It is based on a gradient refolding system based on a decreasing urea gradient to provide a gentle and easily controllable environment for protein refolding. In this process a quick change in urea concentration is avoided. The procedure is gentle, providing a slow change of the environment so that the protein refolds gradually. A refolding study with lysozyme as a model protein using the gradient SEC has been reported (80).

In another study (88), a recombinant scFv fusion protein expressed as IBs in E. coli was refolded on a HiLoad 16/60 Superdex 30 prep-grade column. Figure 13.6 compares different refolding methods. Dilution refolding gave less than 10% recovery. SEC without gradient could increase the recovery to more than 20%. Much better results were found with gradient refolding, especially with the dual gradient of decreasing denaturant concentration and increasing pH. The activity recovery exceeded 50%. The principle of this method is that before feed loading, the column is equilibrated with the refolding buffer, followed by the introduction of a descending gradient of denaturant (e.g., from 6 M Gu-HCl or 8 M urea down to a predetermined concentration in the refolding buffer), sometimes combined with an increasing pH gradient. The gradient is allowed to occupy the upper 60% of the column. The feed in the highest denaturant concentration is then added, followed by a small volume of the same denaturant concentration, in order to avoid uncontrolled dilution of the protein in the rear part of the feed zone. During elution the proteins will be restricted to the void volume only, implying that they will pass through regions with gradually decreasing denaturant concentration, reaching the final refolding buffer concentration just before leaving the column. Table 13.1 presents a summary of operation procedures for refolding by SEC.

13.4.7.2 Refolding by Ion-Exchange Chromatography (IEC)  A very efficient strategy to prevent aggregation is to minimize the chance of intermolecular interactions. This could be achieved by adsorbing the denatured protein molecules onto a solid support, thus effectively separating the individual protein molecules during refolding. IEC, a widely used process for protein separation, can work for this purpose. However, refolding with IEC should be carried out carefully.

Figure 13.7 provides an illustration of IEC refolding. The denatured protein is adsorbed onto the surface of the IEC medium. As the concentration of denaturant in the liquid phase decreases, the protein begins to fold. The folding could take place on the solid surface or away from the surface depending on the protein and its environment. Partial folded protein could also be adsorbed by the solid surface through ionic interaction. The ideal situation is that the protein finishes its refolding process to form a correct structure, and exits the column. Because the adsorption of protein could separate individual molecules, their refoldings do not interfere with one another, and the chance of aggregation is minimized.

In one report, refolding of bovine (x-lactalbumin) with IEC gave less than 10% recovery, and the best result for hen lysozyme was also as low as 10% (113). The soluble refolding intermediates bound tightly to the medium and were very difficult to elute from the column. In order to prevent accumulation of denatured protein during refolding of matrix-adsorbed protein molecules, a new process using a two-buffer system to improve activity yield and mass recovery has been developed (114). The feed was loaded into the ion exchanger and the protein adsorbed onto the solid media in the presence of 8 M urea. A descending urea concentration gradient was introduced in parallel with an increasing

![Figure 13.6](image_url)
ionic strength, allowing the protein to simultaneously structurally rearrange and elute during its migration down through the column. When it entered regions where the salt concentration was low, the protein would be re-adsorbed onto the ion exchanger. The salt concentration increased and the urea concentration decreased gradually in the column, leading to desorption of the protein. Finally, at the column outlet, the urea concentration was 1 M and the salt concentration was high enough for the protein to be eluted. This type of “on/off” cascade process would allow a single protein molecule to refold gradually without much chance of intermolecular interaction and facilitate refolding of the protein to a native, biologically active conformation.

Another factor to be considered in optimizing a refolding process, especially in the formation of disulfide bonds, is the pH of the refolding buffer (115). The most favorable pH value varies from protein to protein. Usually, aggregation

### TABLE 13.1 Refolding by Size-Exclusion Chromatography

<table>
<thead>
<tr>
<th>Operation Procedures</th>
<th>Common Characteristics</th>
</tr>
</thead>
</table>
| **Basic Procedures** | No interaction between protein and stationary phase  
A size-based separation technology. Proteins of different sizes are separated by migrating in the porous media. |
| **Improvement 1: Denaturant Gradient Assisted Refolding**  
1. **Urea gradient**  
Gradually decreasing linear gradient of urea concentration from top to bottom is initially formed in the SEC column. After denatured protein is loaded, protein is refolded in this gradually changing urea environment and finally eluted in the refolding buffer (80, 112, 113).  
2. **Urea and pH dual gradient**  
Dual linear gradients of gradually decreasing urea concentration and increasing pH from top to bottom are initially formed in the SEC column in order to promote the correct formation of disulfide bonds and simultaneously suppress protein aggregation (114).  
3. **Urea and arginine dual gradient**  
Dual linear gradients of gradually decreasing urea concentration and increasing arginine concentration from top to bottom are initially formed in the SEC column (115).  
4. **Urea gradient combined with temperature control**  
Urea gradient is formed and refolding carried out at low temperature (115). |
| **Improvement 2: “Chaperon Solvent Plug” Strategy**  
A little amount of denaturant is introduced in the top of the column before and after the denatured protein is loaded so that protein aggregation is suppressed as contact of the denatured protein with refolding buffer is avoided (116). |
| **Improvement 3: Continuous Annular Chromatography**  
Continuous annular SEC is developed for protein refolding. Protein monomers, aggregates, refolding intermediates, and misfolding species are separated during the long path of migration in the column. Aggregates are then recycled and refolding yield enhanced (117, 118). |

Figure 13.7 Principle of protein refolding by adsorption chromatography. The process is schematic for IEC and HIC.
decreases when the pH of the medium is far away from the protein’s isoelectric point (116). The effect of nearby charged residues on the oxidation potential also makes a difference (117).

In order to accelerate thiol–disulfide exchange, the pH of the renaturation buffer should be at the upper limit that still allows the protein to form its native structure. However, it may be difficult to optimize denaturant concentration and pH simultaneously in a refolding process, especially in a large scale production. Considering the importance of both denaturant concentration and pH in refolding, a dual-gradient IEC process was introduced to enhance the refolding recovery at high protein concentration (114). After the dissolved human lysozyme expressed as IBs was loaded into the column, elution was started by gradually decreasing the urea concentration, combined with a gradual increase of pH of the elution buffer. The dual gradient provides an incremental change of the solution environment for protein refolding and for the formation of disulfide bonds.

Fe-SOD that is lacking disulfide bonds showed an increased refolding yield when a dual-gradient IEC refolding process was applied (118). At high pH, far away from the protein’s isoelectric point, aggregate formation was prevented, while at low pH near the isoelectric point the establishment of a biologically active conformation was facilitated. Table 13.2 presents a summary of operation procedures for refolding by IEC.

### 13.4.7.3 Refolding by Hydrophobic-Interaction Chromatography (HIC)

Hydrophobic interaction chromatography (HIC) is an efficient tool for protein separation. The mechanism involves hydrophobic interaction between immobilized hydrophobic ligands and the proteins in solution (119, 120). Although most hydrophobic amino acids are buried in the interior of globular proteins, some of them are exposed on the protein surface. These can interact with hydrophobic ligands on the HIC gel. The amount of exposed hydrophobic amino acids differs between proteins and so does the ability of proteins to interact with HIC gels. Considering hydrophobic interactions are the dominant forces in protein folding and structure stabilization, we have reason to believe that HIC can be an artificial chaperone system through its interaction with the denatured protein. Furthermore, HIC can directly deal with guanidine-HCl denatured proteins, which is an advantage over IEC refolding, which cannot work in high salt solution.

However, successful polypeptide folding is also dependent on undisturbed hydrophobic interaction forces. This is

<table>
<thead>
<tr>
<th>TABLE 13.2 Refolding by IEC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Operation Procedures</strong></td>
</tr>
<tr>
<td><strong>Basic Procedures: Three-Step Method</strong></td>
</tr>
<tr>
<td><strong>Method 1: Two-Step Elution</strong></td>
</tr>
<tr>
<td><strong>Method 2: Urea and pH Dual Gradient</strong></td>
</tr>
<tr>
<td><strong>Method 3: Expanded Bed Adsorption</strong></td>
</tr>
<tr>
<td><strong>Method 4: Continuous Annular Chromatography</strong></td>
</tr>
</tbody>
</table>
why HIC or RPC interactions should not be so strong as to prevent proper protein refolding. Some binding strength modifying agents might reduce the hydrophobic interaction and improve the refolding when added to the refolding buffer. The additives may influence both the solubility and the stability of the native, denatured, and intermediate states, respectively. They may act by changing the ratio of the rates of proper folding and aggregate formation or they might simply act by solubilizing aggregates already formed.

A new potent system adopting HIC assisted by glycerol was utilized to refold lysozyme at high initial protein concentration (121). Denatured and reduced lysozyme of 50 mg/mL as a model protein was loaded onto a Poros PE perfusion column, a commercially available HIC support, with a ratio of 1 mg protein per mL HIC medium adopting a gradient of urea and salt. Another gradient of urea and glycerol was utilized to remove bound lysozyme from HIC and then refold it, resulting in an activity yield of more than 85% with native special activity. In many cases HIC significantly enhanced protein recovery by minimizing aggregate formation thanks to its capacity to bind unfolded and partially folded protein during the refolding process. However, in some cases HIC caused a decrease in specific activity of the refolded protein, indicating the partial formation of incorrectly refolded structures. Glycerol was able to facilitate the formation of the native structure of lysozyme on HIC due to its hydration ability in refolding proteins accompanied by the adsorption of proteins with a urea gradient. The concentration of glycerol, urea gradient volume, ionic strength, and the concentration of reducing reagent of mercaptoethanol were found to play an important role in determining activity yield. A very mild hydrophobic ligand of PEG immobilized onto agarose microspheres was used successfully for refolding of recombinant Staphylococcus aureus Elongation Factor G (122).

Table 13.3 presents a summary of operation procedures for refolding by HIC.

### 13.4.7.4 Refolding Using Other Chromatographic Techniques

Besides the above mentioned techniques, immobilized metal-ion affinity chromatography (IMAC) has opened new prospects for efficient purification and refolding of proteins equipped with engineered polyhistidine tags. See Table 13.4 for a summary of the operation procedure for refolding by IMAC. Polyhistidine tags form high affinity complexes with immobilized divalent metal ions, even in the presence of high concentrations of chaotropic agents, thereby allowing isolation and refolding of tagged protein. Thus, one-step on-column affinity refolding and purification processes have become quite popular (123). As in the IEC refolding process, a gradual decrease in denaturant concentration induces protein refolding. Elution is achieved by increasing the imidazole concentration or by using a decreasing pH gradient (124). Thus the fusion protein His-TNF expressed in E. coli as IBs was refolded after adsorption to a Ni²⁺-Sepharose 6B column, resulting in >90% refolding yield (125).

### TABLE 13.3 Refolding by HIC

<table>
<thead>
<tr>
<th>Operation Procedures</th>
<th>Common Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basic Procedures</strong></td>
<td>Hydrophobic interaction serves as a double-edged sword in protein refolding: on the one hand it can protect refolding intermediates from aggregating; on the other hand, the exposed hydrophobic surface of denatured protein may bind to solid media on multiple sites, resulting in low recovery by the conventional elution method. Pulse elution by urea can promote protein release and significantly increase recovery. In addition, introduction of osmolytes, such as glycerol and PEG, in the elution buffer can help intramolecular hydrophobic collapse of protein and promote correct folding.</td>
</tr>
</tbody>
</table>

1. **Pulse elution by urea**
   
   Denatured protein is adsorbed on the column equilibrated with high salt concentration buffer, then after decreasing the salt concentration with a low salt concentration buffer, a 0.2–0.4 column volume urea is used to release bound protein, followed by refolding buffer to elute the refolded protein (91).

2. **Urea and glycerol dual gradient**
   
   After decreasing the salt concentration with a low salt concentration buffer, 0.4 column volume urea was used to release bound protein, followed by 0.4 column volume dual gradient of linearly decreasing urea concentration and increasing glycerol concentration to promote released protein refolding in a hydrophilic environment (90).

3. **Urea and PEG dual gradient**
   
   After decreasing the salt concentration with a low salt concentration buffer, 0.15 column volume urea was used to release bound protein, followed by 0.9 column volume dual gradient of linearly decreasing urea concentration and increasing PEG concentration to promote released protein refolding in a hydrophilic environment (131).
Affinity chromatography (AFC) using various ligands can also be used to refold proteins. For example, the strongly negatively charged heparin-Sepharose was used for the binding of a denatured protein containing a polyarginine fusion tag. Renaturation could be achieved under conditions allowing the protein to remain bound to the matrix and resulted in high yields of active protein (126).

Chaperones GroEL and GroES can bind to nascent or unfolded polypeptides and/or their folding intermediates, preventing improper polypeptide chain interactions that lead to aggregation. It is not surprising that these proteins can also affect the in vitro competition between folding and aggregation (127). Because chaperones and foldases are proteins that need to be removed from the renaturation solution at the end of the refolding process and may be costly to produce, their commercial use will require a recovery—reuse scheme (128). GroEL immobilized to agarose gel has been utilized in a lysozyme refolding study (129). See Table 13.5 for a summary of operation procedures for refolding using immobilized chaperones and a variety of artificial chaperones.

As in protein separation and purification, chromatographic refolding can be carried out with a combination of different chromatographic modes, including IEC, IMAC, and SEC. An example is the refolding of NS3, a potential target protein

**TABLE 13.4 Refolding by IMAC**

<table>
<thead>
<tr>
<th>Operation Procedures</th>
<th>Common Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basic Procedures</strong></td>
<td>Single point adsorption of protein on solid media is conducive to protein refolding due to the high degree of freedom; also, refolding and purification can be carried out simultaneously. With the development of genetic engineering, applications of IMAC in refolding are becoming increasingly widespread.</td>
</tr>
<tr>
<td>Denatured protein is adsorbed on the column equilibrated with denaturant, then a gradient of linearly decreasing denaturant concentration is introduced to induce protein refolding, followed by elution of refolded protein in imidazol-containing buffer (92, 93, 132–145).</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 13.5 Refolding by Immobilized Chaperone/Artificial Chaperones, and so on**

<table>
<thead>
<tr>
<th>Operation Procedures</th>
<th>Common Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Refolding by Immobilized GroEL/GroES Media</strong></td>
<td>Refolding by immobilized molecular chaperones and foldases has been simulated in an in vivo folding environment and increased the refolding yield effectively. But the cost of in vivo molecular chaperones is so high that it is not suitable for large scale applications. However, it is a good choice for refolding of protein that has high value and is difficult to refold by conventional methods. The successful applications of artificial molecular chaperones, such as Triton X-100 and β-cyclodextrin, have greatly overcome the above shortcomings, and the development of a cost-effective artificial molecular chaperone system will be a hot research topic in future in vitro protein refolding research.</td>
</tr>
<tr>
<td>Two refolding ways: (a) denatured protein is diluted into the refolding buffer suspended with immobilized GroEL media. After gentle mixing, the gel suspension is centrifuged and refolded protein found in the supernatant; (b) denatured protein is loaded on the immobilized GroEL media equilibrated with refolding buffer, and refolded while flowing through the column.</td>
<td></td>
</tr>
<tr>
<td>• 191–345 residues of GroEL was immobilized on Ni-NTA or CNBr activated agarose (146).</td>
<td></td>
</tr>
<tr>
<td>• 191–345 residues of GroEL, DsbA and peptidyl–prolyl isomerase were immobilized on an agarose gel (146).</td>
<td></td>
</tr>
<tr>
<td>• GroEL and GroES were immobilized on Prosep-9CHO and Prosep-5CHO media (147).</td>
<td></td>
</tr>
<tr>
<td>• GroEL was immobilized on agarose (98, 148).</td>
<td></td>
</tr>
<tr>
<td>• 191–345 residues of GroEL, DsbA, and DsbC were immobilized on N-hydroxysuccinimide activated agarose (149–153).</td>
<td></td>
</tr>
<tr>
<td><strong>Refolding by Immobilized Liposome Chromatography</strong></td>
<td></td>
</tr>
<tr>
<td>Liposome is covalently immobilized on gel media. Denatured protein is loaded on the immobilized liposome media equilibrated with refolding buffer, and refolded while flowing through the column (154, 155).</td>
<td></td>
</tr>
<tr>
<td><strong>Refolding by Immobilized β–Cyclodextrin Chromatography</strong></td>
<td></td>
</tr>
<tr>
<td>β-cyclodextrin is immobilized on allyl-Br activated agarose. Denatured protein is captured by detergent before loading to prevent aggregation, then the detergent is stripped by the immobilized β-cyclodextrin column and protein refolding induced (156, 157).</td>
<td></td>
</tr>
<tr>
<td><strong>Refolding by Immobilized Triton X-100 Chromatography</strong></td>
<td></td>
</tr>
<tr>
<td>Triton X-100 is immobilized on allyl-Br activated agarose. A β- cyclodextrin gradient is used to elute the refolded protein (158).</td>
<td></td>
</tr>
</tbody>
</table>
for HCV therapy (130). The expression of full-length NS3 in *E. coli* results primarily in the formation of inactive aggregates (131). Because it has a histidine tag, partially purification and refolding were initially performed with IMAC after dissolution of the IB in 8 M urea. The IMAC process separated the denatured NS3 from other impurities present in the IB. It also refolded the protein to its intermediate state. Further refolding and purification were followed either with IEC or SEC, or even another IMAC. The comparative results for the second chromatographic refolding process demonstrated that SEC was the best choice because it did not require a pre-desalting procedure, as in the case of IEC after the first IMAC process. Combination of IMAC and SEC chromatographic steps gave 90% of the total activity recovery.

Table 13.6 shows various chromatographic refolding applications, from which one can surmise that lysozyme is the most frequently studied protein. This is due to its relatively low cost and clear molecular structure. About 20 other proteins have also been tested with different chromatographic methods.

13.5 APPLICATIONS

13.5.1 Refolding of Recombinant Human Interferon Gamma (106)

A successful one-step refolding procedure is described in the following application example: the text is a modification from a paper by Zhang and colleagues (106). *E. coli* cells were suspended in 10 volumes of 20 mM phosphate buffer, pH 7.2, containing 0.125 M NaCl and 5 mM EDTA (disodium salt) at 4°C and dispersed using a knife homogenizer. The dispersed cell suspension was passed through an APV Gaulin continuous press for a total of three passages, at 500 bar. The press was kept at 4°C using a circulating coolant. The disintegrated homogenate was centrifuged for 30 min at 1000g (4°C). The sediment obtained was suspended in the same buffer and centrifuged followed by suspension in buffer containing 0.5 M urea. After centrifugation the purified IBs were suspended in three volumes per weight 7 M Gu-HCl in 50 mM borate buffer pH 7.2. Solubilization was achieved by vigorous stirring and the solution was clarified by centrifugation at 17,000g at 4°C.

Refolding took place by 70-fold dilution in one step in the abovementioned phosphate buffer containing 5% (w/v) sucrose and 2 M Gu-HCl. In the next step the solution was further diluted with the same buffer, but without urea, to a protein concentration of 0.1–0.2 mg/mL in 0.2 M Gu-HCl. Final renaturation was obtained after storage overnight at 4°C. The renatured rhIFN-γ solution was clarified by centrifugation at 17,000g for 30 min at 4°C. The supernatant was used as starting material for a purification procedure based on IEC on S Sepharose FF, IMAC on Ni²⁺ saturated Chelating Sepharose FF, and SEC on Superdex 75. This process is currently in practical use as part of the production of rhIFN-γ on an industrial scale.

13.5.2 Refolding of Recombinant Human Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) (110)

The following text is a modification from a paper by Belew and colleagues (110). *E. coli* cells were suspended in ten volumes by weight of 20 mM sodium phosphate pH 7.6, containing 0.125 M NaCl and 5 mM EDTA using a knife homogenizer. After cooling, the cells were disintegrated using an APV Gaulin continuous press at 50 MPa and the homogenate centrifuged at 5000g for 30 min at 4°C. The sediment was suspended in the abovementioned buffer containing 0.1% (v/v) Berol 185 followed by dispersion using the knife homogenizer.

After centrifugation the sediment was further washed by suspension in the same buffer now containing 0.5 M urea followed by dispersion and centrifugation as above. The pellet was again suspended in the original phosphate buffer followed by dispersion and centrifugation at 10,000g for 30 min at 4°C. The supernatant was discarded and the sediment, containing the thoroughly washed IBs, was dissolved in four volumes by weight 50 mM Tris-HCl, pH 7.5, containing 50 mM NaCl, 1 mM EDTA, 100 mM 2-mercaptoethanol, and 7 M Gu-HCl by stirring for 3 h at 4°C. The solution obtained was clarified by centrifugation at 40,000g for 30 min at 4°C.

The denatured protein was then refolded by stepwise dilution with 20 mM Tris-HCl, pH 8.4, containing 1 mM reduced glutathione, 0.1 mM oxidized glutathione, and 0.1% Berol 185 after a total of seven dilution steps. To 1.0 part of the supernatant was added 1.17 parts of the buffer, leading to a decrease in the concentration of Gu-HCl from 7 to 6 M. After stirring for 10 min at 4°C a further amount of the same buffer was added, decreasing the concentration of Gu-HCl to 5 M. The process was repeated until the final concentration of Gu-HCl was 1 M. Finally, a sufficient amount of the same Tris buffer was added, decreasing the concentration of Gu-HCl to 0.1 M (leading to a total 75-fold dilution). The resulting cloudy solution was stirred for 10 min and allowed to stand at 4°C overnight, allowing more time for protein renaturation to occur.

The solution was finally clarified by centrifugation at 17,000g for 30 min at 4°C. The clear supernatant, containing renatured rhGMCSF, was used as starting material for the purification of the active protein based on a combination of HIC on Phenyl SepharoseTM (high sub), IEC on Q SepharoseTM FF, and SEC on Superdex™ 75. The procedure has been scaled up and is currently running for the industrial production of rhGMCSF.
### TABLE 13.6 Chromatographic Refolding Applications

<table>
<thead>
<tr>
<th>Protein</th>
<th>Chromatography Type</th>
<th>Conditions</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme (80)</td>
<td>SEC</td>
<td>Superdex 75 (10/30)</td>
<td>Refolding yield &gt; 90%</td>
</tr>
<tr>
<td>CAB (110)</td>
<td>SEC</td>
<td>Superdex 75 (10/30)</td>
<td>Protein aggregation was inhibited and activity recovery was increased</td>
</tr>
<tr>
<td>scFv57P (81)</td>
<td>SEC</td>
<td>Superdex 200 (16/60)</td>
<td>Refolding yield increased from 40% (single gradient) to 60%</td>
</tr>
<tr>
<td>C1-inhibitor (103)</td>
<td>SEC</td>
<td>Superdex 75</td>
<td>Refolding yield 60–90%</td>
</tr>
<tr>
<td>E. coli IHF (101)</td>
<td>SEC</td>
<td>Superox 6; Superdex 75; Superdex 75 pg</td>
<td>Comparison of effects of sample loading on dilution and SEC refolding</td>
</tr>
<tr>
<td>Platelet-derived growth factor (104)</td>
<td>SEC</td>
<td>Sephacyrl S-100; Sephacyrl S-200; Sephacyrl S-300; Sephacyrl S-400</td>
<td>Comparison of refolding results on different gels</td>
</tr>
<tr>
<td>Transposase Tc1A (106)</td>
<td>SEC</td>
<td>Superdex 200</td>
<td>Simultaneous refolding and separation of multimer, dimmer and active monomer</td>
</tr>
<tr>
<td>Lysozyme (108)</td>
<td>SEC</td>
<td>Superdex 75</td>
<td>Refolding at high protein concentrations</td>
</tr>
<tr>
<td>β-Lactamase (109)</td>
<td>SEC</td>
<td>Sephacyrl S-300</td>
<td>Column diameter and length affected refolding yield</td>
</tr>
<tr>
<td>Lysozyme (124)</td>
<td>IEC</td>
<td>Sephacyrl S-100</td>
<td>Activity recovery was 95%</td>
</tr>
<tr>
<td>Fe-SOD (87)</td>
<td>IEC</td>
<td>Sephacyrl S-300</td>
<td>Refolding yield was increased five times</td>
</tr>
<tr>
<td>NS3 (99)</td>
<td>IEC</td>
<td>Sephacyrl S-300</td>
<td>Refolding yield was higher than that of SEC and IMAC</td>
</tr>
<tr>
<td>Horse cytochrome c, hen ovalbumin, etc (119)</td>
<td>IEC</td>
<td>CM cellulose</td>
<td>Comparative research</td>
</tr>
<tr>
<td>Papilloma virus HPV16 E7MS2 fusion protein (122)</td>
<td>IEC</td>
<td>Mono Q</td>
<td>Inclusion body was solubilized by 0.01M NaOH</td>
</tr>
<tr>
<td>Cyclodextrin glycosyltransferase (159)</td>
<td>IEC</td>
<td>SP Sephacyrl</td>
<td>A polyhistidine tag was added</td>
</tr>
<tr>
<td>A fusion protein of recombinant human growth hormone and a glutathione S-transferase fragment (126)</td>
<td>IEC</td>
<td>Streamline DEAE</td>
<td>Simultaneous particulate removal and refolding by expanded bed adsorption chromatography</td>
</tr>
<tr>
<td>Lysozyme (90)</td>
<td>HIC</td>
<td>Poros PE</td>
<td>Refolding yield was increased by glycerol gradient</td>
</tr>
<tr>
<td>Consensus interferon (131)</td>
<td>HIC</td>
<td>Poros ET 20</td>
<td>Refolding yield was increased by PEG gradient</td>
</tr>
<tr>
<td>Elongation Factor G (91)</td>
<td>HIC</td>
<td>PEG 20 K Sephacyrl HP</td>
<td>Immobilized PEG offered weak hydrophobic interaction</td>
</tr>
<tr>
<td>Elongation Factor G (157)</td>
<td>HIC</td>
<td>Immobilized β-cyclodextrin polymer coupled to an agarose gel</td>
<td>Suppression of protein aggregation</td>
</tr>
<tr>
<td>γ- interferon (130)</td>
<td>HIC</td>
<td>XDF-GM</td>
<td>Protein aggregation was suppressed</td>
</tr>
<tr>
<td>Antigens of <em>Mycobacterium tuberculosis</em> (136)</td>
<td>IMAC</td>
<td>HiTrap chelating column</td>
<td>Refolding yield was 95%</td>
</tr>
<tr>
<td>Bovine prion protein (142)</td>
<td>IMAC</td>
<td>Ni-NTA-agarose</td>
<td>Octarepeat sequences were used as a natural affinity tag</td>
</tr>
</tbody>
</table>

(Continued)
13.5.3 Refolding of Recombinant Single-Chain Antibody Fragment (ScFv57P) by SEC (88)

Single-chain antibody fragments (ScFvs) as potential molecules for the targeted delivery of drugs have attracted a great deal of attention, especially in medical fields. In comparison to the much larger Fab, F(ab')2, and IgG forms of MAb from which they are derived, ScFvs have lower retention times in nontarget tissues, more rapid blood clearance, and better tumor penetration. Various ScFvs have been expressed in E. coli in the form of IB protein. The culture method is common to other E. coli fermentations. After fermentation, the cells were harvested and washed by centrifugation. Cell disruption was the first step. The disruption buffer contained 20 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, 0.1 mmol/L PMSF, DNase (10 μg/g cell). E. coli (2 g) was suspended in 7 mL of the disruption buffer and mixed well. The suspension was passed through a French press at 500 bar for at least two cycles to disrupt the cells.

Cell disruption was the first step. The disruption buffer contained 20 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, 0.1 mmol/L PMSF, DNase (10 μg/g cell). E. coli (2 g) was suspended in 7 mL of the disruption buffer and mixed well. The suspension was passed through a French press at 500 bar for at least two cycles to disrupt the cells.

The cell homogenate, after the disruption process, was centrifuged at 10,000 rpm for 30 min. The supernatant was decanted off. The precipitated material was washed, centrifuged, and resuspended with 50 mL solution of 20 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, containing 0.1% Berol, and 50 mL solution of 1 mol/L NaCl in the same buffer for the same operation. The wash, centrifugation, and resuspension were carried out four times with the two solutions, each used twice.

An alternative way to separate the IBs with cell debris was by gel-filtration chromatography. A Sepharose HP column (16/60) was equilibrated with a solution of 20 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, 0.1% Berol, and 1 mol/L NaCl. The cell homogenate (7 mL) was loaded onto the column. Elution flow rate was 1.5 mL/min. Fraction was collected for the first peak of the elution (88). This process could efficiently separate the cell debris and IBs without conventional centrifugation and washing as mentioned in the previous paragraph.

Solubilization of the IB protein was performed with urea and dithiothreitol (DTT). Solid urea and DTT were added to the IB suspension to an end concentration of urea 8 mol/L and DTT 0.2 mol/L. The mixture was kept at room temperature for 5–6 h, and centrifuged to remove any precipitate. The pH was adjusted to 2 with HCl, DTT removed with a G-25 column equilibrated with 20 mM Tris, 1 mM EDTA, pH 2 (adjust with HCl). The solution was then ready for subsequent refolding procedure.

Gel-filtration refolding was performed on a chromatographic workstation ÄKTA Explorer 100 (GE Healthcare Life Sciences). Buffer A: 20 mM Tris, 1 mM EDTA, pH 7.5, and ratio of 1/1 of GSSG/GSH. Buffer B: 20 mM Tris, 8 M urea, 1 mM EDTA, pH 2 (adjust with HCl), 1 mM EDTA, pH 2. A gel filtration column Superdex 30 (16/60) was first equilibrated with 0.4 CV (column volume) of 5% B followed by a gradient of 0.6 CV from 5% B to 100% B (88). A 200 μL sample containing 1.1 mg dissolved IB protein was applied to the column. Elution was with buffer B, and the flow rate was 0.1 mL/min. Refolded ScFvs appeared as the first elution peak. A recovery of 25% was obtained, in comparison with about 14% of dilution refolding.

### TABLE 13.6 Continued

<table>
<thead>
<tr>
<th>Protein</th>
<th>Chromatography Type</th>
<th>Conditions</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human α-tocopherol transfer protein (138)</td>
<td>IMAC</td>
<td>HiTrap chelating column</td>
<td>Rapid buffer exchange</td>
</tr>
<tr>
<td>Polyhydroxyalkanoate synthase (137)</td>
<td>IMAC</td>
<td>Ni-NTA-agarose</td>
<td>Urea was removed by gradient</td>
</tr>
<tr>
<td>DevS (Rv 3132c) histidine protein kinase (139)</td>
<td>IMAC</td>
<td>Ni-NTA-agarose</td>
<td>Urea concentration was gradually decreased and GSSG-GSH were added</td>
</tr>
<tr>
<td>Exopolysphatase (141)</td>
<td>IMAC</td>
<td>Ni-chelating Sepharose FF</td>
<td>TritonX-100 was added in the refolding buffer</td>
</tr>
<tr>
<td>Scorpion toxin Cn5 (147)</td>
<td>Immobilized molecular chaperone chromatography</td>
<td>Immobilized GroEL fragments, PDI and PPI</td>
<td>This protein has four disulfide bridges and a cis peptidyl–proline bond</td>
</tr>
<tr>
<td>Lysozyme (98)</td>
<td>Immobilized molecular chaperone chromatography</td>
<td>Immobilized GroEL</td>
<td>The effects of denaturant concentration, flow rate and sample loading on refolding were investigated</td>
</tr>
<tr>
<td>scFv (149)</td>
<td>Immobilized molecular chaperone chromatography</td>
<td>Immobilized GroEL fragments, DsbA, DsbC</td>
<td>Oxidoreductase was immobilized as reducing agent</td>
</tr>
<tr>
<td>Glycerol dehydrogenase (148)</td>
<td>Immobilized molecular chaperone chromatography</td>
<td>Immobilized GroEL and GroES fragments on a glass matrix</td>
<td>Elution conditions in the absence and presence of ATP were compared</td>
</tr>
</tbody>
</table>
13.6 REFERENCES


14

PURIFICATION OF PEGYLATED PROTEINS

CONAN J. FEE
Biomolecular Interaction Centre and Department of Chemical and Process Engineering, University of Canterbury, Private Bag 4800, Christchurch 8020, New Zealand

JAMES M. VAN ALSTINE
GE Healthcare Bio-Sciences AB, 751 84 Uppsala, Sweden

14.1 INTRODUCTION
Polymer modified pharmaceuticals represent an important and growing family of pharmaceuticals. However, there is, as yet, no method-of-choice process for their purification. The most famous representatives of this family are proteins covalently modified with the neutral hydrophilic polymer poly(ethylene glycol) (PEG) (1–3). Although this chapter focuses on PEG-modified proteins, much of the chapter relates to the purification of other biopharmaceuticals conjugated with PEG or other polymers (4–6).

PEG polyethers are available from various common commercial sources in relatively controlled average molecular weight (MW). PEG polymers typically consist of \( n \) covalently coupled ethoxy monomers in a linear chain with hydroxyl groups at each terminus, that is, \( \text{HO–[CH}_2\text{CH}_2\text{O}]_n\text{H} \). Di-branched, tetra-branched, and comb PEGs are also commercially available. PEGs exhibit little natural reactivity, but the hydroxyl groups at the ends of the polymer can be readily modified with a variety of functional groups. Functionalized PEG polymers are also available from various commercial sources and come in a variety of MWS and end-group functional reactivities at one or both terminal ends. Monofunctional PEGs typically have a methoxy group at one end that prevents crosslinking. For more details on PEG chemistry, see books edited by J. M. Harris (7), and S. Zalipsky (8), as well as review articles covering PEGylated biopharmaceuticals, including those by

F. M. Veronese, M. D. Bently and other authors (2, 4, 5, 9, 10). Chapman (11), Yang and colleagues (12), and other authors noted below have written specifically on PEG–antibodies and PEG–antibody fragments. Delgado and colleagues (13) have provided a review PEG–protein history through its first 15 years.

14.2 GENERAL CONSIDERATIONS

In the 1970s, various authors, including Merrill and colleagues (14), Sehon (15), and Johansson (16) noted that covalent or other modification of proteins and surfaces with PEG polymers significantly altered various surface properties, reduced nonspecific protein adsorption, reduced surface antigenicity, and altered biodistribution. In 1977, Abuchowski, Davis and colleagues (17) reported that covalent attachment of PEG to proteins results in active conjugates that are nonimmunogenic, nonantigenic, and have greatly increased in vivo circulation half-lives. The term “PEGylated proteins” was first proposed by Derek Fisher in 1985 (18). PEGylation of therapeutic proteins reduces renal clearance rates by increasing the radius of hydration of bioactive substances that are otherwise small enough to be filtered via renal glomeruli. It also protects from proteolytic and other degradation, as well as uptake by phagocytes. As a result, PEGylation often results in enhanced therapeutic efficacy. Other benefits of PEGylation may include improved physical and thermal stability, as well as improved solubility. The latter is particularly important with regard to biopharmaceutical formulation and delivery. These changes appear to be mainly due to significantly increased molecular size (hydrodynamic radius) plus surface alteration, and masking by the neutral, chemically inert, hydrophilic PEG polymer chains (1, 2, 19). Successful PEG protein biopharmaceuticals include PEGylated interferons, for use in the treatment of hepatitis C (e.g., PEGasys® from Hoffman-LaRoche and PEG Intron® from Schering-Plough/Enzon), PEGylated growth hormone receptor antagonist (PEG Somavert® from Pfizer), PEG–asparaginase (Oncospar® from Enzon), adenosine deaminase (ADAGEN® from Enzon), granulocyte colony-stimulating factor (Neulasta® from Amgen), and PEG–oligonucleotide (Macugen® from Pfizer and Eyetech Pharmaceuticals). Total worldwide sales of such substances amount to several billion US dollars a year (1). Many other PEGylated enzymes, hormones, oligopeptides, oligonucleotides, and smaller-MW drug substances are in various stages of development. This includes several new PEGylated biopharms based on hormones such as MirCERA® or antibody fragments such as CIMZIA®.

In addition to articles by Chapman (11) and Yang and colleagues (12) there are several noteworthy references to PEGylation of antibodies, antibody fragments, and other binders (5, 20–23). Protein PEGylation chemistry, reaction engineering, and related pharmaceutical topics have been covered in journal articles (2–5, 25) and in chapters in books such as a review by Zalipsky (24).

As noted later in the chapter, a PEG polymer in solution exists in a dynamic random coil, which, over time courses commensurate with chromatography, can be taken to exhibit a viscosity radius several times larger than that of a protein of similar MW. As a result, the initial grafting of a PEG with MW equal to or greater than 10% of a protein can be expected to significantly alter various properties of the protein, especially if the polymer is attached at the surface near where the protein interacts with its environment. Addition of more polymers (i.e., increasing the PEGylation molar ratio, N) is expected to increase conjugate molecular weight and size and perhaps alter pI (depending on conjugation chemistry). However, physical differences related to additional grafting may not be as significant as those related to the initial grafting event, other than where additional conjugates cause steric hindrance near the active site(s) on the protein, because they only add to the PEG mass at the protein surface. The heavily hydrated PEG chains tend to shield and weaken the strength of the protein’s electrostatic surface-related interactions and may increase noncharge-based interactions. The above changes impart exploitable physical and thus separation behavior differences between the native protein and its PEGylated forms, but less significant differences between PEG–proteins, which vary in degree of PEGylation or sites of PEGylation—although such PEG–proteins may differ dramatically in medical efficacy. Such considerations underpin the general observation that most commercial PEGylated biopharmaceuticals are typically modified with only one or two PEG polymers via reactions chosen to offer controlled (or at least reproducible) site-directed grafting.

PEGylated biopharmaceuticals are normally produced using highly purified native protein (where “native” pertains to its un-PEGylated form, as opposed to its wild versus recombinant or engineered forms). As such, reaction considerations will involve optimizing use of the native protein, as well as choosing reactants and reactions that allow for ready removal of PEGylation reaction byproducts and unconverted reactants. PEGylated reaction products may be heterogeneous with respect to both PEGylation extent and their site of grafting. Reaction conditions that allow elimination or minimization of “positional isomerism” (the positions of the PEG adducts on each molecule) are desirable, as to date it has been very difficult to isolate positional isomers from one another at a preparative scale. They do appear separable for analytical purposes using reverse-phase chromatography or capillary electrophoresis. Naturally, heterogeneity arising from such positional variation is expected to increase with N, as well as in the case of proteins, such as antibodies, composed of more than one polypeptide chain. Two different types of positional variation can be expected. The first is when a reagent directed to a specific type of residue, such
as a protein’s terminal amine, reacts with similar functional groups elsewhere, such as lysine amine groups. The second is when a reagent directed to a specific type of residue reacts with other types of residues. Such nonspecific reactivity occurs due to use of nonspecific reagents, such as triazine functionalized PEGs, and also due to the rich variety of pH and other microenvironments that residues occupy in proteins. As a result of the latter, histidine, serine, and other relatively inert amino-acid residues can be significantly reactive. The chapter reference list provides the reader with several papers related to reaction engineering and medical efficacy aspects of this topic. It only remains to be said that choice of functional PEG reagent will generally involve a trade-off between several considerations, including reagent and solvent costs, reactivity, propensity to generate positional variants, and the ease with which byproducts (including denatured protein) may be removed. This strongly suggests that readers interested in biopharmaceutical production should utilize high throughput reaction condition screening, with both structural and functional characterization of products, as early as possible in product development.

Therapeutic drugs should ideally be homogeneous products with well-defined activities and acceptable side effects. Variations in the position and number of PEG adducts gives rise to variations in characteristics relevant to clinical and other application-related effects. These include enzymatic or other activities, circulation half-life, immunogenicity, and clearance (22, 24–29). Product homogeneity can be improved by maximizing reaction specificity and/or implementing effective product purification. Given the high cost of producing and purifying therapeutic proteins, which are typically the starting reagents for PEGylation, achieving high purity of PEGylated forms at the expense of yield is not an economically feasible option. In many cases neither will be reaction recycling of unreacted protein as it may have been chemically altered in the previous PEGylation reaction. Effective and efficient purification processes are, therefore, of heightened importance when considering PEG–protein process development, especially as the process developer can expect to encounter challenges and require process steps that are not common to the process developed to purify the original native protein. Fortunately, many of the challenges related to processing of one PEGylated protein are common to processing of other PEGylated proteins and from that perspective, such polymer–protein conjugates may be considered a family of proteins.

Similarities between PEG–proteins appear to extend not only to similar proteins modified to a similar degree with polymers of equivalent size. For reasons made clearer later in the chapter, they also extend to proteins modified with branched versus linear PEGs. There appears to be no significant difference between the apparent molecular sizes of PEGylated proteins with branched or linear PEG adducts in regard to size-exclusion chromatography (SEC) (30), and to date there have been no reports of other physicochemical differences between the two that would suggest that one type is easier to separate than the other. However, advantages in terms of reduced plasma clearance rates and increased clinical efficacies have been reported for branched-PEG adducts over linear PEG adducts (31), just as grafting site or size of otherwise similar PEGs can affect PEGylated biopharmaceutical therapeutic efficacy. Intuitively, it would seem that the challenges for purification are virtually identical for various biopharmaceuticals modified with one or more PEG chains, either branched or linear. What appears to be most important is the total MW of the PEG added to the protein, both in regard to chromatographic (19) and also liquid–liquid partition (32). That fact focuses the choice of PEG-biopharmaceutical production processes and applications where it should be—on comparative product functional performance.

Physicochemical properties that are routinely used to purify and analyze proteins can be exploited to varying effectiveness with PEGylated proteins, including molecular size and shape (SEC, membranes, capillary electrophoresis, gel electrophoresis), electrostatic charge (cation- and anion-exchange chromatography, isoelectric focusing, capillary electrophoresis), relative hydrophobicity (hydrophobic interaction and reverse-phase chromatography (RPC)), solubility, and partition in aqueous two-phase systems.

Information provided elsewhere in the chapter and in the referenced works provides insight regarding the various analytical methods used with PEGylated proteins. These can be separated into methods used to routinely follow separation processes and those used to analyze the products of such processes. “On line” methods such as refractive index and UV-visible absorbance spectroscopy can be valuable in following protein and polymer monomers (as well as aggregates) during chromatographic or other separations. “At line” or “off line” methods such as matrix-assisted laser desorption ionization-time of flight (Maldi-TOF) mass spectroscopy (MS), gel electrophoresis, SEC, and capillary electrophoresis are also very useful. Protein stains and analytical methods that are unaffected by PEG or other residual polymers are commercially available, as are antibodies directed to PEG. The antigenicity of PEG is such that the latter require IgM antibodies. Such stains allow for improved gel electrophoresis and enzyme-linked immunoabsorbent assay (ELISA) analytical methods. The authors are not aware of a comprehensive review article covering analysis of polymer modified biopharmaceuticals, but comments related to some specific analytical methodologies such as gel electrophoresis are included in various places throughout the chapter. Monitoring the functional behavior of PEGylated target during processing is recommended. Although it may not be possible to monitor in vivo functionality, it should be possible to monitor function-related enzymatic, antigenic, size, and target binding properties using the above technologies.
or, especially for target binding, using methods such as surface plasmon resonance (SPR) or isothermal calorimetry.

Polymer-modified biopharmaceuticals such as PEGylated proteins will generally be prepared using native protein purified in a USFDA (or similar regulatory authority) approved process. For reasons of speed and ease of governmental approval there is a tendency to try to purify the PEGylated protein using processes developed for the native protein. Depending on time constraints, this may provide a workable but not very efficient process, as one can expect several differences in the processing of a native protein and the corresponding polymer-modified protein. Table 14.1 notes some common polymer-modified protein processing challenges, in relation to the case of PEGylated proteins. Almost all relate to properties of the polymer when both free in solution or coupled to the protein. Other polymers will likely mirror such challenges to a greater or lesser extent. Thus, dextran or starch polysaccharides are less surface active and chemically stable than PEG, but their solutions can be more viscous and the degree of branching per polymer much harder to control.

It is to be remembered that in many cases polymer modification will be carried out in aqueous solution using activated polymers with readily hydrolyzable end-groups such that, as in the cases of ω-hydroxysuccinimide (NHS), succinimidyl-propionate (SPA), or related reagents, protein coupling will compete with hydrolysis. As result, excess molar quantities of polymer are often needed and reaction product mixtures may contain significant concentrations of ungrafted (chemically inert) polymer reagents, as well as native proteins (some of which may be denatured or otherwise altered by the grafting reaction), plus mono-PEGylated proteins (N = 1) and oligo-PEGylated (N ≥ 2) proteins with varying degrees of positional isomerism (Table 14.2). The desired product will

<table>
<thead>
<tr>
<th>Polymer Property or Behavior</th>
<th>Related Bioprocess Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface activity</td>
<td>Foaming</td>
</tr>
<tr>
<td>Greater dependence of viscosity radius (Rg) on mass</td>
<td>Need for large pore media. Low dynamic capacities in chromatography</td>
</tr>
<tr>
<td>Viscosity in solution</td>
<td>Low working loads</td>
</tr>
<tr>
<td>Temperature- and salt-related self-association</td>
<td>Varied temperature and conductivity effects including fouling</td>
</tr>
<tr>
<td>Protein precipitant</td>
<td>Fouling and low working loads</td>
</tr>
<tr>
<td>Chemically stable</td>
<td>Clean-in-place challenges</td>
</tr>
<tr>
<td>Neutral and hydrophilic, although often less hydrophilic than native proteins</td>
<td>Reduced affinity and ion-exchange interaction. Altered hydrophobic interactions and electrophoresis patterns</td>
</tr>
</tbody>
</table>

TABLE 14.2 Classification of some PEGylation Reaction Products by Size

<table>
<thead>
<tr>
<th>Low Molecular Weight</th>
<th>High Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Byproducts from hydrolysis of functionalized PEG</td>
<td>Unreacted functionalized polymers</td>
</tr>
<tr>
<td>Byproducts of PEGylation</td>
<td>Inactive polymer from hydrolysis of reagents</td>
</tr>
<tr>
<td>Buffer components</td>
<td>Native protein possibly in native and altered state</td>
</tr>
<tr>
<td>Other contaminants (e.g., polymer antioxidants, antifoaming agents)</td>
<td>Target PEGylated protein, which may include various forms with varied medical activity</td>
</tr>
</tbody>
</table>

*Low MW is defined here as typically <1000.

typically represent 30–50 mol% (and in some cases site-specific coupling, 70 mol%) of the total protein as measured by absorption at 280 nm. However, appreciable free polymer may be present to a degree that affects purification via foaming, precipitation, fouling, and other problems (Table 14.1). What makes polymer-modified biopharmaceuticals economically viable is that they may be many times more effective per mass dose than their native counterparts and also offer reduced side effects, broader patient tolerance and market base, improved drug delivery and competitiveness, broader delivery options, increased storage life (low proteolysis), and enhanced intellectual property protection (see above-mentioned references).

The main processing challenges one can expect to encounter are as follows. First, the PEGylation reaction will convert the starting pure protein to a mixture of various components (Table 14.2). This mixture will include PEG–protein and unreacted PEG, which are quite viscous and can act as precipitating agents so that some dilution of process streams may be needed to effect early-stage filtration and chromatographic steps. Second, the target PEG–protein will be considerably larger in hydrodynamic volume than the corresponding native form. As a result, diffusivity will be reduced and the pores in some standard chromatography media may not be available to it, so total dynamic capacity will be decreased. The PEGylated protein may not adsorb as efficiently on media surfaces and, in the case of ion-exchange or affinity capture, the surface-localized PEG may significantly reduce the affinity of the conjugate for the media surface. The net result is that one can often expect 10 times lower g/L dynamic capacities on some capture media for PEG–proteins compared to their unmodified protein counterparts. The first and second points indicate that processing of PEG–protein reaction product mixtures will involve much larger volumetric processes and unit operations. The third point suggests some unit operations (e.g., affinity chromatography) that worked well for purifying the native protein may need to be
replaced in order to purify the PEGylated protein. Time will be needed to develop new unit operations. Finally, various methods used previously to analyze the native protein separation process on-, at-, or off-line may need to be modified or replaced.

14.3 NOTES ON SELECTED SEPARATION METHODS

Purification processes are discussed below in terms of the physicochemical properties exploited for separations.

14.3.1 Separation on Basis of Molecular Weight, Size, and Shape

The species to be separated after PEGylation may be classified according to molecular weight, as shown in Table 14.2. Positional isomers having the same $N$, although potentially possessing differentiated biological (therapeutic) activities, are the most difficult to separate, as they have closely similar physicochemical properties.

14.3.1.1 SEC

SEC (or, equivalently, gel filtration or gel permeation) can be used in either group fractionation (desalting) or fractionation modes. Figure 14.1 data are taken from Reference 19 and shows SEC chromatograms for bovine cytochrome $c$ protein (Sigma) modified with different MWs of PEG–succinimidyl propionate (SPA) reagent (Nektar Therapeutics, Huntsville, USA) under conditions generating a significant distribution of PEGylated $N = 1$ to $N = 3$ variants. The PEG40000 reagent was a di-branched 20000 NHS reagent. The SEC media was Superdex™ 200 (GE Healthcare) run with 0.15 M NaCl, 0.01 M NaPhosphate pH 7 (PBS) buffer using a prepacked Superdex HR 10 × 30 column with Vc 23.5 mL run at 40 cm/h. Sample sizes were 50 µL and none of the PEG–protein peaks eluted in the void. Similar results were reproduced with Superose™ media so that the peaks are not thought to reflect any possible coacervate interaction between media dextran and solute PEG. More details are to be found in the reference; however, the following points can be readily seen from this figure:

- The SEC media behaves well with PEG–proteins even up to 80,000 MW added PEG.
- The PEGylation dramatically increases the hydrodynamic size of the protein conjugates.
- One can follow via UV the small MW SPA (or NHS) related chemicals released in the reaction.
- Elution peaks of PEG–proteins of similar total added PEG MW occur in the same volume.

Figure 14.2 shows similar SEC behavior for PEG40000-NHS di-branched reagent (Nektar) modified egg white avidin protein on Superose 6 (GE Healthcare). The protein has a MW of $\sim 66$ kDa and pI of 10, which are values similar to some antibody fragments. Details of the experiment can be found in an Application Note from GE Healthcare (33). Note in terms of resolution how similar the PEG40000 SEC results are for the different protein mixtures on the two different media. Such results suggest that although SEC may be able to readily separate small MW reaction product mixture contaminants, native protein, and PEG–protein fractions, it has a much harder time resolving different

![Figure 14.1](image-url)
PEGylated proteins differing in either protein or in $N$ values. At best, SEC can resolve PEG proteins over a range of $N$ from 1 to 3, and only if the MW of the PEG reagent is relatively small in comparison to the MW of the protein. Other possible drawbacks related to the possible use of SEC include the inherent viscosity of PEG–protein solutions, especially reaction product mixtures.

Desalting with SEC will remove low molecular weight species and allow buffer exchange, such as into a volatile buffer prior to freeze-drying. Low molecular weight hydrolysis and PEGylation reaction byproducts and unconjugated PEG species are generally only weakly retained by ion exchange, so SEC could be used to remove these species. However, ion-exchange binding of PEGylated proteins becomes weaker as PEGylation extent increases. Therefore, higher PEGylated species may well be contained in the flow-through fraction. This is discussed in more detail later in this chapter.

It is well recognized that SEC can be used to separate PEGylated species from other components, but the effectiveness will depend greatly upon the molecular sizes of the species involved. The viscosity radius provides a consistent calibration curve between molecular size and elution volume for both proteins and polymers (specifically dextran) across all SEC media (34–36), while other size- or shape-related parameters such as molecular weight, the second virial coefficient, Stoke’s radius, and radius of gyration have been shown to be inadequate by comparison (37).

Although SEC has generally been thought to be unreliable for identifying PEGylated proteins from their elution profiles (38–41), SEC columns calibrated in terms of molecular size rather than molecular weight give consistent results for protein and PEG standards (19, 42). This is illustrated in Figure 14.3.

For globular proteins, the viscosity radius (in Å) of the molecule (assuming a spherical shape) is related to its molecular weight in daltons, $M_{prot}$, by Equation 14.1 (34):

$$R_{h,prot} \approx (0.82 \pm 0.02)M_{prot}^{1/3} \quad \text{(14.1)}$$

Kuga (43) collated data on the viscosity radius of PEG molecules from a range of sources and a subset of this data gave the following correlation ($r^2 = 0.9995$) with the PEG molecular weight in daltons, $M_{r,PEG}$ (19):

$$R_{h,PEG} = 0.1912M_{r,PEG}^{0.559} \quad \text{(14.2)}$$

Using this technique, Fee and Van Alstine (19) found that the size of PEGylated proteins can be accurately predicted from the radius of a hypothetical PEG molecule having the same molecular weight as the total conjugated PEG and the native protein radius, using the following equation:

$$R_{h,PEGprot} = A \frac{A}{6} + \frac{2}{3A} R_{h,PEG}^{2} + \frac{1}{3} R_{h,PEG} \quad \text{(14.3)}$$

where

$$A = [108R_{h,prot}^3 + 8R_{h,PEG}^3 + 12(81R_{h,prot}^6 + 12R_{h,PEG}^3 R_{h,PEG}^{1/2})^{1/3} \quad \text{(14.4)}$$

where $R_{h,PEGprot}$ is the viscosity radius of the PEGylated protein, $R_{h,prot}$ is the viscosity radius of the native protein, and $R_{h,PEG}$ is the viscosity radius in free solution of a single PEG molecule of the same molecular weight as the total conjugated PEG. Recent experience of the authors is that resolution can decrease with repeated samples, presumably...
due to weak adsorption of PEG on the matrix but that addition of 10% methanol to the mobile phase prevents such fouling.

Thus, by proper calibration using viscosity radii, the PEGylation extent can be determined quantitatively by SEC (Fig. 14.4, in which the approximate sizes of IgG and IgM are shown for comparison). Furthermore, because PEG itself is invisible to UV at 280 nm, and typical amine reactive groups do not contain UV active chromophores, UV adsorption at 280 nm may be used to quantify the concentration of PEGylated species. However, some chromophores, such as maleimide or "fmoc," do have UV active components. For conjugates with these types of linkers, it may be necessary to determine the extinction coefficient after a non-UV method, such as amino-acid analysis, has been used to determine protein concentration.

As a general rule, preparative-scale SEC separates proteins efficiently only if they differ in molecular weight by about 100%. Thus a protein of molecular weight \(x\) can be separated efficiently from one of \(2x\). This corresponds to a size ratio between molecules, calculated from Equation 14.1, of 1.26. In terms of size, a PEG molecule has a much larger hydrodynamic radius than a protein of corresponding molecular weight. In most aqueous environments PEGs exhibit large coils related to one in three chain atoms being oxygen. Various studies suggest that each ethoxy group has a chaotrope effect on many water molecules (7, 8). However, proteins generally contain chemical groups that can interact with PEG groups via covalent, hydrogen-bond, van der Waals, and other interactions even in pure water. Such interactions tend to determine and stabilize their solutions structures, which are also significantly affected by a need to reduce water interaction with protein hydrophobic groups. Conjugating a single PEG polymer of the same molecular weight to a protein increases the protein molecular radius more than is expected from doubling the protein molecular weight. Therefore native and mono-PEGylated proteins should be readily separable by SEC. For low PEGylation extent then, SEC will be effective but the resolution between peaks will be expected to decrease as PEGylation extent increases.

Based on Equations 14.3 and 14.4, Figure 14.5 shows the size ratio between species differing in PEGylation extent by a single PEG adduct for a protein of MW 15 kDa. The normal guideline for separating proteins with acceptable resolution (i.e., a size ratio of 1.26) is shown as a dotted line. All PEGylated species can be separated from the native protein.
in adduct. The dotted line shows the normal guideline for obtaining sufficient resolution for SEC separation. Note that as the molecular weight of the PEG adduct increases, the ability to separate PEGylated species from one another decreases. However, SEC becomes less effective in separating between species differing by a single PEG as the extent of PEGylation increases. The upper limit for good resolution appears to be between di- and tri-PEGylated species even for 40 kDa PEG adducts—the largest functionalized PEGs readily commercially available. Only mono- and di-PEGylated species can be efficiently separated by SEC when a 5 kDa PEG adduct is used. The curves are closer together for the larger molecular weight PEG adducts, so larger PEG adducts will not allow separation of species with \( N > 3 \) from their \( (N - 1) \) counterparts. Increasing the molecular weight of the protein reduces the proportional increase in size with each PEG added. The upper limit for effective SEC separation of PEGylated proteins differing by a single PEG adduct is between di- and tri-PEGylated proteins. This separation will also become more difficult as the ratio of protein to PEG molecular weight increases. Although the sizes of the target PEGylated species are equal, SEC can purify from its under- and over-PEGylated forms a protein that is mono-PEGylated with a 20 kDa PEG but not the same protein tetra-PEGylated with a 5 kDa PEG.

SEC studies (19) suggest that existing SEC media are suitable for separation of PEG–proteins from native proteins and moderate fractionation of PEG–proteins of differing \( N \). This work also suggests that grafting PEGs to proteins results in PEG–protein conjugates where, on the timescales and physical events of SEC, the PEG polymers appear to spread over the surface of the protein so as to maintain the surface-to-volume ratio they normally have in solution. This helps rationalize how modification of proteins with one PEG polymer can have a pronounced effect on both its size (e.g., SEC) and surface properties (11, 32, 44, 45). The relationships described in the above equations can be used as a possible basis for process developers to make logical choices in regard to the sizes of the conjugates with which they will be dealing, so as to make effective chromatographic media choices in regard to resolving power and mean pore size. Although such studies have not yet been published, verifications of the equations have come from independent investigators working with PEG–proteins and other types of chromatographic media, such as Reference 46. A second significant application of SEC results relates to understanding the strengths and limitations of chromatographic separations achieved with PEG–protein reaction product mixtures by ion-exchange (IEC), affinity, and hydrophobic-interaction (HIC) chromatography. We shall return several times to the model these studies present of PEG–protein conjugates which, over chromatographic timescales and conditions, present themselves as proteins coated with a masking PEG surface layer.

Commercial PEGylation reagents are identified by nominal molecular weights that usually underestimate their true molecular weight. For example, PEG reagents with nominal molecular weights of 5 kDa from Nippon Oils and Fats (NOF) Corporation, Japan, 10 kDa (Nektar Therapeutics, Alabama, USA), and 20 kDa (NOF) were found by mass spectroscopy (19) to have actual molecular masses of \( 5589 \pm 56 \) Da, \( 11,555 \pm 116 \) Da, and \( 21,910 \pm 219 \) Da, respectively. In the case of branched PEGs, which are manufactured by combining two identical PEG molecules, the discrepancy between nominal and actual molecular masses can be larger than that for linear PEG chains because branched species are often synthesized by conjugation of two or more linear chains.

Polyacrylamide gel electrophoresis (PAGE), routinely used for protein molecular weight and purity determinations, can also be used for the analysis of PEGylated proteins. However, unlike the case for native proteins, where standard protein ladders can be used to make quantitative determinations of molecular weight, no correlation currently exists for determining PEGylated protein molecular weight by this method.

The migration rates of PEGylated proteins through porous gels are slowed by the large, heavily hydrated, and uncharged PEG chains attached to the proteins, so their apparent molecular masses cannot be determined from their positions relative to standard protein ladders. Such effects have been known for some time and have even been used to achieve affinity electrophoresis using polymer-modified affinity ligands (47). PEGylation extent may often be inferred qualitatively from the band positions in PAGE gels because these positions differ significantly with each PEG chain added to the protein, but this can provide only circumstantial evidence of PEGylation extent. For example, if site-specific PEGylation is obtained through maleimide-PEG reacting with a single, free available cysteine residue on a protein, then a significant shift in the PAGE band position after PEGylation would support the conclusion that PEGylation was successful. On the other hand, if one was using a nonspecific chemistry such as lysine conjugation, the PEGylation extents of the migrating species can only be guessed by assuming that bands are located in the gel in the order of native protein, mono-PEGylated, di-PEGylated, tri-PEGylated protein, and so on, with no missing values of \( N \). In the absence of a native protein band it would be dangerous to make assumptions regarding the values of \( N \) obtained by this method.

PEG tends to interact with sodium dodecyl sulfate (SDS) under reducing conditions, creating a clump of negatively charged chains that move in the gel, despite PEG having no charge. Native SDS-PAGE appears to eliminate PEG interaction with the gel, providing sharper bands and giving consistent band positions depending on the MW of the conjugated PEG, for example, a di-PEGylated 10 kDa PEG–protein having a band at the same position as the mono-PEGylated 20 kDa PEG–protein. In spite of the
above limitations, gel electrophoresis can be a very useful method with which to quantify PEG protein chromatographic fractions (48) or site of coupling (49).

14.3.1.2 Ultrafiltration

Other than for buffer exchange, desalting, or concentration, a few authors have used ultrafiltration for purification of PEGylated proteins (20, 50–52). Maeda and colleagues (53) used hollow-fiber ultrafiltration to remove <40,000 MW species from the product mix after PEGylation of bilirubin oxidase. Bailon and Berthold (54) included diafiltration in their generic purification scheme for PEGylated proteins. Tan and colleagues (55) concentrated PEG–methioninase with 30,000 MW cut-off membranes. Edwards and co-workers (56) used 5 kDa molecular weight cut-off membranes for concentrating PEGylated tumor necrosis factor receptor type I (TNF-RI), noting that unacceptable product losses occurred when using 10 kDa molecular weight cut-off membranes. This is surprising, as TNF-RI has a molecular weight of 55 kDa, and was mono-PEGylated with a 30 kDa PEG. The authors advised that they normally disregard the contribution of the PEG adduct when choosing a suitable pore size for ultrafiltration membranes (56).

A systematic study of ultrafiltration and PEGylated proteins has been carried out by Zydney and colleagues (57–59). Unlike the case in SEC, it is not only the total PEG molecular weight that affects transport through ultrafiltration membranes but other factors also. Molek and Zydney (57) measured a twofold decrease in the sieving coefficient for ovalbumin (43 kDa) PEGylated with two 5 kDa PEGs compared to PEGylation with one 10 kDa PEG. The protein α-lactalbumin (14.2 kDa) tetra-PEGylated with 5 kDa PEG had a sieving coefficient more than an order of magnitude smaller than that di-PEGylated with 10 kDa PEG (see Fig. 14.6). They attributed this dependence on N to possible deformation and/or elongation of PEG chains caused by convective flow into the membrane pores, and pictured a single conjugated PEG chain as a flexible entity, streaming out in advance or trailing behind a globular molecule as it moves through a membrane pore. A higher number of conjugated chains would add more to the effective molecular diameter because of the constraints to movement at the conjugation sites. Such a view of chain flexibility aiding pore transport is consistent with the observation that PEG molecules have greater sieving coefficients than proteins with an equivalent hydrodynamic radius and similar observations regarding differences between PEG and Ficoll (a highly branched, almost spherical, highly crosslinked polysaccharide).

Molek and Zydney also used a two-stage combination of conventional and charged ultrafiltration membranes to achieve separation of PEGylated α-lactalbumin from reaction byproducts and unreacted precursors (58). Conventional (neutral charge) ultrafiltration membranes were used first to remove unreacted native protein and small reaction byproducts from the reaction mixture by diafiltration, taking advantage of the greatly increased hydrodynamic radius of the PEGylated species. In the second stage, a higher molecular weight cut-off, negatively charged membrane was used in diafiltration mode to repel the PEGylated protein, while allowing the (charge neutral) unreacted PEG to pass through. In diafiltration processes there is, of course, a trade-off between purity and yield, which increase and decrease, respectively, with diafiltration volume. In practice, one cannot completely remove unwanted components by diafiltration, but their concentrations can be considerably reduced before final purification using more selective processes.

Ultrafiltration is almost always negatively affected by fouling, caused by the irreversible adsorption and/or deposition of molecules onto the membrane surface and within membrane pores and by concentration polarization, the buildup of a concentrated boundary layer of protein near the membrane surface. Kwon and others (59) recently compared the fouling and concentration behaviors of native and PEGylated bovine serum albumin (BSA) with (hydrophilic) regenerated cellulose and (more hydrophobic) polyethersulfone membranes. There was little fouling with regenerated cellulose membranes, with flux behavior dominated by concentration polarization effects. The increased size of the PEGylated forms of BSA caused increased protein retention and a decrease in the mass transfer coefficient. For polyethersulfone membranes, considerable fouling was observed, with up to eight times higher fouling for the PEGylated form of BSA compared to its native form. This was rationalized in terms of increased target hydrophobicity following PEGylation, as evidenced by RPC.

The consequences for cleaning and membrane lifetime have not yet been adequately explored. It is expected that filtration of PEG–protein reaction product mixtures will
It also suggests it may be possible to use a combination of temperature, pH, and salt (as well as organic or other solution additives) to achieve conditions that offer greater selectivity in some separation techniques—at least in regard to separation of ungrafted PEGs, PEG proteins of differing \( N \), and native proteins. Finally, it suggests the possible dangers of conducting PEGylation reactions at elevated temperatures. What then will be the effect of PEGylation on the precipitation temperature of a protein, such as cytochrome \( c \), which may come out of solution at a temperature lower than the \( T_c \) of PEG in solution? In that case, the \( T_c \) of the PEG–protein may be higher than the precipitation point of the native protein.

To test the above hypotheses, scientists at GE Healthcare in Uppsala, Sweden, conducted a simple experiment in which a sample of horse cytochrome \( c \) from Sigma-Aldrich (St Louis, MO, USA) was modified with PEG20000-NHS reagent (Nektar) under conditions similar to those used by Fee and Van Alstine (19). Cytochrome \( c \) was chosen, as the protein tends to precipitate out of solution at \(~80–90^\circ\text{C}\) and is easy to monitor due to the red color of the protein. A 2 \( \text{mL} \) volume of 10 mg/mL (protein basis) solution (10 mM NaP, pH 8.9) of unmodified protein aggregated and precipitated (as expected) rapidly at \(~89^\circ\text{C}\), but only very slowly (within 1 h) at \(~79^\circ\text{C}\) (e.g., \( T_c \) of 79°C). By contrast, the PEGylated protein conjugate appeared to have a \( T_c \) above 85°C. The protein, PEG–protein, and ungrafted PEG therefore appeared to have \( T_c \) values ranging over a 20°C interval (61).

Unfortunately, self-association of PEG in solution at elevated temperatures may not provide much insight into (or be useful for high throughput scouting of) HIC-based separation of native and PEGylated proteins at lower temperatures. As noted below for the case of cytochrome \( c \), in some (but perhaps not all) HIC media, native protein elutes before PEGylated protein in a descending salt gradient (Fig. 14.7).

14.3.2.2 Hydrophobicity Seely and Richey published a report on the use of IEC and HIC in the preparation and recovery of PEG-linked proteins (62). Use of HIC was noted as the first step in a purification process for growth hormone by Clark and colleagues (63) and to separate PEGylated forms of growth hormone antagonist by Nijs and co-workers (64). In 1999, Vincentelli and colleagues (65) noted that a PEGylated form of \( \alpha \)-lactoglobulin bound more strongly to a hydrophobic interaction resin than either the free PEG or the native protein. Thiol PEGylation has been used with HIC to aid the separation of plant enzymes that were otherwise difficult to resolve, but the objective was the removal of contaminants by selective modification with PEG rather than purification of the PEGylated protein \( \text{per se} \) (66, 67). In keeping with the SEC results of Fee and Van Alstine (19), there appears to be little resolution in HIC chromatography between various PEGylated forms of a protein, although some resolution of low \( N \) forms may be possible.
This may relate to the fact that once there is enough grafted PEG to dominate the surface, addition of more PEG, or grafting PEG polymers at different sites, offers less basis for selectivity.

Figure 14.7 shows idealized descending gradient HIC elution patterns for the main components of a typical protein PEGylation reaction product mixture. Three different possibilities are shown: (a) unreacted polymer more hydrophobic (more resin interactive) than native protein; (b) unreacted polymer less hydrophobic (less resin interactive) than native protein; (c) unreacted polymer less interactive, but the PEG coating on PEGylated protein more interactive than native protein. Other patterns are of course possible, particularly as a PEG-coated protein surface may be more or less interactive than free PEG in solution (32). Based on practical experience, elution pattern (c) is expected to be the most common when dealing with moderately hydrophobic media. However, patterns may change in relation to media hydrophobicity, ligands including use of ethoxy spacer groups, salts, organic or other buffer additives, and of course mobile phase temperatures approaching $T_c$. This suggests broad room to achieve selectivity with HIC provided high throughput screening of media and conditions is used to optimize any unit operation.

Figure 14.8 shows a descending gradient HIC of a PEG20000–SPA modified $\alpha$-lactalbumin reaction product mixture in a prototype HIC media at GE Healthcare. The gradient was run linearly from 0.7 M NH$_4$SO$_4$, 0.1 M KPO$_4$, pH 7 to 0.1 M KPO$_4$, pH 7. The gradient is being followed in units of inverse conductivity, which is why the gradient slope is shown increasing. Note that, under these conditions, neither the unreacted PEGs nor the native protein bind, and there appears to be some resolution of PEG proteins differing in $N$, although it is no more than one can achieve with SEC (see above) or IEC (see below).

RPC can be used (if the protein or peptide is stable in the solvent phase) for analytical as well as preparative separation of PEGylated proteins—including analytical separation of positional isomers (23, 28, 41, 64). Lee and colleagues (68) treated mono-PEGylated salmon calcitonins first by SEC to separate by PEGylation extent and then by RPC to separate into positional isomers. Johnson and colleagues (69) developed a quantitative reversed-phase assay for use during
manufacturing and for stability studies of a PEGylated sta-
phylokinase mutant for use as a thrombolytic agent. Their
assay was able to detect and quantify de-PEGylation events
as well as the presence of host cell protein contaminants.

14.3.2.3 Partitioning in Aqueous Polymer Biphasic
Systems It has been known for 50 years that modification
of proteins by PEG alters their surface properties to cause
extreme increases in their partition into the PEG-rich phase
of aqueous polymer phase systems formed with PEG (16).
Delgado and colleagues (32) used this to explore the relation
between PEG grafting and partition ratio \( K \) of a variety
of proteins in PEG and dextran aqueous two-phase systems.
Their results suggested a direct relationship between log \( K \)
and the mass fraction of PEG in the PEG–protein conjugate,
which is fully in keeping with the SEC results of Fee and Van
Alstine (19). In spite of this, there appears to be little use of
partitioning in fractionating PEG–proteins.

PEG polymers form a polymer–salt biphasic system at
high salt concentrations. Vincentelli and colleagues (65)
examined the salting-out effects of potassium fluoride and
ammonium sulfate on PEG and related these to the effective-
ness of HIC. They showed that PEG 4600 and native bovine
\( \alpha \)-lactoglobulin co-eluted on Fractogel\textsuperscript{TM} TSK-Butyl (EMD
Sciences), but that the conjugate was more tightly bound
to the matrix, concluding that conformational changes to
the protein may contribute to the surface hydrophobicity.
Another explanation is that the PEG may surround the sur-
face of the protein in such a way as to more fully shield hy-
drophilic regions while leaving hydrophobic regions exposed.
Regardless of the mechanisms at play, partition approaches
may be more suitable than solubility when it comes to PEG
proteins for screening potential HIC separations. Hopefully,
more work will be done in this area over the next few years.

14.3.3 Immunoaffinity and Other Specific
Affinity Interactions

PEGylation can affect affinity interactions in two basic ways,
depending on whether the target or the binding protein is
PEGylated. A general observation is that binding constant
logarithms increase directly with PEGylation. A general observation is that binding constant
depending on whether the target or the binding protein is
PEGylation can affect affinity interactions in two basic ways,

14.3.4 Separations on the Basis of Electrostatic
Charge

IEC is the most commonly used technique for purification of
PEGylated proteins, with many examples available in the
literature (Table 14.3). As noted above, common problems
with the purification of PEG–proteins by IEC and other
capture methods include fouling and a need to work with
dilute feed solutions. In addition, adsorption capacities in
terms of (mg protein) per (mL resin) are often 10\( \times \) lower
than is normal for non-PEGylated proteins. However, this
may, to some extent, reflect the fact that much of the volume
of PEG–proteins is related to PEG not protein. Capacities of
media in terms of total mass per mL, rather than protein mass
per mL, may be more comparable. IEC approaches offer the
possibility to achieve some separation of PEGs, native pro-
teins, and PEGylated proteins in one step. Given the generally
satisfactory performance of SEC media within its inherent
limitations, IEC media is the most commonly used media,
which may be in need of improvement. One attempt to do
so is described in the next section of this chapter.

Most purification methods in the literature use ion exch-
ange at some stage in the process. Invariably, it is reported
that interaction weakens with increased PEGylation extent.
Seely and Richey (62) studied the anion- and cation-exchange
behavior of a PEGylated form of an unspecified protein. Their
comparison was between native protein, mono-PEGylated
protein and a protein dimer linked by the same PEG chain.
Their conclusion that a higher PEG:protein ratio results in
weakened binding due to charge shielding is consistent with
other studies, but their comparison of mono-PEGylated
protein with a PEG-linked protein dimer is not directly com-
parable to a comparison between a mono- and a di-PEGylated
protein. Yamamoto and colleagues (93) examined the effects
of PEGylation extent on ion-exchange behavior of PEGylated
lysozyme. They confirmed the presence of charge shielding
effects. As discussed in more detail below, Pabst and col-
leagues (46) recently compared binding capacities of six ion-
exchange media for native BSA and BSA mono-PEGylated
with 12 kDa and 30 kDa PEG at two different linear flow
rates (50 and 250 cm/h).

PEG may affect the charge properties of proteins in three
ways. First, PEG may shield the surface charges of a protein
and weaken electrostatic interactions. Second, the isoelectric
point (pI) may be altered by conjugation of PEG to charged
residues (effectively, neutralization of a single charge with
<table>
<thead>
<tr>
<th>Protein</th>
<th>PEG MW</th>
<th>$N$</th>
<th>Grafting Method</th>
<th>Purification Step</th>
<th>Purification Step Purpose</th>
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<td>SEC</td>
<td>Removal of unreacted PEG and NaCNBH$_4$</td>
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<td>Separation of PEGylated forms</td>
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<td>SEC</td>
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<td>Insulin</td>
<td>750 Da, 2 kDa</td>
<td>1</td>
<td>Succinimidyl propionate</td>
<td>SEC</td>
<td>Analytical separation</td>
<td>81</td>
</tr>
<tr>
<td>Staphylokinase</td>
<td>5 kDa</td>
<td>1</td>
<td>Maleimide</td>
<td>SEC</td>
<td>Analytical separation</td>
<td>69</td>
</tr>
<tr>
<td>Epidermal growth factor</td>
<td>2, 5 kDa</td>
<td>1–3</td>
<td>Succinimidyl propionate, succinimidyl succinamide</td>
<td>Dialysis and lyophilization</td>
<td>Buffer exchange to stop reaction and recover product</td>
<td>82</td>
</tr>
<tr>
<td>Human granulocyte colony-stimulating factor</td>
<td>6, 12, 20, 25, 30 kDa</td>
<td>1</td>
<td>Aldehyde</td>
<td>SEC</td>
<td>Analytical separation</td>
<td>83</td>
</tr>
<tr>
<td>Anti-interleukin-8 F(ab')2</td>
<td>20, 40 kDa</td>
<td>1–4</td>
<td>Succinimidyl propionate, N-hydroxy succinimide</td>
<td>SEC</td>
<td>Analytical separation</td>
<td>22</td>
</tr>
<tr>
<td>Immunoglobulin antigen binding domains (Fv fragments)</td>
<td>2, 3.4, 5, 10, 12, 20 kDa</td>
<td>Various</td>
<td>Hydracid hydrochloride, trichlorophenyl carbonate, N-hydroxy succinimide, succinimidyl carbonate, thiazolidine-2-thione, bifunctional succinimidyl carbonate</td>
<td>SEC</td>
<td>Isolation of PEGylated forms</td>
<td>23</td>
</tr>
<tr>
<td>Salmon calcitonins</td>
<td>12 kDa</td>
<td>1, 2</td>
<td>Succinimidyl carbonate</td>
<td>SEC</td>
<td>Fractionation of native and PEGylated forms</td>
<td>68</td>
</tr>
<tr>
<td>Salmon calcitonin</td>
<td>5 kDa</td>
<td>1</td>
<td>Succinimidyl carbonate</td>
<td>SEC</td>
<td>Isolation of mono-PEGylated forms</td>
<td>68</td>
</tr>
<tr>
<td>Epidermal growth factor</td>
<td>3.4 kDa</td>
<td>1</td>
<td>N-hydroxy succinimide</td>
<td>SEC</td>
<td>Fractionation of mono-PEGylated isomers</td>
<td>28</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>2.3 kDa</td>
<td>1–3</td>
<td>Biotinylated N-hydroxy succinimide</td>
<td>SEC</td>
<td>Fractionation of mono-PEGylated isomers</td>
<td>29</td>
</tr>
<tr>
<td>Ribonuclease A, lysozyme</td>
<td>5 kDa</td>
<td>Various</td>
<td>N-succinimidyl succinate</td>
<td>Capillary electrophoresis</td>
<td>Analytical separation</td>
<td>84</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>5, 10, 20 kDa</td>
<td>2</td>
<td>Maleidophenyl</td>
<td>IEC</td>
<td>Isolation of di-PEGylated forms</td>
<td>85</td>
</tr>
<tr>
<td>Parathyroid hormone</td>
<td>2, 5 kDa</td>
<td>1</td>
<td>Maleimide</td>
<td>SEC</td>
<td>Isolation of mono-PEGylated forms</td>
<td>86</td>
</tr>
<tr>
<td>Protein/Enzyme</td>
<td>Molecular Weight</td>
<td>PEGylation Location</td>
<td>PEGylation Method</td>
<td>Fractionation Method</td>
<td>PEGylation Operation</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------------------</td>
<td>---------------------</td>
<td>-----------------------------------</td>
<td>---------------------</td>
<td>-----------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>5 kDa</td>
<td>Succinimidyl propionate, aldehyde</td>
<td>Dialysis Chromato-focus</td>
<td>Removal of byproduct</td>
<td>Separation of PEGylated forms</td>
<td></td>
</tr>
<tr>
<td>Growth hormone releasing factor 1–29 analog</td>
<td>5 kDa 1, 2</td>
<td>Norleucine succinimidyl ester</td>
<td>Cation exchange</td>
<td>Isolation of mono-PEGylated form</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>Interleukin-2</td>
<td>3, 10, 12 kDa</td>
<td>Enzyme catalyzed alkylamine PEG conjugation with transglutaminase, N-hydroxy succinimidyl PEG</td>
<td>Cation exchange</td>
<td>Isolation of PEGylated forms</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>Uricase</td>
<td>5, 10 kDa</td>
<td>Norleucine succinimidyl ester, lysine succinimidyl ester</td>
<td>SEC/IEC SEC</td>
<td>Removal of reaction by products and unreacted PEG</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>TNF-RI</td>
<td>30 kDa</td>
<td>—</td>
<td>—</td>
<td>Ultrafiltration/dialysis</td>
<td>Analytical separations for dialysis modeling</td>
<td></td>
</tr>
<tr>
<td>Methioninase</td>
<td>5 kDa 2–8</td>
<td>Succinimidyl propionate</td>
<td>Ultrafiltration SEC</td>
<td>Removal of unreacted PEG</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Lysozyme, insulin</td>
<td>5 kDa Various</td>
<td>Norleucine succinimidyl ester</td>
<td>SEC IEC</td>
<td>Removal of native protein</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>BSA, β-lactoglobulin, lysozyme, caricain α-Interferon 2β, interleukin 10</td>
<td>12 kDa 1, 2</td>
<td>Succinimidyl carbonate, dihydroxyridyl</td>
<td>Ultrafiltration/dialysis HIC</td>
<td>Isolation of PEGylated forms</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>Anti-TNF-α scFv fragment</td>
<td>5, 20, 40 kDa</td>
<td>Makimide</td>
<td>SEC</td>
<td>Isolation of mono-PEGylated form</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SEC</td>
<td>Analytical separation</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

* N refers to the molar ratio of PEG polymer to protein.
each PEG group conjugated). Third, PEGs may hydrogen bond with some groups to raise their pKₐ, thus affecting their charge versus pH behavior (32).

The most common observation regarding ion-exchange separation of PEGylated proteins is that more heavily PEGylated species (whether from higher N or higher molecular weight PEG chains) elute at lower ionic strengths than their less heavily PEGylated counterparts. In some cases, highly PEGylated forms are contained in the flow-through fractions of ion exchange, while native and less heavily PEGylated forms bind (33, 46). Such observations are in keeping with SEC results (19) and the concept of the PEG having the possibility under chromatographic conditions to form a weakly associated hydrated polymer “cloud” over the protein surface.

Azarkan and colleagues (66) used thiol PEGylation as a strategy to purify chymopapain for X-ray crystallography and found that PEGylated species bound more weakly to a cation exchanger. Because the pI of the protein was unaltered by PEGylation, they concluded that charge shielding was responsible.

Yamamoto and colleagues (93) demonstrated charge shielding by measuring the elution volumes of randomly PEGylated lysozyme under several salt gradient conditions (Fig. 14.9), showing a correlation between peak salt concentration, Iₛ, and the number of binding sites on the protein, B, according to the law of mass action (Fig. 14.10).

Hecht and colleagues (94) separated PEGylated from native brain-derived neurotrophic factor (BDNF) using a preparative electrofocussing device but were unable to separate BDNF of differing PEGylation extent. The authors suggested the reason for this was that the species with differing degrees of PEGylation probably varied only in the length of PEG chains attached rather than the number, and therefore the pIs of the PEGylated species would not differ. However, the origin of the PEGylated material was from authors (51) who described the variation in the degree of PEGylation as resulting from differing PEGylation extent using a 5000-MW PEG. Nonetheless, it is possible that the pI of the various PEGylated species in Hecht and colleagues’s work did not vary significantly despite their having different degrees of PEGylation. PEGylated proteins, of course, assume net negative and positive charges above and below their isoelectric points, respectively. PEGylation itself may alter the isoelectric point of a protein in cases where each PEG chain binds to and neutralizes a charged residue such as lysine on the protein surface. The change in pI with PEGylation will depend on the number of charged residues on the protein and the shape of the net charge versus pH curve near the isoelectric point itself (30).

Piquet and colleagues (87) described large laboratory-scale chromatographic separation of PEGylated growth hormone release factor (GRF) using a gram-scale column 20 cm × 5.5 cm with stepwise elution of monoPEG-GRF.
Process yield (including PEGylation) was 41% and purity of monoPEG-GRF was ~97%. Hall and colleagues (79) compared conventional anion- and cation-exchange media with monolithic columns and claimed much faster processing in the latter, while analytical separations were faster in monolithic columns but with poorer resolution. This work was for relatively small volume columns. Chapman and colleagues (21) fractionated PEGylated antibody fragments using SP Sepharose High Performance media. The fragments were modified using PEG-maleimide reagents (25 kDa or branched 40 kDa from Nektar Therapeutics, Huntsville, AL, USA) and PEGylated in a controlled fashion at low N, which favored their ease of purification.

Similar to the 1996 success of Kinstler and colleagues with cation exchange (95), Lee and colleagues (96) were recently able to separate mono-, di-, and tri-PEGylated forms of a recombinant granulocyte colony stimulating factor using anion exchange, obtaining a good purity of N-terminal mono-PEGylated product. Yamamoto and colleagues (93) showed that not only the PEG:protein molecular weight ratio, but also the site of conjugation, is important in determining ion-exchange behavior. They were able to separate three mono-PEGylated forms of lysozyme by cation-exchange chromatography. However, the differences in the strengths of electrostatic interactions between positional isoforms are subtle and may not be exploited effectively at the preparative scale.

Aside from charge considerations, ion-exchange capacity will be affected by the size of the molecules, lowering diffusivity and access to internal pores in chromatography media. From Equations 14.3 and 14.4, a 20 kDa protein, PEGylated with a 20 kDa PEG, has viscosity radii of native, mono-, and di-PEGylated forms equivalent to 20, 265, and 730 kDa protein molecular weights, respectively. These size differences are significant and one would expect associated differences in mobility to affect access to ion-exchange binding sites. PEG is commonly used to repel proteins from surfaces (1, 60), so there is a possibility that PEGylated molecules, once bound on the exterior of a chromatography resin, could repel further molecules from approaching, particularly larger, slower-moving, more heavily PEGylated species.

Brumeau and colleagues (20) found that highly PEGylated species were contained in the flow-through fraction during anion exchange, while mildly PEGylated species eluted at lower salt concentrations than native proteins. They also noted that prior removal of free PEG was extremely important in obtaining good resolution, as reported by McGoff and colleagues (40) in charge-reversal capillary zone electrophoresis. Sherman (97) and Olson (98) and colleagues showed that increased PEGylation decreased the conductivity required for elution.

Pabst and co-workers (46) compared six strong anion-exchange resins for separation of native BSA and BSA mono-PEGylated with 12 and 30 kDa PEGs using flow rates and residence times in the ranges typically found in large scale biopharmaceutical purification processes. As expected, the conductivity at elution from all media was lower for PEGylated BSA than for native BSA and the 30 kDa PEG—BSA eluted at a lower salt concentration than 12 kDa PEG—BSA. Some dynamic binding capacity (DBC) data at 50 cm/h is shown in Table 14.4 for these media. Superficial velocity did not have a large affect on DBC, with DBC values at 250 cm/h reduced by 25% for the best performing media. As expected, PEGylation reduced the dynamic binding capacities of the media significantly. Three of the media studied showed virtually no dynamic binding capacity for 30 kDa PEG—BSA, explained by almost total exclusion of the target from the internal pores of the media. Exclusion and hindered diffusion in the internal pores of agarose media also explains the DBC trends observed for those media. Q Sepharose Fast Flow (Q FF) media did not have the highest DBC for native BSA, but it exhibited higher DBCs for 30 kDa PEG—BSA than the six other media tested. The authors noted that Q FF media has a mean pore radius of 288 Å (46) compared to the calculated hydrodynamic radius of 64 Å for 30 kDa PEG—BSA. Capto™ Q had the highest capacity for native BSA and 12 kDa PEG—BSA, but it had a lower DBC than Q FF for 30 kDa PEG—BSA. This was explained by the presence of dextran ligand tethers in Capto Q, which impart very high binding capacities for the native protein but hinder pore diffusion by more overtly PEGylated forms. This is in keeping with the basic phase incompatibilities and dextran and PEG polymers (16, 32, 44, 45). Macroprep™ High Q media had the lowest DBC of all for native BSA, but DBC was not affected by PEGylation.

## TABLE 14.4 Dynamic Binding Capacity of Strong Anion-Exchange Media for PEG—BSA

<table>
<thead>
<tr>
<th>Resin</th>
<th>BSA</th>
<th>PEG 12K—BSA</th>
<th>PEG 30K—BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DBC, mg/mL</td>
<td>DBC, mg/mL</td>
<td>DBC, mg/mL</td>
</tr>
<tr>
<td>Capto™ Q</td>
<td>144</td>
<td>51</td>
<td>8</td>
</tr>
<tr>
<td>Fractopropt™</td>
<td>114</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>TMAE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q Hyper DFT™</td>
<td>61</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Q Sepharose™ FF</td>
<td>53</td>
<td>30</td>
<td>18</td>
</tr>
<tr>
<td>Toyopearl™</td>
<td>36</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Super Q 650C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macroprep™</td>
<td>16</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>High Q</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dynamic binding capacity at 10% breakthrough (DBC) from Pabst et al. (46). BSA, bovine serum albumin. Capto and Sepharose (GE Healthcare), Fractopropt (EMD Sciences), Q Hyper DF (Sigma), Toyopearl (Tosoh Biosciences), Macroprep (Bio-Rad Laboratories). BSA run at 250 cm/h while PEG—BSA samples run at 250 and 50 cm/h.
As pointed out by Pabst, the reduction in DBC for PEGylated forms compared to the native form is not necessarily a fatal flaw. On the contrary, a media such as Q Hyper™ DF or Fractoprep™ TMAE could be used to selectively bind the native form, with PEGylated forms collected in the flow-through, although this would not remove reaction byproducts that do not bind to the ion exchanger. Pabst showed a 97.5% purity of PEGylated BSA in the flow-through fraction using Q Hyper DF media, with native BSA bound onto the column. This approach would be less useful for separating PEGylated forms of differing $N$.

14.4 ION-EXCHANGE MEDIA SPECIFICALLY DESIGNED FOR PEG–PROTEINS

14.4.1 Design Considerations

The above practical and comparative ion-exchange results suggest that both anion-exchange chromatography (AEC) and cation-exchange chromatography (CEC) work with PEGylated proteins and provide good resolution, although often lower than expected dynamic binding capacities. This is especially true when the target is modified with a significant amount of PEG and chromatography is run at moderately high flow rates (>50 cm/h). Such results would appear to be related simply to the hydrodynamic size of PEG–protein conjugates, many of which can have viscosity radii exceeding that of very large proteins such as IgM (Fig. 14.4). Targets diffuse slowly and only have access to a fraction of the pores of most modern chromatographic media. This suggests that two attributes of ion-exchange media optimized for larger PEG–proteins would be larger mean pore size and polymer materials offering fast intraparticle (pore) diffusion rates. With regard to particle size, one has to balance the need for smaller particles (to reduce mass transport distance into the particles) against need for larger particles that have lower pressure drops, especially as PEG–protein solutions may have increased viscosity. The media should be optimized with regard to the strength of ionic interactions with both PEGylated proteins (which suggests higher ligand density) and native proteins (which suggests a ligand density that still allows for low nonspecific adsorption and effective elution of native protein). Finally, the media should offer excellent chemical stability at both high and low pH so as to deal with various clean-in-place regimes. Such a media was recently taken forward by GE Healthcare and launched as MacroCap™ (Table 14.5).

MacroCap is available as SP-ligand-based CEC media and Q-based AEC media. MacroCap SP is available as Bioprocess media and as such is available for direct use in biopharmaceutical processing. MacroCap Q is also commercially available on request from GE Healthcare Life Sciences, Custom Designed Media (GE Healthcare, Uppsala, Sweden).

### Table 14.5 MacroCap SP

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base matrix</td>
<td>Acrylamide–dextran copolmer</td>
</tr>
<tr>
<td>Average particle diameter</td>
<td>50</td>
</tr>
<tr>
<td>Ligand</td>
<td>Sulfopropyl (SP)</td>
</tr>
<tr>
<td>Ionic capacity for Cl⁻, µmol H⁺/mL gel</td>
<td>100–130</td>
</tr>
<tr>
<td>pH stability short term</td>
<td>2–13</td>
</tr>
<tr>
<td>pH stability long term</td>
<td>4–11</td>
</tr>
<tr>
<td>Recommended separation range, kDa</td>
<td>&gt;150 kDa or PEG proteins with PEG &gt;20</td>
</tr>
<tr>
<td>Recommended large column bed height, cm</td>
<td>15 to 30</td>
</tr>
<tr>
<td>Recommended flow rate, cm/h</td>
<td>120 (0.1 MPa for 20-cm-high bed)</td>
</tr>
</tbody>
</table>

14.4.2 MacroCap Ion-Exchange Media

Figure 14.11 illustrates how the larger mean pore size distribution of MacroCap SP allows for better chromatographic performance with native IgM protein. With the other commercial SP media (dotted line), much of the IgM is in the flow-through, even when loading the column with 1 mg/mL solution of protein to total of 2 mg of IgM per mL of media in the column.

Figure 14.12 illustrates ion exchange with an ascending gradient for PEG40000 modified egg-white avidin (Sigma Aldrich) of 66 kDa, pl 10, on SP Sepharose High Performance (HP) and MacroCap SP media (GE Healthcare). Sample loading was at 10 g protein per L media and flow

![Figure 14.11](image-url)
Figure 14.12  Ion-exchange chromatography of PEG40 avidin protein on SP Sepharose HP (SP HP) and on MacroCap SP media. The broader peaks are for the MacroCap media (33). From GE Healthcare, with permission.

Figure 14.13  Ion-exchange chromatography of PEG20–cytochrome c reaction product mixture at 6 g total protein per L of media on MacroCap SP (A) and another commercial acrylate based SP media. SEC analyses of peak fractions are also shown. From GE Healthcare, with permission.
rate was 60 cm/h. Buffer A was 0.05 M NaAcetate while buffer B was A + 0.6 M NaCl. As a glycoprotein with a relatively high pI, and a MW in the range of Fab fragments and other biopharmaceuticals, avidin is a good test protein for IEC. Several points can be noted. First, the elution peak shapes for the native, mono-PEGylated, and diPEGylated reaction mixture components are sharper in the 30-μm-diameter HP particles than in the 50-μm MacroCap media. Second, in spite of the much larger mean pore size and lower SP ligand density in the MacroCap media the ligand surface density in the pores is similar to that of the HP media, with the various peaks eluting in approximately the same conductivities in the gradient. Third, although at low loading an operator might favor the HP media (i.e., normal commercial bioprocess media), at the higher loading shown in the figure, much of the target appears to be lost in the HP flow-through.

Figure 14.13 shows comparative benchmarking for ion exchange of reaction mixture of PEG20 cytochrome c using MacroCap SP, which has an acrylated dextran matrix (Table 14.5) and another commercial SP media, which has an acrylated matrix. Both samples were run with loading at 14 mS/cm so that the oligo-PEGylated protein flowed through. Loading was at 6 g of total protein (native plus mono-PEGylated plus oligo-PEGylated by A280 nm) per L of media with a flow rate of 61 cm/h. Buffer A was 0.05 M sodium phosphate, pH 6.8 and Buffer B was A + 0.4 M NaCl. Column fractions were analyzed by SEC analysis using a Superdex 200 column with A280 nm absorption to give protein concentration related to native, mono-PEGylated, and oligo-PEGylated protein. It can be seen in Figure 14.13 that, based only on the A280 nm absorption, the two media appear quite similar, and based on non-thorough evaluation, MacroCap media appears to offer few advantages with regard to selectivity. However, when the peaks are analyzed, it is seen that MacroCap offers significant advantages with regard to peak purity. Such data reinforce the need to both evaluate IEC media at high loads and use SEC or analogous methods (e.g., Maldi-Tof MS or PAGE) for peak analysis.

One major phenomena functioning in fractionation of PEG–protein reaction mixtures is displacement, which is illustrated in Figure 14.14 and is well known from the work of various scientists involved with adsorption of proteins at surfaces and is often called the “Vroman Effect” (99). Basically, smaller proteins and/or those with greater affinity for the surface will tend to displace other proteins. In the case of PEG–protein reaction product mixtures this means that native proteins tend to bind at the top of the column, while mono-PEGylated proteins tend to be displaced and bind lower down in the column and replace oligo-PEGylated protein. There are two fortuitous effects of this phenomenon. First, as native protein typically binds with 10× higher dynamic binding capacity, the separation of non-PEGylated and PEGylated proteins, which occurs on an IEC column, comes with little loss of total column capacity for the target PEGylated protein. Second, increasing target load can actually lead to an increase in protein purity in the target peak.

Table 14.6 provides peak purity data similar to that noted in Figure 14.13 for chromatographic runs of PEG20–cytochrome c reaction product mixtures on MacroCap SP. Flow rate was 100 cm/h. It can be seen that as the load concentration was increased from 6 to 12 g of total protein per L of media in the column bed, the purity of the target mono-PEGylated protein peak increased from 93 to 98%, which is commensurate with K values increasing from 13 to 99.

<table>
<thead>
<tr>
<th>PEG20-Cyt c MacroCap&lt;sup&gt;TM&lt;/sup&gt; SP</th>
<th>Purity</th>
<th>Oligo Fraction</th>
<th>Mono Fraction</th>
<th>Protein Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 mg/mL media</td>
<td>% Oligo</td>
<td>96</td>
<td>5</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>% Mono</td>
<td>3</td>
<td>93</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>% Protein</td>
<td>1</td>
<td>2</td>
<td>&gt;99</td>
</tr>
<tr>
<td>12 mg/mL media</td>
<td>% Oligo</td>
<td>96</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>% Mono</td>
<td>4</td>
<td>98</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>% Protein</td>
<td>—</td>
<td>&lt;1</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

Data relates to SEC analysis of chromatograms in Fig. 14.13.

**Figure 14.14** Displacement as it relates to a PEGylated protein reaction product mixture. Frome GE Healthcare, with permission.
14.4.3 Development of a MacroCap SP Cation-Exchange-Based Separation Step

We will now see how the above considerations can be used in developing a CEC step for the purification of a PEG40 modified egg-white avidin reaction product mixture using MacroCap SP. Similar considerations would apply independent of the media selected. In the example given in Figure 14.15a–d (33), the column was run under larger scale process conditions of 100 cm/h and 15 g total protein in reaction product mixture loaded per L of media. Buffer A was NaAcetate, pH 5 and B was buffer A plus 0.5 M NaCl. In chromatogram (a), the adsorption conductivity was 20 mS/cm buffer NaAcetate and the resulting target column yield was 77% by $A_{280}$, with a purity of 99% by SEC. Some target loss occurred as the oligo-PEGylated protein fraction in the flow-through was not at baseline. In (b), the adsorption conductivity was 14 mS/cm buffer NaAcetate and the resulting target column yield was 99% with a purity of 89% by SEC. Chromatogram (c) represents a compromise.

Figure 14.15  PEG40–avidin reaction product mixture ion exchange on MacroCap SP at 100 cm/h and reaction product mixture loading of 15 g protein per L of media (33). From GE Healthcare, with permission.
between (a) and (b), with the adsorption conductivity at 17 mS/cm buffer NaAcetate and the resulting target column yield of 95% with a purity of 95% by SEC. In (d), a step gradient has been added and the result is baseline resolution for all three fractions and yield and purity both \( \gg 95\% \). These results indicate that significant unit operation performance may reflect small changes in operating conditions and that it is best to optimize an operation with linear gradients before adding in step gradients.

### 14.5 SUMMARY

Separation of PEGylated proteins is challenging because PEG itself is a relatively inert, neutral, hydrophilic polymer and the starting point for PEGylation is a pure protein. Thus, other than molecular weight and size, differences in the physicochemical properties typically used to fractionate proteins may be slight between different PEGylated forms of a protein. The usual properties of electrostatic charge and molecular weight (size) form the basis of the most commonly used separation techniques, particularly IEC, SEC, and ultrafiltration. The main effect of PEGylation on ion-exchange separations is to shield the electrostatic charges on the protein surface and to reduce the strength of interactions with higher PEG chain molecular weight or higher PEGylation extent. Thus, ion exchange can be used very effectively to separate on the basis of PEGylation extent for low extents, but as \( N \) increases, the effectiveness of separation rapidly diminishes. Separation of positional isomers is possible by RPC or ion exchange at analytical scale, but it is problematic at the preparative scale due to the small size of the differences in electrostatic interactions between isomers.

PEGylation imparts significant changes in molecular weight with each chain added to a protein and there are corresponding increases in molecular size, so SEC and ultrafiltration (and dialysis) are effective methods for separating native and PEGylated proteins. However, the relative size difference between variants differing in PEGylation extent by one adduct reduces with \( N \), so that efficient SEC separation between PEGylated species differing by one PEG chain is not likely to be economical at the preparative scale for \( N > 3 \). This holds true even for PEG proteins produced with large PEG polymers \( (M_r \geq 20 \text{kDa}) \). For small PEG polymers \( (M_r = 2 \text{kDa}) \), only native and PEGylated species can be separated effectively. At the analytical scale, with proper calibration, SEC can provide valuable information on PEGylation extent. Membranes can be used to reduce the concentration of smaller molecular weight species by dialysis but cannot fully remove them, and an operational trade-off between purity and yield is required. Gel electrophoresis can confirm PEGylation reactions have proceeded and indicate the relative purity of products, but its use to confirm PEGylation extent requires further research. The main drawback of separations based solely upon molecular size is that they cannot differentiate between positional isomers. Capillary electrophoresis is an exception, quantitatively combining any or all of size, shape, conformational freedom, and small differences in protein surface properties to separate by both PEGylation extent and positional isomerism.

Relative hydrophobicity is a useful property for analytical separations using RPC, but HIC, which is used routinely for production-scale purification of proteins, does not appear to be particularly useful for separation of PEGylated species.

### 14.6 REFERENCES


PART IV

ELECTROPHORESIS
15

ELECTROPHORESIS IN GELS

REINER WESTERMEIER
SERVA Electrophoresis GmbH, Carl-Benz-Strasse 7, D-69115 Heidelberg, Germany

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15.1 INTRODUCTION
Electrophoresis is the migration of charged particles and molecules in an electric field. Because the speed of migration differs depending on charge and size, the components of a sample are separated into zones and can be displayed as a characteristic pattern. The electrophoretic principle was discovered by Arne Tiselius (1) and developed into the so-called “moving boundary electrophoresis” in free solution. The technique allowed him to separate human serum into its four major components: albumin and the α-, β-, and γ-globulins. This separation is celebrated on a stamp of the Swedish mail service as a reminder that he was awarded the Nobel prize in 1948 (Fig. 15.1).

The easiest way to carry out protein electrophoresis is in a gel, which serves as an anticonvective medium. The separation results are easily visualized by staining the gels with dyes, which specifically bind to proteins. Electrophoresis of proteins in gels has become a widespread separation technique, because it has a very high resolving power, is fast, easy to perform in relatively simply designed instruments, and the consumables do not cost very much. The principle of electrophoresis is also relatively easy to understand.

15.2 PRINCIPLE
Each protein has a specific electrophoretic mobility, \( m \), which determines its migration velocity, \( v \), in an electric field \( E \) (measured in V/cm), and is therefore decisive for the separation:

\[
v = m \times E.
\]

Electrophoretic mobility is dependent on the net charge and the size of the molecule. Proteins with different
electrophoretic mobilities migrate in the electric field with different migration velocities and form discrete zones.

15.2.1 A Few More General Parameters

It may be useful to understand a few more parameters to explain the process of migration.

In the electric field \( E \), an accelerating electric force, \( F_e \), acts on a protein molecule with a net charge \( q \):

\[
F_e = q \times E.
\]

There is also a countering retardation force, \( F_r \), which is dependent on the migration velocity of the protein molecule \( v \), and the frictional coefficient \( f \), which is dependent on the viscosity of the medium, the temperature, and the pore size of the gel:

\[
F_r = v \times f.
\]

The balance of the two accelerating forces causes the migration of the protein molecule with a constant velocity:

\[
F_e = F_r; \quad q \times E = f \times v; \quad v = \frac{q \times E}{f} = m \times E.
\]

The longer the separation distance, the higher voltage necessary to reach certain electric field strength. It is the electric field strength that drives the migration, not the current.

The electric current is dependent on the composition, and thus the conductivity of the buffer. A minimum concentration of buffer is necessary to maintain constant charges on the proteins.

The relative mobility \( m \) is determined by relating the distance of migration of a sample component to the distance of migration of the tracking dye, often Bromophenol blue.

15.2.2 Behavior of Proteins in Electrophoresis

Because proteins are amphoteric molecules, they will have different net charges that depend on their pH environment. Figure 15.2 presents a schematic of a net charge curve of a protein.

The shape of the curve and its intersection with the x-axis describe the behavior of a protein at a certain buffer pH value and in the electric field. It should be mentioned that the net charge curves of proteins can be determined by a simple-to-perform electrophoretic technique called titration curve analysis (2), which is very valuable for ion-exchange chromatography (IEC, see Chapter 4) and isoelectric focusing. The procedure will be explained in Chapter 16 (on isoelectric focusing).

There are several electrophoretic separation principles that can be applied on proteins: moving boundary electrophoresis, isotachophoresis, isoelectric focusing, and zone electrophoresis. This chapter deals only with zone electrophoresis methods (often called just electrophoresis). The separation principle of zone electrophoresis is shown in Figure 15.3.
each other, will not aggregate, and all migrate toward the anode. Glycine is very hydrophilic; it does not bind to proteins. Furthermore, polyacrylamide gels are easy to polymerize at basic pH with TEMED and ammonium persulfate (see below).

The anions and cations of the buffer migrate in the electric field toward the anode and cathode, respectively, transporting the electric current. The higher the concentration of buffer, the higher the current, and the more Joule heat developed. The production of Joule heat is the reason why electrophoretic separations are mainly used for analytical purposes and cannot be upgraded to preparative procedures, as is possible with chromatography. Joule heat production is proportional to the applied electric power $P$, measured in watts, which is the product of voltage $U$ and current $I$:

$$P = U \times I.$$  

To limit heat production, the gels are either cooled during the run or the run is performed with a limited electric field strength and hence has longer separation times. If the heat dissipation from a slab gel is insufficient and/or the gel is run too fast, the zones in the center of the gel will migrate faster than on the two sides, resulting in a curved front. This phenomenon is called the “smiling effect.”

The migration of the buffer components in the electric field has a second consequence. In order to maintain constant pH and buffer conditions, the supplies of buffer ions must be large enough for the electrophoretic run, so buffer reservoirs are needed between the electrodes and the gel ends.

An alternative to such “mobile” buffers are “stationary” buffers, which were—in principle—first used in titration curve analysis (2). An amphoteric buffer with a high buffering capacity at its own isoelectric point will have a net charge of zero and thus does not migrate in the electric field. It is however important, that all charged components have been removed from the gel, for instance by washing the gel with distilled water. Such a buffer will behave like a single homolog of carrier ampholytes during titration curve analysis. Very low Joule heat will be developed and buffer tanks reservoirs are not needed. However, the sample proteins will be charged and migrate electrophoretically. The separation can be run with a very high electric field, very quickly, without thermal problems. Such an electrophoresis in a stationary buffer can be realized in a polyacrylamide gel, from which the charged catalysts TEMED and ammonium persulfate have been removed by pre-electrophoresis or by washing with deionized water. Some examples and applications will be described later.

15.3 GELS

Polyacrylamide gels are predominantly used for protein electrophoresis; in some exceptional cases agarose gels are used. The structures of these gel matrices are presented in Chapter 2 (Introduction to Chromatography).

15.3.1 Agarose

Agarose gels are used for protein electrophoresis in clinical diagnostics and when large pores for the analysis of large proteins over 800 kDa are needed. Agarose is a polysaccharide obtained from red seaweed. By removal of the agarpectin, gels of varying electroendosmosis and degrees of purity can be obtained. They are characterized by their melting point (35–95°C) and the degree of electroendosmosis (see below). The electroendosmosis factor $m_e$ is dependent on the number of polar groups left. The pore size depends on the concentration of agarose. In general, gels with a pore size from 150 nm [from 1% (w/v) agarose] to 500 nm (at 0.16%) are used. Agarose is dissolved in boiling water or buffer, and the gel forms upon chilling. Agarose gels are mainly run in horizontal flatbed systems, because they have a tendency to slide down between the glass plates in a vertical set-up. For a mechanical support the gels are cast on glass plates or plastic films, which have been precoated with a solution of agarose. Usually 1-mm-thick gels are used.

15.3.2 Polyacrylamide Gels

Polyacrylamide forms a much more restrictive gel, is mechanically and chemically more stable, has much lower electroendosmosis, and is clearer than agarose. It is formed by a
chemical copolymerization of acrylamide monomers and crosslinker, mostly \(N,N'\)-methylenebisacrylamide (Bis). The pore size can be exactly and reproducibly controlled by the total acrylamide concentration \(T\) and the crosslinking factor \(C\) (3):

\[
T = \frac{a + b}{V} \times 100 \text{ (%)}; \quad C = \frac{b}{a + b} \times 100 \text{ (%)}
\]

where \(a\) is the mass of acrylamide in grams, \(b\) is the mass of Bis in grams, and \(V\) is the volume in mL.

With increasing total acrylamide concentration \(T\), the pore size will decrease. With increasing crosslinking factor \(C\) at constant \(T\), the pore size follows a parabolic function with a minimum at \(C \approx 5\). These gel structures have been visualized with electron microscope images by Rüchel and colleagues (4), as shown in Figure 15.4.

In the practice of protein electrophoresis, total acrylamide values of between 7 and 18\% \(T\) are used; lower concentrations of gels are unstable and/or show poor sieving, and more concentrated gels modify the structures of some proteins. Gels with high crosslinking factors are brittle and relatively hydrophobic. Thus, in protein electrophoresis, \(C\) values of 2.5–3\% are generally used. A comprehensive overview of differently crosslinked gels can be found in Righetti’s book on isoelectric focusing (5).

The polymerization is initiated by radicals, which split off from ammonium persulfate in the presence of tertiary amines, which are supplied by TEMED in the monomer solution. Oxygen interrupts the chain formation and must, therefore, be excluded. The polymerization of polyacrylamide gels is performed in closed cassettes, and the monomer solutions should be deaerated for optimal polymerization effectiveness. The polymerization kinetics is dependent on oxygen content, monomer concentration, temperature, and pH value.

This catalyst system works only at neutral and basic pH. For the polymerization of acidic gels, alternative reagents are used, such as ascorbic acid with ferrous sulfate. It is very tricky to perform this polymerization, because the gelation process runs very quickly and is difficult to control. It is by far easier to prepolymerize the film-supported gels at neutral pH, and wash and rebuffer them with acidic buffer subsequently.

In general, polyacrylamide gels should not be used immediately after gelation, because there is a silent polymerization going on, which forms the final matrix. The best measure is to leave the gels at room temperature overnight before using them. Gels containing an alkaline buffer have a limited shelf life, because at high pH values the matrix begins to hydrolyze after a few weeks.

15.3.3 Electroendosmosis

Electroendosmosis is a phenomenon that occurs in an electric field, when a gel medium contains fixed charged groups. This can happen in polyacrylamide gels, when impure acrylamide is used for polymerization: acrylic acid will be built into the matrix, thus adding carboxylic groups. Agarose still contains sulfonic and carboxylic groups from the remains of agarpectin. In a basic buffer these acidic groups become deprotonated, which means that these moieties become negatively charged. These negative charges are attracted by the anode. However, they cannot migrate, because they are fixed. This fact causes a counteraction: the migration of protonated water molecules in the cathodal direction. The phenomenon can be observed during electrophoresis as shrinking of gels in the anodal area and sweating, sometimes even water flooding in the cathodal region. The results show blurred zones with a loss of sensitivity of detection.

High quality acrylamide reagents should therefore be used for gel polymerization. Acrylic acid can be removed from an

Figure 15.4 Polyacrylamide gel structures dependent on \(T\) and \(C\) values. Electron microscope images. Horizontal series: varying \(T\) values, constant \(C\) values. Vertical series: constant \(T\) values, varying \(C\) values. From Reference 4, with permission.
acrylamide monomer solution with the help of a mixed-bed ion exchanger such as Amberlite IRN-150. Highly purified agarose shows reduced electroendosmosis effects, defined as the $m_r$ factor. Electroendosmosis is highly disturbing when high voltages are applied, as in isoelectric focusing. For such high performance separations the remaining negative charges are counterbalanced with positively charged groups, as in the “Agarose IEF” from GE Healthcare Life Sciences. Electroendosmosis can also occur on glass surfaces, because they carry charged silicium oxide groups. This can cause problems during electrophoresis in glass or quartz capillaries.

In the following, polyacrylamide gel electrophoresis techniques are mainly described.

15.4 GEL GEOMETRY AND EQUIPMENT

Two different ways of running electrophoresis gels are possible: vertical and horizontal (Fig. 15.5). The electrodes are usually made from platinum wire, because all other material will disintegrate after a few runs.

15.4.1 Vertical Systems

15.4.1.1 Gel Rods Polyacrylamide electrophoresis carried out in cylindrical gel rods in glass tubes, as shown in Figure 15.5A, is history. Sometimes, this method has been confused with “disc electrophoresis,” because the stained...
protein bands are disc-shaped. In the past, disc electrophoresis, which means in reality “discontinuous electrophoresis,” had often been performed in such gel rods. Discontinuous electrophoresis will be described later. The gel rod technique is very work-, time-, and skills-demanding, and the results are complicated to evaluate. Blotting is practically impossible.

15.4.1.2 Vertical Slab Gels  Vertical slab gels are mostly polymerized between glass plates and lateral spacers, which determine the gel thickness. The gels are left in these cassettes from casting to the end of the run. The samples are loaded in slots on the top edge (Fig. 15.5B,C), which have been formed by inserting a comb during polymerization. Combs with different thicknesses and numbers of teeth are available. Prefabricated gels are shipped in the cassette, including the inserted combs, and they are then inserted into the appropriate apparatus. So-called “preparative” combs, which form a wide opening across the gel width, are used when subsequent blotting—electrophoretic transfer of the proteins onto an immobilizing membrane—for the preparation of probing strips is intended (see Chapter 18).

The upper and lower gel edges are in direct contact with the electrode buffers. To prevent mixing of the samples with the upper buffer, \( \approx 20\% \) glycerol or sucrose is added to the sample buffer in order to increase the density of the samples markedly.

In the simplest design for vertical electrophoresis (B) the gels cannot be cooled. The applied electrophoretic field has therefore to be limited accordingly, and also when the run is carried out in a cold room. Heat dissipation via air contact is not very efficient.

It is possible to cool the gel just from one side via the upper buffer tank (not shown). In this case the set-up looks similar to design (B), but with the upper buffer tank reaching the lower edge of the gel. Either the upper buffer tank is used as a heat sink or it is actively cooled from the other side with a heat exchanger plate. This design works best with gels thinner than 1.5 mm, because the heat is removed only from one surface. If the two surfaces of gel have markedly different temperatures, the protein zones on the warm side migrate faster than on the cool side. This leads to the so-called “venetian blinds” effect, where the bands are slanted within the gel layer. When silver staining is employed for protein detection, double bands can occur, because it stains mainly the proteins close to the surface. The effect can be avoided by reducing the electric field.

The best concept for a vertical system is realized in an apparatus that cools the gels via the lower buffer and a heat exchanger (C). By circulation of the lower buffer the heat dissipation can be made so efficient that two double sandwiches of gels can be run at a time.

For all vertical designs it is important to prevent leakage of the upper buffer tanks. Figure 15.6 shows a discontinuous sodium dodecyl sulfate (SDS) vertical gel after staining with Coomassie Brilliant Blue.

15.4.2 Horizontal Systems

The gels are polymerized in glass cassettes that are built from two glass plates, one with a glued-on U-shaped gasket and clamps (Fig. 15.7).

The gels are usually 0.5 mm thick and polymerized on a polyester support film GelBond PAG Film™. The sample wells are formed by self-adhesive embossing tape glued on the glass plates. Such gels are available prefabricated under the name ExcelGel™ SDS from GE Healthcare. Alternatively, for the purpose of blotting (see Chapter 18), the gels can be polymerized on a polyester net and big sample application wells can be formed. In Figure 15.8, such a gel—stained with Coomassie Brilliant Blue—is shown.

Gels for horizontal flatbed electrophoresis are removed from the casting cassette before use. Prefabricated gels are packed in aluminum bags; glass plates are not needed.

A horizontal gel is placed on a cooling plate, which is connected to a thermostatic circulator. Thin gels, \( \approx 0.5 \) mm, are preferred, because the gels are cooled from only one side. The samples are easy to apply with a pipette; it is not necessary to add sucrose or glycerol to the sample.

Figure 15.5D shows different types of electrode buffer reservoirs: (a) lateral buffer tanks containing the electrode...
wires, connected to the gel edges with paper wicks, which are soaked in electrode buffer; prefabricated disposable polyacrylamide strips containing electrode buffers in high concentration [this concept reduces the chemical and—if applicable—radioactive waste considerably (6)]; (c) thick filter cardboard strips soaked with concentrated electrode buffer. For (b) and (c), electrodes designed for isoelectric focusing gels are applied on top of the electrode strips.

Figure 15.5E shows the set-up for small gels used in the PhastSystem® (from GE Healthcare). For short separation distances—4 cm in this case—agarose gel strips can be used as buffer reservoirs, because electroendosmosis effects will not show up during the short separation time required for such small gels.

15.5 TECHNIQUES

When a simple buffer like Tris–borate–EDTA or Tris–glycine is used for gel electrophoresis of proteins, a problematic effect will happen: the proteins in the liquid sample are highly charged and will thus start to migrate in the electric field with high velocity. When they enter the gel edge at the bottom of the sample well, they will be slowed suddenly and concentrated because of the strong retardation effect of the gel. The sudden concentration of a protein mixture leads very often to the formation of aggregates and precipitates. This phenomenon can be observed as a strong protein zone at the bottom edge of the sample well, and smearing proteins in the lane instead of bands.

15.5.1 Disc Electrophoresis

Ornstein (7) and Davies (8) created the concept of discontinuous (disc) electrophoresis to solve the problem described above. In the example of the most frequently used Tris-chloride/HCl buffer system, displayed in Table 15.1, the four discontinuities are shown.

The stacking gel is polymerized on top of the resolving gel (Fig. 15.9). It has large pore sizes with almost no retardation effect on the proteins and the pH value of pH 6.8 is close to the isoelectric point of glycine (pH 6.7 at 20°C). Thus, glycine has only a very low electrophoretic mobility; it has almost no negative charges. The chloride ions have a very high electrophoretic mobility.

The sample protein mixtures are applied into the wells in the stacking gels, located between the “leading ion” Cl− with high mobility and the “trailing ion” glycine with

Figure 15.7 Casting a polyacrylamide gel for horizontal flatbed electrophoresis.

Figure 15.8 Blotting gel polymerized on polyester net for horizontal electrophoresis with a large sample application well for probing strips preparation. SDS PAGE of bean seed protein extract, hot Coomassie Brilliant blue staining. Central lane: molecular weight standards. From HP Schickle, ETC, Kirchentellinsfurt, Germany, with permission.
very low mobility. When the electric field is applied, all negatively charged molecules will start to migrate toward the anode. The migration velocity will be very slow, because the glycine has a very low mobility. The ions with higher mobilities—the chlorides and proteins—cannot migrate faster, because they would leave an “ion gap” between the glycine and the protein with lowest mobility. An “ion gap” cannot exist, because it would interrupt the electric field. Therefore all ions migrate slowly with the same velocity. This phenomenon is called “isotachophoresis” (Greek word for migration with the same speed).

The isotachophoretic migration causes a “stacking” effect: in the area of low mobility the electric field strength will be high (to move the lowly charged molecules), in the area of high mobility the field strength will be low (otherwise the highly mobile ions would start to migrate quickly). The field strength gradient causes a sorting of the ions according to their mobilities: the protein with the highest mobility will directly follow the chloride; the protein with the lowest mobility will migrate directly in front of the glycine; the proteins in between will follow each other directly in the sequence of highest to lowest mobility. They will form sharp zones, because there is a regulation and a concentration effect: ions that would migrate more slowly will be accelerated forward by the higher field strength, ions that would migrate faster will be slowed by the lower field strength at the front. The concentration of the “leading ion” will determine the concentrations of the following zones, leading to a concentrating effect.

When the stack of protein zones reaches the border with the resolving gel, the conditions will suddenly change: the gel with the small pore size will execute a strong retardation of the (relatively large) protein molecules, but not on the (small) glycine molecules. The pH value is much higher with a higher buffering power because of the high ionic strength. Suddenly the glycine gains a higher mobility than the protein molecules following the chloride. This means that the glycine ions will overtake all the proteins and follow the chloride directly. The protein ions are suddenly located in a homogeneous Tris-glycine buffer and will follow the migration law of zone electrophoresis.

Disc electrophoresis improves the results considerably, because the proteins are slowly introduced into the gel matrix and will not aggregate; they are concentrated and pre-separated at their entrance into the resolving gel and form sharp zones.

The limitation of native disc electrophoresis arises from the fact that only those proteins that have mobilities between

<table>
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<tr>
<th>TABLE 15.1 Properties of a Disc Electrophoresis Gel, One Example</th>
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<tbody>
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<td>Stacking Gel</td>
</tr>
<tr>
<td>Gel composition</td>
</tr>
<tr>
<td>Buffer ions</td>
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<tr>
<td>pH value</td>
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<td>Ionic strength of Tris-base</td>
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Figure 15.9 Schematic of disc electrophoresis. From Reference 9, with permission.
those of the Cl⁻ and glycine⁻ will be separated. In the buffer system described above, proteins with isoelectric points above pH 6.8 will not enter the gel and will thus not be included. The separation of basic proteins and those with a neutral isoelectric point requires alternative buffer systems.

15.5.2 The Ferguson Plot

In native polyacrylamide gel electrophoresis (PAGE), the migration distance is dependent on both charge and size of the molecule. The first approaches to measuring physicochemical parameters with gel electrophoresis were explored by Ferguson (10). He separated proteins under identical buffer, time, and temperature conditions, but in differently concentrated gels. When the logarithms of the relative mobilities \( m_r \) are plotted against gel concentrations \( %T \), straight lines are obtained (Fig. 15.10).

The slope of a protein line is a measure of the molecular size and is called retardation coefficient \( K_R \). In the case of globular proteins the relationship between \( K_R \) and the Stokes radius (molecular radius) \( r \) is linear: this allows calculation of the molecular size from the slope of the Ferguson plot. The net charge of a protein can be calculated, when the free mobility and the Stokes radius are known (11).

Instead of running a series of gels, transverse gradient gel electrophoresis in one porosity gradient gel can be employed for such measurements (12).

15.5.3 Gradient Gel Electrophoresis

Porosity gradient gels are used because of their band sharpening effect, and in some cases for a pore limit electrophoresis for the measurement of the Stokes radius of a native protein (13). Gradient gels are obtained by continuously changing the acrylamide concentration in the monomer solution during casting the gel. A gradient maker and two starting solutions are required (Fig. 15.11). About 25% glycerol is added to one of the solutions to stabilize the gradient before polymerization occurs. The compensation bar is inserted into the reservoir to raise the fluid level because of the density difference of the solutions and the volume displacement of the magnetic bar. In the case shown in Figure 15.11, the glycerol is added to the solution with the high acrylamide content.

When casting gels for horizontal systems it is better to add the glycerol to the low acrylamide solution, because a high glycerol content in the sample application area prevents the open gel surface from drying, the gradient is not disturbed by thermal convection, and the leveling of the gradient is improved in the lower viscous, but higher acrylamide-containing area, where the sieving effect is more pronounced.

When gels are prepared in a multicasting box, the solutions are introduced through the bottom: in this case the dense solution is in the reservoir and the light solution in the mixing chamber, and no compensation bar is used.

Nonlinear, exponential gradients are obtained with a plug inserted into the mixing chamber to keep the volume there constant. The volume flowing from the reservoir will then be the same as that flowing from the mixing chamber into the cassette.

15.5.4 SDS Electrophoresis

SDS is a very strong anionic detergent, which solubilizes almost all protein, including very hydrophobic ones. It denatures the proteins by opening the hydrogen bonds and canceling the secondary and tertiary structures. SDS and proteins form complexes with a necklace-like structure composed of protein-decorated micelles connected by short flexible polypeptide segments (14). About 1.4 g SDS/g protein are incorporated in a SDS–protein complex. The
mobility of a SDS–protein complex in a polyacrylamide gel is solely dependent on the molecular size; the charges of the proteins are masked by the SDS. The negative charge per mass unit of the polypeptide is constant. When, additionally, the disulfide bridges between cysteines are opened with a reductant like 2-mercaptoethanol (2ME) or dithiothreitol (DTT), the molecular size will become directly proportional to the molecular weight of the polypeptide.

When the SDS–protein complex mixture is separated in a SDS-containing polyacrylamide gel, the logarithms of the molecular weights will have a linear relation to the relative mobilities of the respective polypeptides.

Usually, the sample is boiled for 3 min in the sample buffer containing 2% SDS, 50 mM Tris-Cl pH 8.8, 0.01% Bromophenol blue, and 1% (w/v) DTT. For vertical gels the sample buffer must contain 25% glycerol. After heating, the same amount of DTT has to be added again to the sample to avoid back-folding and aggregation of the subunits, which can occur because of oxidation of the reductant. Alternatively, the sample can be alkylated by adding iodoacetamide to a final concentration of 2.5% after heating.

Alkylation results in higher stability of the samples and prevents stainable artefactual lines across the gel, caused by excess DTT migrating into the gel together with acrylamide oligomers.

For some clinical applications it might be necessary to run nonreduced samples to keep the quaternary structure of the immunoglobulins intact. In this case the molecular weights cannot be estimated, because some of the proteins are not completely unfolded. For instance, nonreduced human albumin will migrate to a position where a 55 kDa protein would be; in the reduced form it shows a molecular weight of 67 kDa. Figure 15.12 shows (among other samples) the separation lanes of reduced and nonreduced human serum in a prefabricated, film-supported horizontal SDS polyacrylamide gel with 12.5% T and 3% C.

For SDS gels and buffers, the relationship between the logarithm of the molecular weight of a polypeptide and its relative mobility is only linear in a limited range, depending on the acrylamide concentration. For small proteins the linear relationship occurs in highly concentrated gels; for large proteins in more diluted gels. Gradient gels show a wide linear relationship.

The most used buffer system for SDS electrophoresis is the Laemmli buffer (15), which is in principle the above described discontinuous buffer with 0.1% SDS added to the gel and the electrode buffer. The gels should be used within 10 days after polymerization, because the high pH value of 8.8 in the resolving gel will cause hydrolysis of
gel electrophoresis method. There are several reasons for this: 0.4% SDS. Tris-acetate instead to save costs. The buffer strips contain tricine is not needed in the anode, so the anodal strips contain works best, when polyacrylamide buffer strips are used. This buffer system is sufficient for a good stacking effect. This buffer system contains a Tris-acetate buffer pH 6.4 (and 0.1% SDS). Tris-tricine buffer at the cathode is used for an adequate separation, containing a Tris-acetate buffer pH 6.4 (and 0.1% SDS). Tris-tricine buffer at the cathode is used for an adequate separation, as glycine does not work. Stacking and resolving gel contain the same buffer; the discontinuity between acetate and tricine is sufficient for a good stacking effect. This buffer system works best, when polyacrylamide buffer strips are used. Tricine is not needed in the anode, so the anodal strips contain Tris-acetate instead to save costs. The buffer strips contain 0.4% SDS.

SDS electrophoresis is by far the most frequently applied gel electrophoresis method. There are several reasons for this:

- SDS solubilizes almost all proteins.
- The separations are rapid, because SDS–protein complexes are highly charged.
- Because all fractions are negatively charged, all proteins are included.
- Sharp zones and high resolution are obtained, because the polypeptides are unfolded and do not diffuse much in the restrictive gels.
- The proteins are easy to fix and stain in the gel.
- It is an easy method for protein molecular weight estimation.
- Charge heterogeneities of isoenzymes are masked, resulting in one band for one enzyme.
- Proteins separated with SDS bind dyes better, resulting in higher sensitivity of detection than native gels.
- It is the ideal separation method prior to blotting, because all proteins are included, and SDS–protein complexes leave the gel in one direction during the electrophoretic transfer.

There are some special cases. For example, glycoproteins migrate considerably slower than polypeptides of the same molecular weight, because the sugar part does not bind SDS, resulting in a lower charge/mass unit relation. Preparing the sample with Tris-borate and using Tris-borate in the cathodal buffer can improve the result, because borate ions stick to the sugars and add more negative charges to the SDS–glycoprotein complexes.

Peptides with molecular weight smaller than 10 kDa are not displayed in Tris-glycine gels, because they co-migrate with the SDS front. Schägger and von Jagow (16) have developed a gel and buffer system that separates and displays peptides and proteins in the range between 1 and 100 kDa. The resolving gel is composed of 16% T and 6% C; the stacking and resolving gel contain the same high molar (1 M) Tris-chloride buffer pH 8.4, and tricine is used instead of glycine in the upper (cathodal) buffer. Also, the Tris-acetate/Tris-tricine buffer of the ExcelGel™ SDS separates smaller proteins and peptides much better than the Tris-glycine buffer.

15.5.5 Cationic Detergent Electrophoresis

An alternative to SDS electrophoresis is cationic detergent electrophoresis in an acidic buffer system. The migration direction is reversed. Cationic detergents are less denaturing than SDS; the separation pattern is different from a pattern obtained with SDS electrophoresis. The technique is applied on strongly acidic proteins, which do not bind SDS, and very basic nucleoproteins, which behave abnormally in SDS gels. It is also successfully used as a first dimension of two-dimensional electrophoresis of very hydrophobic proteins like membrane proteins.

The technique is more complicated to perform than SDS electrophoresis. Polymerization of acidic gels is more difficult than basic gels. In the past, cetyltrimethylammonium bromide (CTAB) has mainly been used (17, 18). More recent applications use benzylidimethyl-n-hexadecylammonium chloride (16-BAC) (19).

15.5.6 Blue Native Electrophoresis

This method is applied either for the separation of highly hydrophobic proteins, like membrane proteins, or for the separation of hydrophobic membrane protein complexes in an enzymatically active form (20). The membrane proteins and complexes are extracted with the help of a nonionic detergent like Triton X-100 or dodecyl-β-D-maltoside. Depending on the protein sample type, Coomassie Brilliant blue G-250, which is more hydrophilic than R-250 or R-350, is added to the cathodal buffer or to both the cathodal buffer and the sample for native PAGE. During electrophoresis, the anionic dye competes with the nonionic detergent and binds to the proteins and complexes. All proteins become negatively charged as with SDS, and—under the mild condition of pH 7.5—they all migrate into the anodal direction, the basic proteins included. The blue bands are visible during the separation. The dye–protein complexes are soluble in detergent-free solution. The formation of aggregates is minimized, because all protein surfaces are negatively charged and repel each other.

The blue protein complexes can be blotted or electroeluted from cut-out bands and further analyzed (20–22).
Alternatively, the separation lanes are cut into 1-cm-broad strips, briefly dipped into a 2-mercaptoethanol solution, placed into a vertical electrophoresis cassette, and "connected" to a freshly cast discontinuous SDS polyacrylamide gel. With the second dimension, electrophoresis under denaturing conditions, the individual subunits are separated, and can be assigned to the respective complexes.

15.5.7 Native Electrophoresis in Amphoteric Buffers

As already explained in Section 15.2.3, amphoteric buffers can be used as stationary buffers in media that do not contain any other ionic compounds. Hjerten and colleagues (23) and Righetti and co-workers (24) have successfully applied this concept in capillary electrophoresis. In gel electrophoresis the procedure works best in film-supported gels that have been washed with deionized water after polymerization. The gels are dried and can be reconstituted in the desired buffer before use. Depending on the desired pH value this can be HEPES, MES, MOPS, or another amphoteric buffer. Practical experiments have shown, that for adequate protein separation, a stacking effect is necessary during the starting phase of electrophoresis. For acidic electrophoresis in HEPES, acetic acid in a very low concentration is used as a counter ion; arginine is used as the leading ion and ε-aminocaproic acid as the trailing ion (9).

With this technique, acidic electrophoresis separations are no problem and very fast separations are achieved. In practice it is mainly applied for the routine analysis of basic proteins, such as alcohol soluble crop proteins (25) or fish sarcoplasma proteins (26). Figure 15.13 shows an example of acidic electrophoresis in an amphoteric buffer of barley Hordeins. A short separation time of 2 h can be achieved for a distance of 10 cm in a native gel, because the buffer is not migrating and does not contribute to Joule heating.

![Figure 15.13](image)

Figure 15.13 Acidic electrophoresis in amphoteric buffer of 48 single barley grain extracts for raw material control in food industry. The lanes marked with arrows represent cultivars that are detrimental to production. Separation time, 2 h. Hot Coomassie Brilliant blue staining. From HP Schickle, ETC, Kirchentellinsfurt, Germany, with permission.

15.6 PROTEIN DETECTION TECHNIQUES

15.6.1 General Staining

15.6.1.1 Coomassie Brilliant Blue Coomassie Brilliant blue staining is the most frequently applied method. Preferably, nonalcoholic protocols should be used, because during destaining the alcohol of the destaining solution competes with the proteins for the dye. Thus the bands become partly destained, depending on the dye-binding properties of the individual proteins. In order to achieve reliable quantification, the bands should not become destained. The most reliable methods are colloidal staining with Coomassie Brilliant blue G-250 (26) and hot staining in 10% acetic acid with Coomassie brilliant blue R-350 (9). The collagen hydrolysate bands in Figure 15.12 and the bands of the alcohol soluble proteins in Figure 15.13 would not be detected with alcohol-containing staining solutions. The detection limit is dependent on the protein type and goes down to the low nanogram range. If colloidal staining is applied on a gel repeatedly over several days, the sensitivity of detection can be considerably increased.

15.6.1.2 Reversible Imidazole Zinc Staining This technique, introduced by Fernandez-Patron and colleagues (28), is similar in sensitivity to colloidal Coomassie Brillant blue, but is a negative stain. Its advantages lie in the speed of detection and the possibility of removing the white background for blotting and subsequent immunodetection. Unfortunately it is not usable for quantification, because it stains the background of the gel, not the proteins.

15.6.1.3 Silver Staining The first silver staining was applied on agarose electrophoresis gels for the detection of oligoclonal immunoglobulins (29). A modified staining protocol showed that silver staining is particularly sensitive in polyacrylamide gels (30). Of the high number of different protocols available, the modification according to Heukeshoven and Dernick (31) is the most useful and sensitive procedure. In order to obtain a high sensitivity of detection, it is very important to use high quality reagents and good quality of distilled or deionized water. The sensitivity of silver staining is in the picogram range. Unfortunately, quantification with silver staining is not very reliable.

15.6.1.4 Fluorescent Staining Fluorescence stains are almost as sensitive as silver staining; their wide linear dynamic range makes them very useful for quantification. A UV table or fluorescent scanner is needed for the detection of bands, and the costs for the dye are higher than with visible dyes. When glass plate- or film-supported gels are used, the glass and film material must be of low or nonfluorescent quality. The most sensitive dyes are Deep Purple™ (from GE Healthcare) (32) and SYPRO Ruby™ (from Molecular
Probes) (33). Deep Purple™ is a naturally occurring fluorophore free from heavy metals, which is easy to dispose of and environmentally friendly. It works particularly well in SDS gels.

### 15.6.2 Specific Staining

After native electrophoresis, enzymes can be specifically detected via zymogram techniques. The gels are submerged in a substrate-containing buffer, and the enzyme–substrate reaction in the gel is then coupled to a dye reaction. Almost all zymogram protocols are derived from histology. Collections of recipes for gel electrophoresis are found in the books by Rothe (34) and Manchenko (35).

### 15.7 REFERENCES

## 16

### CONVENTIONAL ISOELECTRIC FOCUSING IN GEL SLABS AND CAPILLARIES AND IMMOBILIZED pH GRADIENTS

**Pier Giorgio Righetti, Elisa Fasoli, and Sabina Carla Righetti**

*Department of Chemistry, Materials and Chemical Engineering “Giulio Natta,” Politecnico di Milano, Via Maancinelli 7, 20131 Milano, Italy*

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16.1 INTRODUCTION

This chapter is organized as follows. We will first treat conventional isoelectric focusing in soluble, carrier ampholyte (CA) buffers, as originally envisaged by Svensson-Rilbe. In this Section, we will give a brief historical survey of its evolution, from a preparative-scale approach, to the extremely popular gel-slab version. This part will also contain a description of the properties of CA buffers, especially in regard to their buffering capacity ($\beta$) and conductivity, because these basic concepts have been found to be fundamental in capillary zone electrophoresis (CZE). We will then carry on with a description of immobilized pH gradients (IPGs), the novel version of focusing launched in 1982 (1). In a third part, we will describe IEF in a capillary format (2–4). As a final part, we will describe CZE separations exploiting isoelectric buffers as a background electrolyte. Although this is not a focusing technique per se, it is the natural evolution of the know-how developed in IEF, and it appears to hold a unique separation potential in CZE.

16.1.1 A Brief Historical Survey

In 1960–1962 as we, as freshmen in the University, went down to the beach to the tune of *Surfin’ Safari* (Beach Boys) others spent their time trying to surf the rough seas of separation science, in the hope of some major developments. A major breakthrough was indeed the discovery of conventional IEF in CAs (soluble, ampholytic buffers), as reported by Svensson-Rilbe in a series of now classical articles (5–7). At just about the same time, Meselson and colleagues (8) described isopycnic centrifugation (IPC), a related, high resolution technique, in fact another member of a family called by Kolin (9) “isoperichoric focusing.” Unlike conventional chromatographic and electrophoretic techniques available up to those times, which could not provide any means for avoiding peak decay during the transport process, IEF and IPC had built-in mechanisms opposing entropic forces trying to dissipate the zone. As the analyte reached an environment (the “perichoron,” in Greek) in which its physicochemical parameters were equal (iso) to those of the surroundings, it focused, or condensed, in an ultrathin zone, kept stable and sharp in time by two opposing force fields: diffusion (tending to dissipate the zone) and external fields (voltage gradients, in IEF, or centrifugal fields, in IPC) forcing the “escaping” analyte back into its “focusing” zone. It was truly a magic event in separation science, through which all of us, general practitioners in the field, and humble Clark Kents in the lab, all at once felt (and acted) like Superman.

According to the original idea of Svensson-Rilbe, CA-IEF was born as a preparative technique, in vertical, hollow columns exploiting two colinear gradients: a sucrose density gradient acting as an anticonvective medium and a pH gradient generated by the current during the focusing step (10). A typical experiment took several days to complete and the subsequent analyses of fractions eluted from the gradients were tedious and laborious. It was the advent of gel IEF (11–13) that rendered the technique so popular. The evolution of CA-IEF can be followed in a series of meetings, starting with the cornerstone symposium in New York (14) soon followed by Glasgow (15), Milan (16), Hamburg (17), and Cambridge (MA, USA) (18). At the latter meeting, the specific interest in IEF and isotachophoresis (ITP, note that ITP is also a steady-state technique with this proviso: in IEF all zones have identical mobilities all equal to zero, whereas in ITP all zones acquire the same, nonzero, velocity) moved to the general field of electrophoresis (in which, however, IEF still played the role of prima donna). There followed a series of meetings in Munich (19), Charleston (20), Tokyo (22), Göttinngen (23), London (24), and Copenhagen (25). With this last meeting, the publication of proceedings ceased, because the official journal of the International Electrophoresis Society was launched, that is, the journal *Electrophoresis*, which appeared to be the proper forum for such events. Nevertheless, this collection of proceedings is an interesting means for following the evolution of IEF and, later on, of general electrophoretic techniques.
It was in 1975 that IEF took another, important turn. In that year O’Farrell (26) introduced two-dimensional (2D) electrophoresis (2-DE) and demonstrated that, by sequentially running IEF in the first dimension, followed by sodium dodecyl sulphate (SDS) electrophoresis at right angles, >1100 individual polypeptides could be resolved in an Escherichia coli lysate. These 2D maps became the nightmare of chromatographers: they still had to accept the hard reality that, even in the best gas-chromatographic separation, it was hard to resolve barely 100 distinct peaks (27). Soon 2-DE excited grandiose projects, like the Human Protein Index System of Anderson and Anderson (28), who started the far-reaching goal of mapping all possible phenotypes expressed by any and all different cells in our organism. This started a series of meetings of the 2-DE group, particularly strong in the field of clinical chemistry (29, 30). A number of books were devoted to this 2-DE issue (31–34). Later on, 2-DE found a proper forum in the journal Electrophoresis, which began hosting individual papers dealing with variegate topics in 2-D maps. Starting in 1988, Electrophoresis launched special issues devoted to 2-D maps, not only in clinical chemistry and human molecular anatomy, but in fact in every possible living organism and tissue. This collection of “Paper Symposia” is worth a perusal, because it is a gold mine of new information and on novel evolutionary steps on the IEF technique. Today, however, 2D map analysis and proteomic research are properly hosted in a number of new journals, such as Proteomics, Journal of Proteome Research, Molecular and Cellular Proteomics, and Journal of Proteomics.

16.2 CONVENTIONAL ISOELECTRIC FOCUSING IN AMPHOTERIC BUFFERS

16.2.1 General Considerations

All fractionations that rely on differential rates of migration of sample molecules, for example, along the axis of a chromatographic column or along the electric field lines in electrophoresis, generally lead to concentration bands or zones that are essentially always out of equilibrium. The narrower the band or zone, the steeper the concentration gradients, and the greater the tendency of these gradients to dissipate spontaneously. This dissipative transport is thermodynamically driven; it relates to the tendency of entropy to break down all gradients, to maximize dilution, and, during this process, to thoroughly mix all components (27). Most frequently, entropy exerts its effects via diffusion, which causes molecules to move down concentration gradients and so produces band broadening and component intermixing.

The process of isoelectric focusing (IEF) in CAs (5–7) and in IPGs (35) provides an additional force that counteracts CAs’ diffusion and so maximizes the ratio of separative to dissipative transports. This substantially increases the resolution of the fractionation method. The sample focuses at its isoelectric point (pI), driven by the voltage gradient and by the shape of the pH gradient along the separation axis (Figs. 16.1 and 16.2). The separation can be optimized by using thin or ultrathin matrices (36, 37) (0.5 mm or less in thickness) and by applying very low sample loads (as permitted by high sensitivity detection techniques, such as silver and gold staining, radioactive labeling, immunoprecipitation, followed by amplification with peroxidase- or alkaline phosphatase-linked secondary antibodies).

16.2.1.1 The Basic Method

IEF is an electrophoretic technique by which amphoteric compounds are fractionated according to their pIs along a continuous pH gradient (38). In contrast to zone electrophoresis, where the constant (buffered) pH of the separation medium establishes a constant charge density at the surface of the molecule and causes it to migrate with constant mobility (in the absence of molecular sieving), the surface charge of an amphoteric compound in IEF keeps changing, and decreasing, according to its titration curve, as it moves along a pH gradient until it reaches its equilibrium position, that is, the region where the pH matches its pI. There, its mobility equals zero and the molecule comes to a stop.

The gradient is created, and maintained, by the passage of an electric current through a solution of amphoteric compounds that have closely spaced pIs, encompassing a given pH range. The electrophoretic transport causes these CAs to stack according to their pIs, and a pH gradient, increasing from anode to cathode, is established. After this stacking process is completed, some CAs still enter zones of higher, or lower, pH by diffusion, where they are no longer in isoelectric

![Image](359x182 to 574x355)

Figure 16.1 Illustration of the forces acting on a condensed zone in isoelectric focusing (IEF). The focused zone is represented as a symmetric Gaussian peak about its focusing point (pI; y = 0). Migration of sample towards the pI position is driven by the voltage gradient and by the slope of the pH gradient. σ is the standard deviation of the peak. Courtesy of Dr O. Vesterberg.
equilibrium. As soon as they enter these zones, however, the CAs become charged and the applied voltage forces them back to their equilibrium position (Fig. 16.1). This pendulum movement, diffusion versus electrophoresis, is the primary cause of the residual current observed under isoelectric steady-state conditions. Finally, as time progresses, the sample protein molecules also reach their isoelectric point.

16.2.1.2 Applications and Limitations The technique only applies to amphoteric compounds and more precisely to good ampholytes with a steep titration curve around their pi, *conditio sine qua non* for any compound to focus in a narrow band. This is very seldom a problem with proteins but it may be so for short peptides, which need to contain at least one acidic, or basic, amino-acid residue, in addition to the –NH₂ and –COOH termini. Peptides that have only these terminal charges are isoelectric over the entire range of ≈pH 4 and 8, and so do not focus. Another limitation with short peptides is encountered at the level of the detection methods: CAs are reactive to most peptide stains. This problem may be circumvented by using specific stains, when appropriate (39, 40), or by resorting to immobilized pH gradients (IPG) which do not give background reactivity to ninhydrin and other common stains for primary amino groups (e.g., dansyl chloride, fluorescamine) (41).

In practice, notwithstanding the availability of CAs covering the pH 2.5–11 range, the practical limit of CA-IEF is in the pH 3.5–10 interval. Because most protein pIs cluster between pH 4 and 6 (42), this may pose a major problem only for specific applications.

When a restrictive support like polyacrylamide (PAA) is used, a size limit is also imposed for sample proteins. This can be defined as the size of the largest molecules that retain an acceptable mobility through the gel. A conservative evaluation sets an upper molecular mass limit of about 750,000 when using standard techniques. The molecular form in which the proteins are separated strongly depends upon the presence of additives, such as urea and/or detergents. Moreover, supramolecular aggregates or complexes with charged ligands can be focused only if their $K_D$ is lower than 1 μM and if the complex is stable at pH ≈ pI. An aggregate with a higher $K_D$ is easily split by the pulling force of the current.

16.2.1.3 Specific Advantages First, IEF is a steady-state technique; therefore the results do not depend (within reasonable limits) upon the mode of sample application, the total protein load, or the time of operation. Second, an intrinsic physicochemical parameter of the protein (its pI) may be measured. Third, IEF requires only a limited number of chemicals, is completed within a few hours, and is less sensitive than most other techniques to the skill (or lack of it) of the operator. Finally, IEF allows excellent resolution of proteins with pIs differing by only 0.01 pH units (with immobilized pH gradients, up to about 0.001 pH units); the protein bands are very sharp due to the focusing effect.

16.2.1.4 Carrier Ampholytes Table 16.1 lists the general properties of CAs, that is, of the amphoteric buffers used to generate and stabilize the pH gradient in IEF. The fundamental and performance properties listed in this table are usually required for a well behaved IEF system, whereas the “phenomena” properties are in fact the drawbacks or failures inherent to the technique. For instance, the “plateau effect” or “cathodic drift” is a slow decay of the pH gradient with time.
All species have been assessed by measuring the
and Ampholine, Bio-Rad with Bio-Lyte and Serva with
by three companies (GE Healthcare with Pharmalyte
lyzing all the narrow (2 pH unit wide) intervals produced
by capillary electrophoresis–mass spectrometry (MS) by ana-
CAs have been decoded via Rotofor fractionation followed
on the market: the GE Healthcare Ampholines (formerly
phosphonate. Accordingly, there are three types of products
in both PAA and agarose).

In chemical terms, CAs are oligoamino, oligocarboxylic
acids, available from different suppliers under different
trade names. There are three basic synthetic approaches:
Vesterberg’s approach, which involves reacting different oli-
goaamines (tetra-, penta-, and hexa-amines) with acrylic acid
(43); the Söderberg and colleagues (44) synthetic process,
which involves the copolymerization of amines, amino acids,
and dipeptides with epithophorhydrin, and the Grubhofer–
Borja approach (45), which utilizes ethyleneimine and propy-
lenediameine for subsequent reaction with 1,3-propanesultone
(IUPAC name: oxathiolane 2,2-dioxide, chemical formula:
C₃H₆O₃S), sodium vinylsulfonate, and sodium chloromethyl
phosphonate. Accordingly, there are three types of products
on the market: the GE Healthcare Ampholines (formerly
LKB-Produkter AB) and Bio-Rad Biolytes, which belong
to the first class; the GE Healthcare Pharmalytes, which
should be listed in the second class, and the Novex-
Servalyt Ampholytes and Genomic Solutions pH 3–10
ampholytes, which should be classified into the third
category.

Only recently, the basic physicochemical parameters of
CAs have been decoded via Rotofor fractionation followed
by capillary electrophoresis—mass spectrometry (MS) by ana-
lyzing all the narrow (2 pH unit wide) intervals produced
by three companies (GE Healthcare with Pharmalyte
and Ampholine, Bio-Rad with Bio-Lyte and Serva with
Servalyt). All species have been assessed by measuring the
types of pH gradient produced, the total number of individual
chemicals (with $M_r$ values) and isoforms, and their focusing
behaviour (“good” or “poor” ampholytes) (46–51). Servalyt
contains a grand total of 686 chemical entities and no less
than 3899 isoforms; Pharmalyte 643 and 2211; Bio-Lyte
255 and 1192; Ampholine 294 and 1182, respectively. In
terms of $M_r$ distribution, although all 2 pH unit ranges start
with the same low $M_r$ values (~200), their upper bounds are
quite different. Thus, Pharmalyte reaches an upper $M_r$
value of 1179 (in the pH 4–6 range), versus 907 for Servalyt, 835
for Bio-Lyte, and 893 for Ampholine. In general, in going
towards the more alkaline pH intervals (e.g., pH 8–10) the
molecular mass of CAs is reduced to as low as 491 (Bio-
Lyte), indicating that the alkaline species are probably made
with shorter oligoamines and are, in general, less substituted.
All acidic pH intervals (up to pH 6–8) appear to be constituted
by a very large proportion of well focusing species, indicating
small values of $\Delta pK$ across their $pI$. Above pH 8, all brands of
CAs worsen, the vast majority being unable to focus properly
and sustain adequately the pH gradient. Figure 16.2 gives the
pH gradients (panel a), $M_r$ distribution (panel b), and focusing
properties (panel c) of four CA brands nominally focusing in
the pH 4–6 interval. CAs should be readily separable (unless
they are hydrophobically complexed to proteins) from macro-
molecules by gel filtration. Dialysis is not recommended due
to the tendency of CAs to aggregate. Salting out of proteins
with ammonium sulfate seems to completely eliminate any
contaminating CAs. A further complication arises from the
chelating effect of acidic CAs, especially towards Cu$^{2+}$ ions,
which may inactivate some metallo-enzymes. In addition,
focused CAs represent a medium of very low ionic strength
(less than 1 mEq/L at the steady state). Because the isoelectric
state involves a minimum of solvation, and thus of solubility,
for the protein macroion, there is a tendency, for some proteins
(e.g., globulins) to precipitate during the IEF run near their $pI$
position. This is a severe problem in preparative runs. In
analytical procedures it can be minimized by reducing the
total amount of sample applied.

The hallmark of a “carrier ampholyte” is the absolute
value of $pI - pK_{pox}$ (or $1/\Delta pK$): the smaller this value,
the higher the conductivity and buffering capacity (at $pH =
pl$) of the amphoter. A $\Delta pK$ of log 4 (i.e., $pI - pK = 0.3$
would provide an incredible molar buffering power ($\beta$) at
the pl of 2.0. A $\Delta pK$ of log 16 (i.e., $pI - pK = 0.6$
offers a $\beta$ value of 1.35 at $pH = pl$. A practical example is given
by Lys and His, two amino acids that can be considered good
CAs for IEF. For Lys, the $pI$ value (9.74) is nested on a
high saddle between two neighboring protolytic groups (the
$\epsilon$- and $\alpha$-amino; $pI - pK = 0.79$), thus providing an excel-
lent $\beta$ power (~1.0). The situation is not so brilliant with
His: the $pI$ value (7.57) is located in a valley with a $\beta$ value
of only 0.24 (due to a $pI - pK$ of 1.5). When plotting the
molar $\beta$ power of a weak protolyte along the $pH$ axis, one
reaches a maximum of $\beta = 1.0$ at $pH = pI$. If one accepts,

<table>
<thead>
<tr>
<th>TABLE 16.1 Properties of Carrier Ampholytes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fundamental “Classical” Properties</strong></td>
</tr>
<tr>
<td>Buffering ion has mobility of zero at pI</td>
</tr>
<tr>
<td>Good conductance</td>
</tr>
<tr>
<td>Good buffering capacity</td>
</tr>
<tr>
<td><strong>Performance Properties</strong></td>
</tr>
<tr>
<td>Good solubility</td>
</tr>
<tr>
<td>No influence on detection systems</td>
</tr>
<tr>
<td>No influence on sample</td>
</tr>
<tr>
<td>Separable from sample</td>
</tr>
<tr>
<td>“Phenomena” Properties</td>
</tr>
<tr>
<td>“Plateau” effect (i.e., drift of the pH gradient)</td>
</tr>
<tr>
<td>Chemical change in sample</td>
</tr>
<tr>
<td>Complex formation</td>
</tr>
</tbody>
</table>


whereby, upon prolonged focusing at high voltages, the pH
gradient with the focused proteins drifts towards the cathode
and is eventually lost in the cathodic compartment. There
seems to be no remedy to this problem (other than abandon-
ing CA-IEF in favor of the IPG technique), because there are
complex physicochemical causes underlying it, including a strong electrosomotic flow generated by the covalently bound negative charges of the matrix (carboxyls and sulfate in both PAA and agarose).
as a still reasonable $\beta$, a value of one-third of this maximum, this is located at $pH - pK = \pm 0.996$. It is thus seen that even His, generally considered a good CA, is in fact barely acceptable and falls just below this one-third limit of acceptance (52).

### 16.2.2 Equipment

#### 16.2.2.1 Electrophoretic Equipment

Three major items of apparatus are required; an electrophoretic chamber, a power supply, and a thermostating unit.

The optimal configuration of the electrophoretic chamber is for the lid to contain movable platinum wires (e.g., in the Multiphor II, in the GE Healthcare FBE3000, or in the Bio-Rad chambers mod. 1045 and 1415). This allows the use of gels of various sizes and the application of high field strengths across just a portion of the separation path. A typical chamber is shown in Figure 16.3.

The most suitable power supplies for IEF are those with automatic constant power operation and with voltage maxima as high as 5000–6000V. The minimal requirements are a limiting voltage of 1000 V and a reliable amperometer with a full scale not exceeding 50 mA. Lower field strengths cause the protein bands to spread (resolution is proportional to $\sqrt{E}$).

Efficient cooling is important for IEF because it allows high field strengths to be applied without overheating, thus the need for a thermostating unit. Tap water circulation is adequate for 8 M urea gels, but is not acceptable for gels lacking urea. Placing the electrophoretic apparatus in a cold room may be beneficial to prevent water condensation around the unit in very humid climates, but it is inadequate as a substitute for coolant circulation.

#### 16.2.2.2 Polymerization Cassette

The polymerization cassette is the chamber that is used to form the gel for IEF. It is assembled from the following elements: a gel supporting plate, a spacer, a cover (molding) plate, and some clamps (Fig. 16.4). For thin, soft gels a permanent support is required. Thin plastic sheets designed to bind polyacrylamide gels firmly (e.g., Gel Bond PAG by Marine Colloids, PAG foils by GE Healthcare, Gel Fix by Serva) are by far preferred today. The plastic sheet is applied to a supporting glass plate and the gel is cast onto this. The binding of the PAA matrix to these substrata, however, is not always stable and so care should be taken in using them, especially for detergent-containing gels and when using aqueous staining solutions. For good adherence, the best procedure is to cast “empty” gels (i.e., PAA gel lacking CAs), wash and dry them, and then reswell with the solvent of choice.

U-gaskets of any thickness between 0.2 and 5 mm can be cut from rubber sheets (para-, silicone-, and nitrile-rubber) and used as spacers. For thin gels, a few layers of Parafilm (each about 120 $\mu$m thick) can be stacked and cut with a razor blade. The width of such U-gaskets should be $\sim 4$ mm. In addition, cover plates with a permanent frame are commercially available. Mylar foil strips or self-adhesive tape may be used as spacers for 50–100- $\mu$m-thick gels. Rubber- or tape-gaskets should never be left to soak in soap (which they absorb) but just rinsed and dried promptly.

Clean glass, glass coated with dimethyl dichloro silane (Repel Silane), or a thick Perspex sheet are all suitable materials for the cover plate. If you wish to mold sample application pockets into the gel slab during preparation, attach Dymo tape pieces to the plate, or glue small Perspex blocks to the plate with drops of chloroform. Perspex should never be exposed unevenly to high temperatures (e.g., by being rinsed in running hot water) because it bends even if cut in thick slabs.

### 16.2.3 The PAA Gel Matrix

#### 16.2.3.1 Reagents

Stocks of dry chemicals (acrylamide, Bis, ammonium persulfate) may be kept at room temperature,
provided they are protected from moisture by being stored in air-tight containers. Very large stocks are better sealed into plastic bags, together with Drierite (Merck), and stored in a freezer. TEMED stocks should also ideally be kept in a freezer, in an air-tight bottle, or, better, under nitrogen. Avoid contaminating acrylamide solutions with heavy metals, which can initiate its polymerization. Acrylamide and Bis for IEF must be of the highest purity to avoid poor polymerization and strong electrosmosis resulting from acrylic acid. Recently, novel monomers, endowed with extreme resistance to alkaline hydrolysis and with higher hydrophilicity, have been reported: they are N-acryloylamino ethoxy ethanol (53) and N-acryloyl amino propanol (54). Table 16.2 lists the most commonly used additives in IEF.

**TABLE 16.2** Common Additives for IEF

<table>
<thead>
<tr>
<th>Additive</th>
<th>Purpose</th>
<th>Concentration</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose, glycerol</td>
<td>To improve the mechanical properties of low % gel and to reduce water transport and drift</td>
<td>5–20%</td>
<td>The increased viscosity slightly slows the focusing process</td>
</tr>
<tr>
<td>Glycine, taurine</td>
<td>To increase the dielectric constant of the medium. This increases the solubility of some proteins (e.g., globulins) and reduces ionic interactions</td>
<td>0.1–0.5 M</td>
<td>Glycine is zwitterionic between pH 4 and 8, taurine between pH 3 and 7; their presence somewhat slows the focusing process and shifts the resulting gradient</td>
</tr>
<tr>
<td>Urea</td>
<td>Disaggregation of supramolecular complexes</td>
<td>2–4 M</td>
<td>Unstable in solution especially at alkaline pH</td>
</tr>
<tr>
<td></td>
<td>Solubilization of water-insoluble proteins, denaturation of hydrophilic proteins</td>
<td>6–8 M</td>
<td>Urea is soluble at $\geq 10^\circ$ C; it accelerates polyacrylamide polymerization, so reduces the amount of TEMED added</td>
</tr>
<tr>
<td>Nonionic and zwitterionic detergents</td>
<td>Solubilization of amphiphilic proteins</td>
<td>0.1–1%</td>
<td>To be added to the polymerizing solutions just before the catalysts to avoid foaming; they interfere with polyacrylamide binding to reactive substrata; they are precipitated by TCA and require a specific staining protocol</td>
</tr>
</tbody>
</table>
16.2.3.2 Gel Formulations

In order to allow all the sample components to reach their steady-state position at essentially the same rate, and the experiment to be terminated before the pH gradient decay process adversely affects the quality of the separation, it is best to choose a nonrestrictive anticonvective support. There are virtually no theoretical but only practical lower limits for gel concentration (the minimum being ≈ 2.2% T, 2% C). Large pore sizes can be obtained both by decreasing % T and by either decreasing or increasing % C from the critical value of 5%. Although the pore size of PAA can be enormously enlarged by increasing the percentage of crosslinker, two undesirable effects also occur in parallel, namely increased gel turbidity and proneness to syneresis. In this respect, N,N'- (1,2-dihydroxyethylene)bisacrylamide (DHEBA), with its superior hydrophilic properties, appears superior to bisacrylamide. In contrast, N,N'-diallyltartardiamide (DATD) inhibits the polymerization process and so gives porous gels just by reducing the actual % T of the matrix. Because unpolymerized acryloyl monomers may react with –NH₂ and react readily with –SH groups on proteins and, once absorbed through the skin, act as neurotoxins, the use of DATD should be avoided altogether.

16.2.3.3 Choice of CAs

A simple way to extend and stabilize the extremes of a wide (pH 3–10) gradient is to add acidic and basic (natural) amino acids. Thus lysine, arginine, aspartic acid, and glutamic acid are prepared as individual stock solutions containing 0.004% sodium azide and stored at 0–4°C. They are added in volumes sufficient to give 2–5 mM final concentration. To cover ranges spanning between 3 and 5 pH units, a few narrow cuts of CA need to be blended, with the proviso that the resulting slope of the gradient will be (over each segment of the pH interval) inversely proportional to the amount of ampholytes isoelectric in that region.

Shallow pH gradients are often used to increase the resolution of sample components. However, longer focusing times and more diffuse bands will result unless the gels are electrophoresed at higher field strengths. Shallow pH gradients (shallower than the commercial 2 pH unit cuts) can be obtained in different ways:

- By subfractionating the relevant commercial carrier ampholyte blend; this can be done by focusing the CAs at high concentration in a multicompartment electrolyzer.
- By allowing trace amounts of acrylic acid to induce a controlled cathodic drift during prolonged runs; this is effective in the acidic pH region, but it is rather difficult to obtain reproducible results from run to run.
- By preparing gels containing different concentrations of carrier ampholytes in adjacent strips or with different thickness along the separation path.
- By adding specific amphoteric compounds (spacers) at high concentration.

In the last case, two kinds of ampholytes may be used for locally flattening the pH gradient: “good” and “poor.” Good CAs, those with a small pI (i.e., possessing good conductivity and buffering capacity at the pI) are able to focus in narrow zones. Low concentrations (5–50 mM) are sufficient to induce a pronounced flattening of the pH curve around their pIs. A list of these CAs is given in Table 16.3. Poor CAs, on the other hand, form broad plateaux in the region of their pI, and should be used at high concentrations (0.2–1.0 M). Their presence usually slows the focusing process. Some are listed in Table 16.4.

A note of caution: in an IEF system, the distribution of acids and bases is determined according to their dissociation curve, in a pattern that may be defined as protonation (or

<table>
<thead>
<tr>
<th>CA</th>
<th>pI</th>
<th>CA</th>
<th>pI</th>
<th>CA</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>2.77</td>
<td>p-Aminobenzoic acid</td>
<td>3.62</td>
<td>Lysyl-glutamic acid</td>
<td>6.10</td>
</tr>
<tr>
<td>Glutathione</td>
<td>2.82</td>
<td>Glycyl-aspartic acid</td>
<td>3.63</td>
<td>Histidyly-glycine</td>
<td>6.81</td>
</tr>
<tr>
<td>Aspartyl-tyrosine</td>
<td>2.85</td>
<td>m-Aminobenzoic acid</td>
<td>3.93</td>
<td>Histidyl-histidine</td>
<td>7.30</td>
</tr>
<tr>
<td>α-Aminophenylarsonic acid</td>
<td>3.00</td>
<td>Diodotyrosine</td>
<td>4.29</td>
<td>Histidine</td>
<td>7.47</td>
</tr>
<tr>
<td>Aspartyl-aspartic acid</td>
<td>3.04</td>
<td>Cystinyl-diglycine</td>
<td>4.74</td>
<td>L-Methylhistidine</td>
<td>7.67</td>
</tr>
<tr>
<td>p-Aminophenylarsonic acid</td>
<td>3.15</td>
<td>α-Hydroxyasparagine</td>
<td>4.74</td>
<td>Carnosine</td>
<td>8.17</td>
</tr>
<tr>
<td>Picolinic acid</td>
<td>3.16</td>
<td>α-Aspartyl-histidine</td>
<td>4.92</td>
<td>α,β-Diaminopropionic acid</td>
<td>8.20</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>3.22</td>
<td>β-Aspartyl-histidine</td>
<td>4.94</td>
<td>Anserine</td>
<td>8.27</td>
</tr>
<tr>
<td>β-Hydroxyglutamic acid</td>
<td>3.29</td>
<td>Cysteinyl-cysteine</td>
<td>4.96</td>
<td>Tyrosyl-arginine</td>
<td>8.38–8.68</td>
</tr>
<tr>
<td>Aspartyl-glycine</td>
<td>3.31</td>
<td>Pentaglycine</td>
<td>5.32</td>
<td>L-Ornithine</td>
<td>9.70</td>
</tr>
<tr>
<td>Isonicotinic acid</td>
<td>3.44</td>
<td>Tetruglycine</td>
<td>5.40</td>
<td>Lysine</td>
<td>9.74</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>3.44</td>
<td>Triglycine</td>
<td>5.59</td>
<td>Lysil-lysine</td>
<td>10.04</td>
</tr>
<tr>
<td>Anthranilic acid</td>
<td>3.51</td>
<td>Tyrosyl-tyrosine</td>
<td>5.60</td>
<td>Arginine</td>
<td>10.76</td>
</tr>
</tbody>
</table>
TABLE 16.4 Poor CAs Acting as Spacers

<table>
<thead>
<tr>
<th>CA</th>
<th>pK₁</th>
<th>pK₂</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mes</td>
<td>1.3</td>
<td>6.1</td>
<td>3.70</td>
</tr>
<tr>
<td>Pipes</td>
<td>1.3</td>
<td>6.8</td>
<td>4.05</td>
</tr>
<tr>
<td>Aces</td>
<td>1.3</td>
<td>6.8</td>
<td>4.05</td>
</tr>
<tr>
<td>Bes</td>
<td>1.3</td>
<td>7.1</td>
<td>4.20</td>
</tr>
<tr>
<td>Mops</td>
<td>1.3</td>
<td>7.2</td>
<td>4.25</td>
</tr>
<tr>
<td>Tes</td>
<td>1.3</td>
<td>7.5</td>
<td>4.40</td>
</tr>
<tr>
<td>Heps</td>
<td>1.3</td>
<td>7.5</td>
<td>4.40</td>
</tr>
<tr>
<td>Epps</td>
<td>1.3</td>
<td>8.0</td>
<td>4.65</td>
</tr>
<tr>
<td>Taps</td>
<td>1.3</td>
<td>8.4</td>
<td>4.85</td>
</tr>
</tbody>
</table>

“Good’s” Buffers with Acidic pIs

- Aminocaproic acid 4.42 11.66 8.04
- Aminovaleric acid 4.26 10.77 7.52
- Aminobutyric acid 4.03 10.56 7.30
- Alanine 3.55 10.24 6.90

The gravity procedure uses a vertical cassette with a rubber gasket U-frame glued to the cover plate (Fig. 16.4a). Because the cover plate has V-indentations along its free edge as shown in Figure 16.4a, the gel mixture can be transferred simply by using a pipette or a syringe with its tip resting on one of these indentations (Fig. 16.4d). Filling the mold too fast should be avoided, as this will create turbulence and trap air bubbles. If an air bubble appears, stop pouring the solution and try to remove the bubble by tilting and knocking the mold. If this maneuver is unsuccessful, displace the bubble with a 1-cm-wide strip of polyester foil. For preparing ultrathin gels (100 μm and lower), the flap technique, as described by Radola (67), is mainly used.

16.2.4.3 Gel Polymerization CA-IEF should preferably run in rather “soft” gels, for example, 4–5%T, containing 2–4% CAs of the desired pH interval and, if needed, additives such as urea and surfactants. As a variant of this protocol, one can polymerize “empty” gels (i.e., devoid of CAs and of any leachable additive), wash and dry them, and reswell them in the appropriate CA solution (including any additive, as needed). This is a direct application of the IPG technology (1). After polymerization (as above, but in the absence of CAs) wash the gel three times, in 100 mL distilled water each time, in order to remove catalysts and unreacted monomers. Equilibrate the washed gel (20 min with shaking) in 1.5% glycerol and finally dry it onto Gel Bond PAG foil. Drying must be at room temperature, in front of a fan. Finally, mount the dried gel back in the polymerization cassette and allow it to reswell in the appropriate CA (and suitable additives) solution. In ultrathin gels, pH gradients are sensitive to the presence of salts, including TEMED and persulfate. Moreover, unreacted monomers are toxic and noxious to proteins. The preparation, washing and re-equilibration of “empty” gels removes these components from the gel and so avoids these problems.

16.2.4.4 Sample Loading and Electrophoresis

The gel is placed on the cooling plate of the electrophoresis chamber (Fig. 16.3). It is necessary to perform the electrophoresis at a constant temperature and with well defined conditions, because the temperature influences the pH gradient and consequently the separation positions of the proteins. Also, the presence of additives (e.g., urea) strongly affects the separation positions by changing the physicochemical parameters of the solution trapped inside the gel matrix. Electrode strips filled with electrode solutions are placed on the surface of the gel (at anodic and cathodic extremes). We much prefer weak acids (acetic acid) as anolyte or bases (Tris) as catholyte. Amphoteric compounds could also be used: for example, at the anode aspartic acid (pI 2.77 at 50 mM concentration) or iminodiacetic acid (pI 2.33 for a 100 mM solution) and at the cathode lysine free base (pI 9.74).
The electrophoretic procedure is divided into two steps. The first is a pre-electrophoresis, which allows prefocusing of CAs and the elimination of all contaminants or unreacted catalysts from the gel matrix. The protein sample is then loaded. In IEF gels, the sample might be applied along the whole pH gradient in order to determine its optimum application point. The second electrophoretic step is then started. This is carried out at constant power; low voltages are used for the sample entrance, followed by higher voltages during the separation and bands sharpening. The actual voltage used in each step depends on gel thickness.

### 16.2.5 General Protein Staining

Table 16.5 gives a list, with pertinent references, of some of the most common protein stains used in IEF. Extensive reviews covering general staining methods in gel electrophoresis have appeared (74, 75). Merrill and Washart (76) have also given a very extensive bibliography covering all aspects of polypeptide detection methods, including enzyme localization protocols (>350 citations). Additionally, although not specifically reported for IEF, some recent developments include the use of Eosin B dye (77), a mixed-dye technique comprising Coomassie Blue R-250 and Bismark Brown R (78), Stains-All for highly acidic molecules (79), fluorescent dyes for proteins, such as SYPRO Orange and SYPRO Red (80, 81), and a two-minute Nile Red staining (82).

A general comment on staining protocols seems appropriate here. Rabilloud (83) has evaluated all possible stains adopted for IEF and 2D maps according to key issues such as sensitivity (detection threshold), linearity, homogeneity (i.e., variation from one protein to another), and reproducibility. Accordingly, four steps have been considered:

- affixing the label before IEF
- labeling between IEF and SDS-PAGE

### TABLE 16.5 Protein Staining Methods

<table>
<thead>
<tr>
<th>Stain</th>
<th>Application</th>
<th>Sensitivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie Blue G-250 (micellar)</td>
<td>General use</td>
<td>Low</td>
<td>68</td>
</tr>
<tr>
<td>Coomassie Blue R-250/CuSO₄</td>
<td>General use</td>
<td>Medium</td>
<td>69</td>
</tr>
<tr>
<td>Coomassie Blue R-250/ Sulfoalicylic acid</td>
<td>General use</td>
<td>High</td>
<td>70</td>
</tr>
<tr>
<td>Silver stain Coomassie Blue R-250/CuSO₄</td>
<td>General use In presence of detergents</td>
<td>Very high</td>
<td>71</td>
</tr>
<tr>
<td>Light Green SF</td>
<td>General use</td>
<td>Medium</td>
<td>37</td>
</tr>
<tr>
<td>Fast Green PCF</td>
<td>General use</td>
<td>Low</td>
<td>72</td>
</tr>
<tr>
<td>Coomassie Blue R-250 at 60°C</td>
<td>In presence of detergents</td>
<td>High</td>
<td>73</td>
</tr>
</tbody>
</table>

We will briefly review these four steps, with the understanding that they apply not only to plain IEF and IPG, as in this Chapter, but also to a variety of other electrophoretic steps (e.g., SDS, 2D PAGE).

#### 16.2.5.1 Affixing the Label before IEF

In principle, this approach would be very convenient, because it could be done in a minimal volume with a high concentration of reactants. However, any noncovalently bound tag will be disrupted. On the other hand, any prelabeling method that alters the charge state of the protein, either by removing a charged group or by adding a spurious one, is not compatible with 2D maps. Thus, amine or thiol alkylation with reactive acidic dyes such as Remazol (84) must be avoided because the proteins will gain negative charges. Covalent fluorescent tagging would appear to be superior, provided that fluorophors with intense light absorption, high quantum yield, large Stokes shifts, and limited fading can be found. Because fluorescein derivatives carry negative charges, pre-IEF labeling has been limited for a long time to thiol alkylation with electrically neutral probes (85), although these species offer limited sensitivity. Use of cyanine-based probes (86), with attachment via amine acylation, improves the process and does not alter the protein charge (except for rather basic proteins), because the positive charge lost upon covalent bonding is replaced by the quaternary ammonium on the cyanine fluorophore.

Radioactive labeling, followed by fluorography (87) or by phosphor storage (88), appears to be the most sensitive and IEF- and 2D-compatible method. The latter technique, in which the β-radiation induces an energy change in an europium salt, which, via laser excitation, is converted into visible light, appears to offer a 20- to 100-fold higher sensitivity than autoradiography, coupled to a linear dynamic range of four orders of magnitude. Another modern detection method is based on amplification detectors similar to those used in high energy physics, and is compatible with dual-isotope detection (89). The conclusions: radioactive detection probably offers the best signal-to-noise ratio and the best ultimate sensitivity.

#### 16.2.5.2 Labeling between IEF and SDS-PAGE

This stage offers much greater flexibility, because the charge state of the proteins can be altered provided that the apparent $M_r$ is kept constant. Covalent labeling dominates at this point and thus fluorescent tags are preferred. However, because protein nucleophiles (amine and thiol groups) are the preferred targets for such a grafting, care to remove CAs should be exerted, because these compounds could consume most of the label. This process is usually achieved with a few aqueous-acid alcohol baths or with 10% trichloroacetic acid (TCA) (90). When tagging with fluorophores, it should
be remembered that detection is an important point. In 2D maps, a scanning device should be used for acquiring all the spots in the gel. Because laser-induced fluorescence offers the highest sensitivity, fluorophors having an excitation maximum close to an available laser source (e.g., fluorescein and rhodamine) are preferred. In another set-up, illumination is done in the gel plane, preferably by UV excitation, and the fluorescent light is collected by a charged coupled device (CCD) camera (91).

16.2.5.3 Tagging after SDS-PAGE After this step, which can be either mono- or bi-dimensional (2D maps), any solution can be adopted, according to the sensitivity desired. Noncovalent detection with organic dyes, the most common being the Coomassie Brilliant Blue G and R, is quite popular, due to the simple protocol, although the sensitivity is of the order of \( \sim 1 \mu g \) (92). These kinds of stains are of the regressive type; that is, the gel is first saturated with dye and then destained to remove the dye unreadable to the protein. Alternatively, in dilute dye solutions, the gel can be stained to the end point, which, however, requires very long staining times (93). As an additional option, micellar or colloidal stainings can be adopted, with sensitivities of \( \sim 100 \) ng. Noncovalent, fluorescent probes are also very popular after this stage. Generally, such probes are nonfluorescent in water but highly fluorescent in apolar media, such as detergent, thus they take advantage of SDS binding to proteins to create a microenvironment promoting fluorescence at the protein spot. Typical labels of this type are naphthalene derivatives (94), SYPRO dyes (81), and Nile Red (95).

Detection by differential salt binding is also an attractive procedure, although negative stains are problematic if photographic documentation is required. All negative stains use divalent cations (Cu, Zn) for forming a precipitate with SDS. Submicrogram sensitivities are claimed, the staining is very rapid, and destaining is achieved by simple metal chelators such as EDTA. Increased sensitivities are obtained when the precipitate in the gel is no longer Zn\(-SDS\), but a complex salt of zinc and imidazole (96, 97). In this last case, nanogram sensitivities can be obtained. Perhaps one of the most popular stains, however, is metal ion reduction, that is, silver staining, although it is one of the most complex detection procedures. What makes a silver stain highly sensitive is the strong autocatalytic character of silver reduction (98). This condition is achieved by using a very weak developer (e.g., dilute formaldehyde) and sensitizers between fixation and silver impregnation. Silver staining protocols are divided into two families. In one, the silversing agent is silver nitrate and the developer is formaldehyde in an alkaline carbonate solution. In the second, the silversing agent is a silver–ammonia complex and the developer formaldehyde in dilute citric acid. The sensitivity with silver staining in modern protocols is in the low nanogram range for both procedures. This is 100-fold better than classical Brilliant Blue staining, 10-fold better than colloidal Brilliant Blue tags and about twofold better than zinc staining, with the extra benefit of a much better contrast. All silver staining protocols that utilize aldehydes, however, are in general not compatible with subsequent MS analysis. Formaldehyde in general leads to alkylation of \( \alpha -\) and \( \epsilon -\) amino groups (99–101), which hinders identification of proteins using MS analysis. When fixation is avoided, good results are often obtained with methods in which the proteins are digested, such as in peptide mass fingerprinting. In this last case, protocols that include silver nitrate (102) or silver ammonia (103) have been reported to be successful in peptide mass identification. Also, destaining silver-stained gels with ferricyanide and thiosulfate seems to greatly improve subsequent analysis by MS (104).

16.2.5.4 Labeling after Blotting Staining on blots with organic dyes is done almost exclusively by regressive protocols. Because most dyes bind weakly to neutral membranes (nitrocellulose and polyvinylidene difluoride), and due to the concentration effect afforded by blotting, high nanogram sensitivities are easily reached (105). Noncovalent fluorescence detection is also feasible on blots. In this case, lanthanide complexes are used (106), because they have the advantage of providing time-lapse detection with a very good signal-to-noise ratio. In the case of PVDF membranes, not wettable with water, fluorescein or rhodamine can be used, because they will bind only to the wetted protein spots (107). Under these conditions, the detection limit on blots appears to be \( \sim 10 \) ng protein. At the end of this long excursion, some interesting conclusions have been drawn by Rabilloud (83). According to this author, most staining methods have reached a plateau close to their theoretical maximum. For organic dye staining, the maximum occurs when the dye is bound to all available sites on the protein, as is the case with colloidal Brilliant Blue staining. A maximum also seems to have been reached for silver staining, because no increase of sensitivity has been reported in the last five years. The only method left with much potential for improvement is fluorescence. Recently, in fact, an ultrasensitive protocol utilizing SYPRO Ruby IEF Protein Gel Stain (Molecular Probes, Eugene, OR, USA) has been reported. This luminescent dye can be excited with 302 or 470 nm light and it has been optimized for protein detection in IEF (and IPG) gels. Proteins are stained in a ruthenium-containing metal complex overnight and then simply rinsed in distilled water for 2 h. Stained proteins can be excited by UV light at \( \sim 302 \) nm (UV-B transilluminator) or with visible wavelengths at \( \sim 470 \) nm. Fluorescent emission of the dye is maximal at 610 nm. The sensitivity of SYPRO Ruby is superior to colloidal Coomassie stains and the best silver staining protocols by a factor of 3–30 times. SYPRO Ruby is suitable for staining proteins in both non-denaturing and denaturing CA IEF and IPGs. The unique advantage of this stain is that it does not contain the
extraneous chemicals (formaldehyde, glutaraldehyde, Tween-20) that frequently interfere with peptide identification in MS. In fact, successful identification of stained proteins by peptide mass profiling has been demonstrated, rendering this stain procedure a most promising tool for 2D mapping (108). Although it has been reported that Nile Red (109), as well as SYPRO Red and SYPRO Orange, can also stain IEF gels, the gels must be first incubated in SDS, because all three of these lipophilic dyes bind to proteins indirectly through the anionic detergent. Moreover, with these three dyes, the sensitivity is often poorer than with standard Coomassie staining. On all these accounts, it would appear that this novel SYPRO Ruby stain could represent a major revolution in detection techniques.

16.2.6 Troubleshooting

16.2.6.1 Waviness of Bands near the Anode This may be caused by the following:

- Carbonation of the catholyte: in this case, prepare fresh NaOH with degassed distilled water and store properly.
- Excess catalysts: reduce the amount of ammonium persulfate.
- Too long sample slots: fill them with dilute CAs.
- Too low concentrations of CAs: check the gel formulation.

To alleviate the problem, it is usually beneficial to add low concentrations of sucrose, glycine, or urea, and to apply the sample near the cathode. To salvage a gel during the run, as soon as the waves appear, apply a new anodic strip soaked with a weaker acid (e.g., acetic acid versus phosphoric acid) inside the original one, and move the electrodes closer to one another.

16.2.6.2 Burning along the Cathodic Strip This may be caused by the following:

- Formation of a zone of pure water at pH = 7: add to your acidic pH range a 10% solution of either the 3–10 or the 6–8 range ampholytes.
- Hydrolysis of the acrylamide matrix after prolonged exposure to alkaline pH: choose a weaker base, if adequate, and, unless a prerun of the gel is strictly required, apply the electrode strips after loading the samples.

16.2.6.3 pH Gradients Different from Expected This may be caused by the following:

- For acidic and alkaline pH ranges, the problem is alleviated by the choice of anolytes and catholytes with pH values close to the extremes of the pH gradient.
- Alkaline pH ranges should be protected from carbon dioxide by flushing the electrophoretic chamber with moisture-saturated nitrogen (or better with argon) and by surrounding the plate with pads soaked in NaOH. It is worth remembering that pH readings on unprotected alkaline solutions become meaningless within about half an hour.
- A large amount of a weak acid or base, supplied as sample buffer, may shift the pH range (β-mercaptoethanol is one such base). The typical effect of the addition of urea is to increase the apparent pIs of the CAs.
- It may be due to cathodic drift. There are several options to counteract this: reduce the running time to the required minimum (as experimentally determined for the protein of interest, or for a colored marker of similar $M_r$); increase the viscosity of the medium (with sucrose, glycerol, or urea); reduce the amount of ammonium persulfate; remove acrylic acid impurities by recrystallizing acrylamide and Bis, and treating the monomer solution with a mixed-bed ion-exchange resin; for a final cure, incorporate into the gel matrix a reactive base, such as 2-dimethylamino propyl methacrylamide (Polyscience) (110) (its optimal concentration (of the order of 1 μM) should be experimentally determined for the system being used).

16.2.6.4 Sample Precipitation at the Application Point If large amounts of material precipitate at the application point, even when the $M_r$ of the sample proteins is well within the porosity limits of polyacrylamide gels, the trouble is usually caused by protein aggregation. Some remedies include the following:

- Try applying the sample in different positions on the gel, with and without prerunning; some proteins might be altered only by a given pH.
- If you have evidence that the sample contains high $M_r$ components, reduce the value of $%T$ of the PAA gel.
- If you suspect protein aggregation brought about by the high concentration of the sample (for example when the problem is reproduced by disc electrophoresis runs) do not prerun and set a low voltage (100–200 V) for several hours to avoid the concentrating effect of an established pH gradient at the beginning of the run. Also, consider decreasing the protein load and switching to a more sensitive detection technique. The addition of surfactants and/or urea is usually beneficial.
- If the proteins are only sensitive to the ionic strength or the dielectric constant of the medium (in this case they perform well in disc electrophoresis and are precipitated if dialyzed against distilled water), increasing the CA concentration, adding glycine or taurine, and sample application without prerunning may overcome the problem.
• The direct choice of denaturing conditions (8 M urea, detergents, β-mercaptoethanol) very often minimizes these solubility problems, dissociating proteins (and macromolecular aggregates) to polypeptide chains.

### 16.2.7 Some Typical Applications of IEF

IEF is a fine-tuned analytical tool for investigating post-translational processing and chemical modification of proteins. Protein processing may be grouped into two types. The first covers changes in primary structure, with proteolysis at peptide bonds, for example, for removal of intervening sequences or of leader peptides, as in intracellular processing. Such modifications are typically identified by size fractionation techniques, such as SDS-PAGE. A second class covers another mode of processing, in which the size is only marginally affected and no peptide bond is cleaved, but the surface charge is modified. Such post-translational processing is the typical realm for IEF analysis. It includes attachment of molecular ligand. In extreme cases, such interactions could be a protein with a small ligand or a protein with a macromolecular ligand, to name just a few. But there are also a number of modifications occurring at the NH₂ groups (e.g., acetylation), at the COOH groups (e.g., deamination), and at the SH groups as well as a number of chemical modifications occurring in vitro, such as accidental carbamylation due to the presence of urea. All of these modifications can be properly investigated by IEF, as amply discussed in a broad review dedicated to this topic (111).

IEF, as a fractionation tool, coupled to MS, can be a formidable hyphenated method for assessing the extent of modification involved, the stoichiometry of the reaction, as well as the reaction site. IEF can also be used as a probe for interacting systems, such as protein–ligand interactions. If the interacting species are stable in the electric field, IEF enables determination of intrinsic ligand-binding constants for statistical binding of a charged ligand, binding to heterogeneous sites, and cooperative binding. The interacting systems can be a protein with a small ligand or a protein with a macromolecular ligand. In extreme cases, such interactions could be engendered by isomerization (pH-dependent conformational transition) or by interaction with the CA buffers. An excellent review on all these aspects is now available (112).

### 16.2.8 Examples of some Fractionations

It is impossible here to cover the vast body of literature on the use of IEF as a probe for protein microheterogeneity and for interacting systems. A case in point is the hemoglobin (Hb) saga, for which the reader is referred to the book by Righetti (113). We will only give here an example on the separation of active fragments of the human growth hormone (hGH), synthesized by the solid-phase method of Merrifield (114). In the early days of IEF it was thought that IEF of peptides would not be feasible, first, because they have a higher diffusion coefficient than proteins, and second, because they would not be precipitated and fixed into the gel matrix by the common protein stains, like alcoholic solutions of Coomassie Blue. Figure 16.5 dispelled both myths and proved that IEF of peptides was highly feasible and would indeed produce very sharp bands (68). The problem of fixation was solved by adopting a leuco-stain, a micellar suspension of Coomassie Brilliant Blue G-250 (G stands for its greenish hue, which cannot be appreciated in monomeric solutions, but is clearly visible in the micellar state) in TCA: as the focused gel is bathed directly in this solution, the peptides adsorb the dye from the micelle and are fixed by the dye molecules, which probably act as crosslinks over the different peptide chains, thus forming a macromolecular aggregate that is trapped in the gel matrix fibres. This experiment was very important for peptide chemists, because it helped in redirecting their synthetic strategies. As it turned out from Figure 16.5, when medium length peptides were produced via the Merrifield approach, the amount of failed and truncated sequences were in large excess over the desired product, to the point that the latter could no longer be recognized. Although this had been theoretically predicted, it had not been experimentally verified up to that time, due to lack of high resolution techniques. As news spread, in vitro synthesis of peptides took an important turn: it was done only in short sequences (5–6 amino acids at most), which were at the end joined together by splicing.

### 16.2.9 Preparative Aspects

In a book on protein purification, preparative aspects of IEF cannot be overlooked. One of the oldest methods is preparative IEF on a horizontal bed of granulated gels (typically Sephadex G 200) (115). The trough consists on a glass or quartz plate at the bottom of a lucite frame (40 × 40 cm or 20 × 20 cm, with a gel layer thickness of up to 10 mm). As much as 5–10 mg protein per mL gel suspension may be handled. Radola (115) fractionated 10 g pronase in 800 mL of gel suspension and obtained excellent band resolution even at this remarkable load capacity. Once focusing is achieved, 30 fractions are collected by compartmentation of the gel with the aid of a fractionation grid and by scraping off the gel with a spatula (Fig. 16.6). The protein is then recovered by placing the Sephadex gel fraction into a small column and eluting with plain water of suitable buffer. The technique has been recently resurrected for prefractionation in proteome analysis.

The other remarkable gadget is the mini-Rotofor, a Milan Bier creature (116). The device is assembled from 20 sample chambers, separated by liquid-permeable nylon screens, except at the extremities, where cation- and anion-exchange membranes are placed against the anodic and cathodic
compartments, respectively, so as to prevent diffusion within the sample chambers of noxious electrodic products (Fig. 16.7). During sample loading (panel a) the apparatus is at rest; during the IEF it rotates on its axis, so as to prevent electro-decantation (panel b). At the end of the preparative run, the 20 focused fractions are collected simultaneously by piercing a septum at the chambers’ bottom via 20 needles connected to a vacuum source (panel c). The narrow-pI range fractions can then be used to generate conventional 2D maps or to go directly to MS analysis after CA removal. A number of applications in proteome analysis have been reported. A micro-Rotofor, with only 10 chambers and reduced sample volume, for proteome analysis of minute samples, is also available.

16.2.10 Artefacts or not?
This question has been the object of hot debate, at the inception of the technique, and there were just as many reports on compartments, respectively, so as to prevent diffusion within the sample chambers of noxious electrodic products (Fig. 16.7). During sample loading (panel a) the apparatus is at rest; during the IEF it rotates on its axis, so as to prevent electro-decantation (panel b). At the end of the preparative run, the 20 focused fractions are collected simultaneously by piercing a septum at the chambers’ bottom via 20 needles connected to a vacuum source (panel c). The narrow-pI range fractions can then be used to generate conventional 2D maps or to go directly to MS analysis after CA removal. A number of applications in proteome analysis have been reported. A micro-Rotofor, with only 10 chambers and reduced sample volume, for proteome analysis of minute samples, is also available.

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artefacts as additional ones denying them. The final consensus was that artefacts, when reported, occurred only in extreme cases and with “extreme” structures. One of the most glorious examples was the suspicious report that heparin gave a large number (up to 21) of bands focusing in the pH 4–5 range (121). Now, how could a pure polyanion, bearing no positive counterions on its polymeric backbone, exhibit an “isoelectric point” remained a mystery to anybody with a minimum knowledge of chemistry. It took some extensive work (122, 123) to find out that these 21 fractions indeed represented 21 different complexes of the same heparin polymer with 21 different Ampholine molecules. This was the “catch 22”: in those days, when binding of small molecules to macromolecules was suspected, all theoreticians had worked out a bimodal distribution, that is, the bound versus the unbound species! After our report, it was clear that multimodal distribution could be the real trend in IEF, so theoreticians like Cann (124) hurried to change their models. It then became apparent that, in a specular fashion, polycations would also produce such an artefactual binding pattern (125). Other than these artefacts generated by peculiar structures, there was no practical evidence that CAs would elicit the same multimodal distribution by interacting with proteins, so general users widely accepted the technique.

16.3 IMMOBILIZED pH GRADIENTS

16.3.1 General Considerations

As illustrated below, IPGs represent perhaps the ultimate development in all focusing techniques, a big revolution in the field, in fact. Owing to the possibility of engineering the pH gradient at whim, from the narrowest (which, for practical purposes, has been set at 0.1 pH units over a 10 cm distance) to the widest possible one (a pH 2.5–12 gradient), IPGs permit the highest possible resolving power, in the one hand, and the widest possible collection of spots (in 2D maps) on the other hand. The chemistry is precise and amply developed; so are all algorithms for implementing any possible width and shape of the pH gradient. Because of their unique performance, IPGs represent now the best possible first dimension for 2D maps and are increasingly adopted for this purpose.

16.3.1.1 The Problems of Conventional IEF

Table 16.6 lists some of the major problems associated with conventional IEF using amphoteric buffers. Some of them are quite severe; for example, low ionic strength often induces near-isoelectric precipitation and smearing of proteins, even in analytical runs at low protein loads. The problem of uneven conductivity is magnified in poor ampholyte mixtures, like the Poly Sep 47 (a mixture of 47 amphoteric and nonamphoteric buffers, claimed to be superior to CAs) (126). Due to their poor composition, huge conductivity gaps form along the migration path, against which proteins of different pIs are stacked. The results are simply disastrous (127). Cathodic drift is also a major unsolved problem of CA-IEF, resulting in extensive loss of proteins at the gel cathodic extremity upon prolonged runs. For all these reasons, in 1982

<table>
<thead>
<tr>
<th>TABLE 16.6 Problems with CA Focusing</th>
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<tbody>
<tr>
<td>1. Medium of very low and unknown ionic strength</td>
</tr>
<tr>
<td>2. Uneven buffering capacity</td>
</tr>
<tr>
<td>3. Uneven conductivity</td>
</tr>
<tr>
<td>4. Unknown chemical environment</td>
</tr>
<tr>
<td>5. Not amenable to pH gradient engineering</td>
</tr>
<tr>
<td>6. Cathodic drift (pH gradient instability)</td>
</tr>
</tbody>
</table>
Bjellqvist and colleagues (35) launched the technique of immobilized pH gradients (IPGs).

16.3.1.2 The Immobiline Matrix  IPGs are based on the principle that the pH gradient, which exists prior to the IEF run itself, is copolymerized, and thus insolubilized, within the fibers of a PAA matrix (see Fig. 16.8 for a pictorial representation). This is achieved by using, as buffers, a set of six nonamphoteric, weak acids and bases, having the following general chemical composition: \( \text{CH}_2 = \text{CH}–\text{CO}–\text{NH}–R \), where R denotes either two different weak carboxyl groups, with a \( pK_a \) of 3.6 and 4.6, or four tertiary amino groups, with a \( pK_a \) of 6.2, 7.0, 8.5, and 9.3 (available under the trade name Immobiline from GE HealthCare). Their synthesis has been described by Chiari and colleagues (128, 129). A more extensive set, comprising 10 chemicals (a \( pK_a \) 3.1 acidic buffer, a \( pK_a \) 10.3 basic buffer, and two strong titrants, a \( pK_a \) 1 acid and a \( pK_a \) > 12 quaternary base) is available as “pI select” from Fluka AG, Buchs, Switzerland (see Tables 16.7 and 16.8 for their formulas) (130). All of the above chemicals have been reported by our group: for example, the synthesis of the \( pK_a \) 3.1 buffer was utilized (131) by Righetti and colleagues for the separation of isoforms of very acidic proteins (pepsin); the \( pK_a \) 10.3 species was first adopted by Sinha and Righetti (132) for creating alkaline gradients for separation of elastase isoforms. Over the years, we have reported the synthesis of a number of other buffering ions, produced with the aim of closing some gaps between the available Immobilines, especially in the pH 7.0–8.5 interval. These are a \( pK_a \) 6.6, 2-thiomorpholinoethylacrylamide and a \( pK_a \) 7.4, 3-thiomorpholinopropylacrylamide (133); a \( pK_a \) 6.85, 1-acryloyl-4-methylpiperazine (134); an alternative \( pK_a \) 7.0, 2-(4-imidazolyl)ethylamine-2-acrylamide (135); and a \( pK_a \) 8.05, \( N,N\)-bis(2-hydroxyethyl)\( N^\prime\)-acryloyl-1,3-diaminopropane (136). Additional species have also been described by Bellini and Manchester (137).

During gel polymerization, these buffering species are efficiently incorporated into the gel (84–86% conversion efficiency at 50°C for 1 h) (138). Immobiline-based pH gradients can be cast in the same way as conventional PAA gradient gels, using a density gradient to stabilize the Immobiline concentration gradient, with the aid of a standard, two-vessel gradient mixer. As shown in their formulas, these buffers are no longer amphoteric, as in conventional IEF, but are bifunctional. At one end of the molecule is located the buffering (or titrant) group, and at the other end is an acrylic double bond, which disappears during immobilization of the buffer on the gel matrix. The three carboxyl Immobilines have rather small temperature coefficients (dpK/dT) in the 10–25°C range, due to their small standard heats of ionization.

**Figure 16.8** Isoelectric focusing in immobilized pH gradients (IPG). A hypothetical gel structure is depicted, where the strings represent the neutral acrylamide residues, the cross-over points the Bis crosslinking, and the positive and negative charges the grafted Immobiline molecules. Courtesy of GE Healthcare.
2-acrylamido-2-N\textsubscript{2}methylpropane sulfonic acid

TABLE 16.7 Acidic Acrylamido Buffers

| pK | Formula | Name | M
<table>
<thead>
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<tbody>
<tr>
<td>1.2</td>
<td>CH\textsubscript{3}</td>
<td>2-acrylamido-2-methylpropane</td>
<td>207</td>
</tr>
<tr>
<td>3.1</td>
<td>CH\textsubscript{3}\textsubscript{2}SOH</td>
<td>2-acrylamido-glycolic acid</td>
<td>145</td>
</tr>
<tr>
<td>3.6</td>
<td>CH\textsubscript{3}\textsubscript{2}NH-N\textsubscript{2}CH\textsubscript{2}COOH</td>
<td>N\textsubscript{2}-acryloyl-glycine</td>
<td>129</td>
</tr>
<tr>
<td>4.6</td>
<td>CH\textsubscript{3}\textsubscript{2}NH-N\textsubscript{2}CH\textsubscript{2}COOH</td>
<td>4-acrylamido-butyric acid</td>
<td>157</td>
</tr>
</tbody>
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(
~1 \text{ kcal/mol}) and thus exhibit negligible pK variations in this temperature interval. On the other hand, the five basic Immobilines exhibit rather large \( \Delta pK \)s (as much as \( \Delta pK = 0.44 \) for the pK 8.5 species) due to their larger heats of ionization (6–12 kcal/mol). Therefore, for reproducible runs and pH gradient calculations, all the experimental parameters have been fixed at 10°C. The largest pK changes are due to the presence of urea: acidic Immobilines increase their pK in 8 M urea by as much as 0.9 pH units, while the basic Immobilines increase their pK by only 0.45 pH units (139). Detergents in the gel (2%) do not alter the Immobiline pK, suggesting that they are not incorporated into the surfactant micelle. For generating extended pH gradients, we use two additional chemicals that are strong titrants having pK ranging extended pH gradients, we use two additional chemicals.

As shown in Figure 16.8, the proteins are placed on a gel with a preformed, immobilized pH gradient (represented by carboxyl and tertiary amino groups grafted to the PAA chains). When the voltage is applied, only the sample molecules (and any ungrafted ions) migrate in the electric field. Upon termination of electrophoresis, the proteins are separated into stationary, isoelectric zones. Owing to the possibility of designing stable pH gradients at will, separations have been reported in only 0.1 pH unit-wide gradients over the entire separation axis, leading to an extremely high resolving power (\( \Delta pI = 0.001 \) pH unit).

16.3.1.3 Narrow and Ultranarrow pH Gradients

We define the gradients from 0.1 to 1 pH unit as narrow (towards the 1 pH unit limit) and ultranarrow (close to the 0.1 pH unit limit) gradients. Within these limits we work on a tandem principle; that is, we choose a buffering Immobiline, either a base or an acid, with its pK within the pH interval we want to generate, and a nonbuffering Immobiline, then an acid or a base, respectively, with its pK at least 2 pH units removed from either the minimum or maximum of our pH range. The titrant will provide equivalents of acid or base to titrate the buffering group but will not itself buffer in the desired pH interval. For these calculations, we used to resort to modified Henderson–Hasselbalch equations and to rather complex nomograms (1). A list of 58 gradients, each 1 pH unit wide, starting with the pH 3.8–4.8 interval and ending with the pH 9.5–10.5 range, separated by 0.1 pH unit increments, can be found in Righetti (1).

16.3.1.4 Extended pH Gradients: General Rules for their Generation and Optimization

Linear pH gradients are obtained by arranging for an even buffering power throughout. The latter could be ensured only by ideal buffers spaced apart by \( \Delta pK = 1 \). In practice, there are only eight buffering Immobilines with some wider gaps in \( \Delta pK \)s, so other approaches must be used to solve this problem. Two methods are possible. In one approach (constant buffer concentration), the concentration of each buffer is kept constant throughout the span of the pH gradient and “holes” of buffering power are filled by increasing the amounts of the buffering species bordering the largest \( \Delta pK \)s. In the other approach (varying buffer concentration) the variation in concentration of different buffers along the width of the desired pH gradient results in a shift in each buffer’s apparent pK, together with the \( \Delta pK \) values evening out. The second approach is by far preferred, because it gives much higher flexibility in the computational approach. In a series of papers (140–150) we have described a computer approach able to calculate and optimize any such pH interval, up to the most extended one (which can cover a span of pH 2.5–11). Tables for these recipes can be found in the book by Righetti (1) and in many of the above references. In addition, a user-friendly computer program, written by Giaffreda and colleagues (151), allows easy calculation of any desired pH interval. However, we will give here

TABLE 16.8 Basic Acrylamido Buffers

| pK | Formula | Name | M
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>6.2</td>
<td>CH\textsubscript{3}\textsubscript{2}N\textsubscript{2}</td>
<td>2-morpholino ethylacrylamide</td>
<td>184</td>
</tr>
<tr>
<td>7.0</td>
<td>CH\textsubscript{3}\textsubscript{2}N\textsubscript{2}</td>
<td>3-morpholino Propylacrylamide</td>
<td>199</td>
</tr>
<tr>
<td>8.5</td>
<td>CH\textsubscript{3}\textsubscript{2}N\textsubscript{2}</td>
<td>N,N-dimethyl aminoethyl acrylamide</td>
<td>142</td>
</tr>
<tr>
<td>9.3</td>
<td>CH\textsubscript{3}\textsubscript{2}N\textsubscript{2}</td>
<td>N,N-dimethyl aminopropyl acrylamide</td>
<td>156</td>
</tr>
<tr>
<td>10.3</td>
<td>CH\textsubscript{3}\textsubscript{2}N\textsubscript{2}</td>
<td>N,N-diethyl aminopropyl acrylamide</td>
<td>184</td>
</tr>
<tr>
<td>&gt;12</td>
<td>CH\textsubscript{3}\textsubscript{2}N\textsubscript{2}</td>
<td>N,N,N-triethyl aminoethyl acrylamide</td>
<td>198</td>
</tr>
</tbody>
</table>
general guidelines for the use of such program and optimization of various recipes:

- When calculating recipes up to 4 pH units, in the pH 4–9 interval, there is no need to use strong titrants. As most acidic and basic titrants, the pK 3.1 and 10.3 Immobilines can be used, respectively.
- When optimizing recipes >4 pH units (or close to the pH 3 or pH 11 extremes) strong titrants are preferably used, otherwise it will be quite difficult to obtain linear pH gradients, because weak titrants will act as buffering ions as well.
- When calculating recipes of 4 pH units, it is best to insert in the recipe all the eight weak buffering Immobilines. The computer program will automatically exclude the ones not needed for optimization.
- The program of Giaffreda and colleagues (151) can calculate not only linear, but also concave or convex exponential gradients (including sigmoidal ones). In order to limit consumption of Immobilines (at high concentration in the gel they could give rise to ominous reswelling and interact with the macromolecule separand via ion-exchange mechanisms), one should limit the total Immobiline molarity (e.g., to only 15–20 mM) and the average buffering power (β) (these two bits of information are specifically asked when preparing any recipe). In particular, please note that recipes with an average β value of only 2–3 milliequiv/L/pH are quite adequate in IPGs. The separand macroions, even at concentration >10 mg/mL, rarely have β values greater than 1 milliequiv/L/pH.
- When working at acidic and alkaline pH extremes, however, the average β power of the recipe should be progressively higher, so as to counteract the β value of bulk water. Additionally, at such pH extremes, the matrix acquires a net positive or negative charge and this gives rise to strong electroosmotic flow (EOF). In order to quench EOF, the washed and dried matrix should be reswollen against a gradient of viscous polymers (e.g., liquid linear PAA, hydroxyethyl cellulose) (152).

16.3.1.5 Nonlinear, Extended pH Gradients Although, originally, most IPG formulations for extended pH intervals had been given only in terms of rigorously linear pH gradients, this might not be the optimal solution in some cases. The pH slope might need to be altered in pH regions that are overcrowded with proteins. This is particularly important in the general case involving the separation of proteins in a complex mixture, such as cell lysates, and is therefore imperative when performing 2D maps. We have computed the statistical distribution of the pl of water-soluble proteins and plotted them in the histogram of Figure 16.9. From the histogram, given the relative abundance of different species, it is clear that an optimally resolving pH gradient should have a gentler slope in the acidic portion and a steeper profile in the alkaline region. Such a course has been calculated by assigning to each 0.5 pH unit interval in the pH 3.5–10 region a slope inversely proportional to the relative abundance of proteins in that interval. This generated the ideal curve (dotted line) in Figure 16.9.

16.3.1.6 Extremely Alkaline pH Gradients Recipes can also be calculated for producing an extremely alkaline immobilized pH gradient, covering nonlinearly the pH 10–12 interval, for separation of very alkaline proteins, such as subtilisins and histones (153, 154). Successful separations were obtained in 6% T, 4% C PAA matrices, reswollen in 8 M urea, 1.5% Tween 20, 1.5% Nonidet P-40, and 0.5% Ampholine pH 9–11. Additionally, in order to quench the very high conductivity of the gel region on the cathodic side, the reswelling solution contained a 0–10% (anode to cathode) sorbitol gradient (or an equivalent 0–1% hydroxyethyl cellulose (HEC) gradient). Best focusing was obtained by running the gel at 17°C, instead of the customary 10°C temperature. In the case of histones, all their major components had pl values between pH 11 and 12, and only minor components (possibly acetylated and phosphorylated forms) focused below pH 11. This same recipe could be used as a first dimension run for a 2D separation of histones (155).

16.3.2 IPG Methodology

Note that the basic equipment required is the same as for conventional CA-IEF gels. In addition, as we essentially use
the same PAA matrix, the reader is referred to Section 16.2.3 for a description of its general properties.

16.3.2.1 Casting an Immobiline Gel When preparing for an IPG experiment, two pieces of information are required: the total liquid volume needed to fill the gel cassette, and the required pH interval. Once the first is known, this volume is divided into two halves: one half is titrated to one extreme of the pH interval, the other to the opposite extreme. As the analytical cassette usually has a thickness of 0.5 mm and, for the standard 12 × 25 cm size, (Fig. 16.4) contains 15 mL of liquid to be gelled, in principle two solutions, each of 7.5 mL, should be prepared. However, because the volume of some Immobilines to be added to 7.5 mL might sometimes be rather small (<50 μL), we prefer to prepare a double volume, which will be enough for casting two gel slabs. The polymerization cassette is filled with the aid of a two-vessel gradient mixer and thus the liquid elements that fill the vertically standing cassette have to be stabilized against remixing by a density gradient. The two solutions are, by convention, called “acidic dense” and “basic light” solutions.

Figure 16.10 gives the final assembly for cassette and gradient mixer. As for the gradient mixer, it should be noted that one chamber contains a magnetic stirrer, while in the reservoir is inserted a plastic cylinder having the same volume, held by a trapezoidal rod. The latter, in reality, is a “compensating cone” needed to raise the liquid level to such an extent that the two solutions (in the mixing chamber and in the reservoir) will be hydrostatically equilibrated. In addition, this plastic rod can also be utilized for manually stirring the reservoir after addition of TEMED and persulfate.

It is preferable to use “soft” gels, that is, with a low %T. Originally, all recipes were given for 5%T matrices, but today we prefer 3.5–4%T gels (156). These “soft” gels can be easily dried without cracking and allow better entry of larger proteins. In addition, the local ionic strength along the polymer coil is increased, and this permits sharper protein bands due to increased solubility at the pI. A linear pH gradient is generated by mixing equal volumes of the two starting solutions in a gradient mixer. It is a must, for any gel formulation removed from neutrality (pH 6.5–7.5), to titrate the two solutions to neutral pH, so as to ensure reproducible polymerization conditions and avoid hydrolysis of the five alkaline buffering Immobilines. If the pH interval used is acidic, add Tris, if it is basic, add formic acid. If the same gradient is to be prepared repeatedly, the buffering and non-buffering Immobiline and water mixtures can be prepared as stock solutions and stored according to the recommendations for Immobiline. Prepared gel solutions must not be stored.

16.3.2.2 Reswelling Dry Immobiline Gels Precast, dried Immobiline gels, encompassing a number of ranges, are now available from GE Healthcare, Bio-Rad, and Serva. They all contain 4%T and they span the following pH narrow ranges: pH 3.5–4.5, pH 4.0–5.0, pH 4.5–5.5, pH 5.0–6.0, pH 5.5–6.7. In addition, there are a number of wide pH ranges: pH 4–7L, pH 6–9, pH 6–11, pH 3–10L, and pH 3–10NL (L, linear; NL, nonlinear). Some of them are available in 7, 11, 13, and 18 cm (now even in 24 cm) in length. All of them are 3 mm wide and, when reswollen, 0.5 mm thick (gel layer). Precast, dried IPG gels in alkaline narrow ranges should be handled with care, because at high pHs the hydrolysis of both the gel matrix and the Immobiline chemicals bound to it is much more pronounced. Because, even under isionic conditions, acidic ranges swell 4–5 times faster than alkaline ones (157), it is preferable to reswell dried Immobiline gels in a cassette similar to the one for casting the IPG gel.
CONVENTIONAL ISOELECTRIC FOCUSING

16.3.2.3 Electrophoresis  A common electrophoresis protocol consists of an initial voltage setting of 500 V, for 1–2 h, followed by an overnight run at 2000–2500 V. Ultrannarrow gradients are further subjected to a couple of hours at 5000 V, or better at about 1000 V/cm across the region containing the bands of interest.

16.3.2.4 Staining and pH Measurements  IPGs tend to bind strongly to dyes, so the gels are better stained for a relatively short time (30–60 min) with a stain of medium intensity, such as colloidal Coomassie Blue. For silver staining, a recipe optimized for IPG gels has been published (158). A novel fluorescent staining protocol, using the dye SYPRO Ruby, has been reported (108) and is valid for both conventional IEF and IPGs. It appears to have the highest possible sensitivity so far reported for any staining procedure. Accurate pH measurements are virtually impossible by equilibration between a gel slice and excess water, and not very reliable with a contact electrode, although, in mixed Immobiline–Ampholine gels this is feasible, due to elution of the soluble CA buffers into the supernatant (159–161). One can preferably either refer to the banding pattern of a set of marker proteins, or elute CAs from a mixed-bed gel, when applicable.

16.3.2.5 Storage of Immobiline Chemicals  There are two major problems with Immobiline chemicals, especially with the alkaline ones: hydrolysis and spontaneous autopolymerization. Hydrolysis is quite a nuisance because then only acrylic acid is incorporated into the IPG matrix, with a strong acidification of the calculated pH gradient. Hydrolysis is an autocatalyzed process for the basic Immobilines, because it is pH-dependent. For the pK 8.5 and 9.3 species, such a cleavage reaction on the amido bond can occur even in the frozen state, at a rate of about 10% per year (162). Autopolymerization is also quite deleterious for the IPG technique. Again, this reaction occurs particularly with alkaline Immobilines, and is purely autocatalytic, as it is greatly accelerated by deprotonated amino groups. Oligomers and n-mers are formed that stay in solution and can even be incorporated into the IPG gel. These products of autopolymerization, when added to proteins in solution, are able to bridge them via two amidate bonds (163–165). One fact has emerged: the species most prone to autopolymerization is the pK 7.0 Immobiline and, in present-day Immobilines, we could detect, by MS, only traces (0.5%) of barely dimers (166). As a remedy to these drawbacks, it has been shown that, when dissolved in anhydrous n-propanol (containing a maximum of 60 ppm water), these species are stabilized against both hydrolysis and autopolymerization for a virtually unlimited period of time (less than 1% degradation per year even when stored at +4°C). Thus, present-day alkaline Immobiline chemicals are now supplied as 0.2 M solutions in n-propanol. The acidic Immobilines, being much more stable, are available as water solutions laced with 10 ppm of an inhibitor (167).

16.3.2.6 Mixed-Bed, CA-IPG Gels  In CA-IPG gels the primary, immobilized pH gradient, is admixed with a secondary, soluble CA-driven pH gradient. It sounds strange that, given the problems connected with the CA buffers (discontinuities along the electrophoretic path, pH gradient decay, and so on), which the IPG technique was supposed to solve, one should resurrect this past methodology. In fact, when working with membrane proteins (168) and with microvillar hydrolases (45), we found that the addition of CAs to the sample and IPG gel would increase protein solubility, possibly by forming mixed micelles with the detergent used for membrane solubilization or by directly complexing with the protein itself. On a relative hydrophobicity scale, the five basic Immobilines (pK 6.2, 7.0, 8.5, 9.3, and 10.3) are decidedly more hydrophobic than their acidic counterparts (pK 3.1, 3.6, and 4.6). Thus CAs might quench the direct hydrophobic protein–IPG matrix interaction. It has been additionally found that addition of CAs to the sample protects it from strongly acidic and alkaline boundaries originating from the presence of salts in the sample zone (especially “strong” salts, such as NaCl, phosphates, and so on) (169). In general, only a minimum amount of CAs (in general ~0.5%) should be added, to avoid sample precipitation in the pocket and to produce sharply focused bands.

16.3.3 Trouble Shooting

We will only list here the major problems encountered with the IPG technique and we refer the readers to Table 16.9 for all the possible causes and remedies suggested. We highlight the following points:

- When the gel is gluey and there is poor incorporation of Immobilines, the biggest offenders are generally the catalysts (e.g., too old persulfate, crystals wet due to adsorbed humidity, wrong amounts of catalysts added to the gel mix). Also check the polymerization temperature and the pH of the gelling solutions.
- Bear in mind the last point in Table 16.9: if you have done everything right, and still you do not see any focused protein, you might have simply positioned the platinum wires on the gel with the wrong polarity. Unlike conventional IEF gel, in IPGs the anode has to be positioned at the acidic (or less alkaline) gel end.
16.3.4 Some Analytical Results with IPGs

We will limit this section to some examples of separations in ultranarrow pH intervals, where the tremendous resolving power ($\Delta pI$) of IPGs can be fully appreciated. The $\Delta pI$ is the difference, in surface charge, in pI units, between two barely resolved protein species. Rilbe has defined $\Delta pI$ as

$$\Delta(pI) = 3.07 \sqrt{\frac{D(d(pH)/dx)}{E[-du/d(pH)]}}$$

where $D$ and $du/d(pH)$ are the diffusion coefficient and titration curve of proteins, $E$ is the voltage gradient applied, and $d(pH)/dx$ is the slope of the pH gradient over the separation distance. Experimental conditions that minimize $\Delta pI$ will maximize the resolving power. Ideally, this can be achieved by simultaneously increasing $E$ and decreasing $d(pH)/dx$, an operation for which IPGs seem well suited. With conventional IEF it is very difficult to engineer pH gradients that are narrower than 1 pH unit. One can push the $\Delta pI$, in IPGs, to the limit of 0.001 pH unit; the corresponding limit

Table 16.9 Troubleshooting Guide for IPGs

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Cause</th>
<th>Remedy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drifting of pH during measurement of basic starting solution</td>
<td>Inaccuracy of glass pH electrodes (alkaline error)</td>
<td>Consult information supplied by electrode manufacturer</td>
</tr>
<tr>
<td>Leaking mold</td>
<td>Dust or gel fragment on the</td>
<td>Carefully clean the gel plate and gasket</td>
</tr>
<tr>
<td>The gel consistency is not firm, gel does not hold its shape after</td>
<td>Inefficient polymerization</td>
<td>Prepare fresh ammonium persulfate and check that the</td>
</tr>
<tr>
<td>removal from the mold</td>
<td></td>
<td>recommended polymerization conditions are being used</td>
</tr>
<tr>
<td>Plateau visible in the anodic and/or cathodic section of the gel</td>
<td>High concentration of salts in</td>
<td>Check that the correct amounts of ammonium persulfate</td>
</tr>
<tr>
<td>during electrofocusing, no focusing proteins seen in that part of the</td>
<td>the system</td>
<td>and TEMED are used</td>
</tr>
<tr>
<td>gel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overheating of gel near sample application when beginning electrofocusing</td>
<td>High salt content in the</td>
<td>Reduce salt concentration by dialysis or gel filtration</td>
</tr>
<tr>
<td>Nonlinear pH gradient</td>
<td>sample</td>
<td></td>
</tr>
<tr>
<td>Refractive line at pH 6.2 in the gel after focusing</td>
<td>Backflow in the gradient mixer</td>
<td>Find and mark the optimal position for the gradient</td>
</tr>
<tr>
<td>Curved protein zones in that portion of the gel at the top of the mold</td>
<td>Unincorporated polymers</td>
<td>mixer on the stirrer</td>
</tr>
<tr>
<td>polymerization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uneven protein distribution across a zone</td>
<td>Slot or sample application</td>
<td>Place the slot or sample application pieces perfectly</td>
</tr>
<tr>
<td></td>
<td>not perpendicular to running</td>
<td>perpendicular to the running direction</td>
</tr>
<tr>
<td>Diffuse zones with unstained spots, or drops of water on the gel</td>
<td>Incomplete drying of the gel</td>
<td>Dry the gel until it is within the 5% of its original</td>
</tr>
<tr>
<td>surface during the electrofocusing</td>
<td>after the washing step</td>
<td>weight</td>
</tr>
<tr>
<td>No zones detected</td>
<td>Gel is focused with the wrong</td>
<td>Mark the polarity on the gel when removing it from the</td>
</tr>
<tr>
<td></td>
<td>polarity</td>
<td>mold</td>
</tr>
</tbody>
</table>

Figure 16.11 Focusing of umbilical cord lysates from an individual heterozygous for fetal hemoglobin (HbF) Sardinia (only the HbF bands are shown, because the two other major components of cord blood, that is, HbA and HbFa, are lost in very shallow pH gradients). In an IPG gel spanning 0.1 pH unit, over a standard 10-cm gel length, two bands are resolved, identified as the Gly versus Ala gene products. The resolved Aγ/Gγ bands are in a 20:80 ratio, as theoretically predicted from gene expression. Their identity was established by eluting the two zones and fingerprinting the γ chains. From Reference 170, with permission.
in CA-IEF being only 0.01 pH units. Resolving neutral mutants, which carry a point mutation involving amino acids with nonionizable side chains and are, in fact, described as “electrophoretically silent,” is a challenging proposition. A remarkable example of the resolving power of IPGs is given in Figure 16.11, which shows the separation of two fetal hemoglobins, Aγ and Gγ, carrying a Gly → Ala mutation in γ-136. These two tetramers, normal components during

Figure 16.12  Exploded view of the miniaturized multicomartment electrolyzer operating with isoelectric membranes. The buffering, fixed-pI membranes between the various chambers are not shown. An assembly with only five chambers is displayed. Courtesy of B. Herbert and P.G. Righetti, unpublished.

Figure 16.13  Experimental set-up used to perform Off-Gel separations with multi-cup devices, composed of either 10 (a) or 22 (b, c) wells. The pH gradients used in each case are presented under the gels. From Reference 171, with permission.
fetal life, are found in a ratio of \( \approx 80:20 \). If one adopts a 0.1 pH unit gradient (over a standard 10-cm migration length), even these two tetramers can be separated, with a resolution close to the practical limit of \( \Delta pI = 0.001 \) (170).

### 16.3.5 Preparative Aspects of IPGs

Just as stated for CA-IEF, preparative separations are important in a book devoted to “protein purification.” We will show here just two instruments, which have been favorably received in proteomic analysis: the multicompartment electrolyzer (MCE) with isoelectric, Immobiline membranes and the Off-Gel apparatus. Figure 16.12 gives an exploded view of the MCE, in which two terminal electrode chambers are used to block between three sample chambers (the instrument is modular and can accommodate up to eight chambers) (171). Between each chamber there is an isoelectric membrane, made with Immobilines, defining a given pH (pl) value and thus buffering and titrating any ion crossing it. Each pair of membranes thus acts as an isoelectric “trap” capturing only proteins having intermediate pl values. The various fractions can then be collected and analyzed in 2D maps having as a first dimension an IPG strip covering the same pH interval as that of the collected proteins, thus ensuring maximum spread on the pl/\( M_r \) plane of the polypeptide chains and permitting very high resolution separations. The Off-Gel apparatus (Fig. 16.13) is built on a similar principle, except that the various cups are pressed directly against an IPG strip spanning a given pH interval, rather than onto membranes of fixed pl value (172). Here too, narrow-pl fractions are collected, as shown in the analysis of an \( E. coli \) lysate presented in Figure 16.14. Both instruments are user-friendly and have found important applications in proteome prefractionation. However, strong competition to both instruments might come from the novel technique of solid buffers, where isoelectric beads of different pl values are put in contact, in a cascade fashion, with anion or cation exchangers, thus also capturing proteins of given pl intervals, but in a very fast time frame (dozens of minutes versus up to one day in the two electrophoretic devices) and a highly simplified set-up (173).

### 16.4 CAPILLARY ISOELECTRIC FOCUSING (cIEF)

Although cIEF is still beleaguered by some problems (notably how to cohabit with the ever present hazard of electroosmotic flow), the technique is gaining momentum and is becoming quite popular, especially in the analysis of r-DNA products and of heterogeneity due to differential glycosylation patterns and to “protein ageing,” that is, Asn and Gln deamidation in vitro. A spin-off of cIEF is zone electrophoresis in zwitterionic, isoelectric buffers, a technique that exploits all the basic concepts of IEF and offers unrivalled resolution due to fast analysis in high voltage gradients.

#### 16.4.1 General Considerations

In addition to the reviews already suggested (2–4), we recommend, as further reading, another set of reviews, amply covering the field (174–180).
Capillary electrophoresis offers some unique advantages over conventional gel-slab techniques: the amount of sample required is truly minute (a few μL at the injection port, but only a few nL in the moving zone); the analysis time is in general very short (often just a few minutes) due to the very high voltages applicable; and analyte detection is on-line and is coupled to a fully instrumental approach (with automatic storage of electropherograms on a magnetic support). A principal difference between IEF in a gel and in a capillary is that, in the latter, mobilization of the focused proteins past the detector has to be carried out if an on-line imaging detection system is not being used. Three main techniques are used: chemical and hydrodynamic flow mobilization (in coated capillaries) and mobilization utilizing EOF (in uncoated or partially coated capillaries).

### 16.4.2 cIEF Methodology

Table 16.10 gives a typical methodology for cIEF in a coated capillary. Because a tremendous number of procedures for silanol deactivation have been reported and good coating practice is very difficult to achieve in a general biochemical lab, we recommend buying precoated capillaries.

#### 16.4.2.1 General Guidelines for cIEF

The following general guidelines are additionally suggested:

- All solutions should be degassed.
- The ionic strength of the sample may influence dramatically the length of the focusing step and also completely ruin the separation; therefore, sample desalting prior to focusing or a low buffer concentration (ideally made of weak buffering ion and weak counter-ion) is preferable. Easy sample desalting can be achieved via centrifugation through Centricon membranes (Amicon).
- The hydrolytic stability of the coating is poor at alkaline pH; therefore, mobilization with NaOH may destroy such coatings after a few runs.
- Ideally, nonbuffering ions should be excluded in all compartments for cIEF. This means that in the electrolytic reservoirs one should use weak acids (at the anode) and weak bases (at the cathode) instead of phosphoric acid and NaOH, as adopted today by most cIEF users. This includes the use of zwitterions (e.g., Asp pI = 2.77 or Glu pI = 3.25 at the anode and Lys pI = 9.74 or Arg pI = 10.76 at the cathode).
- When moving the focused bands past the detector, resolution is better maintained by a combination of salt elution (e.g., adding 20 mM NaCl or Na phosphate to the appropriate compartment) and a siphoning effect, obtained by having a higher liquid level in one compartment and a lower level in the other. The volumes to use will depend on the apparatus. For the BioFocus 2000 apparatus from Bio-Rad, the volumes used are 650 and 450 μL, respectively.

#### 16.4.2.2 Increasing the Resolution by Altering the Slope of the pH Gradient

Methods have not yet been devised for casting IPGs in a capillary format and so it is difficult in cIEF to achieve the resolution typical of IPGs, namely ΔpI = 0.001. Nevertheless, one efficient way for incrementing the resolving power of CA-IEF is to add “spacers” to a regular 2 pH unit interval. For instance, for screening for β-thalassemia by measuring the relative proportions of three major Hbs present in cord blood of newborns: adult (A), fetal (F), and acetylated fetal (Fac) spacer can be proficiently added for incrementing resolution. We have applied this know-how to cIEF of cord blood. As shown in Figure 16.15a, the addition of 100 mM β-Ala brought about excellent resolution among the three species, thus allowing proper quantitation and correct diagnosis of thalassemic conditions (181). Similar results have been obtained by the same procedure for screening of glycated Hbs (Hb A1c; Fig. 16.15b) (182), important for evaluation of some pathological alterations of the glycosidic metabolic pathways, such as for assessing the degree of diabetes.

Another interesting study lies in the field of protein–protein interaction. This is usually done by immobilizing in the capillary one of the reactants and letting the interacting species move through the immobilized zone by electrophoretic transport. However, there is another efficient and simple procedure: one of the two reactants could be immobilized “as a temporal” event, in a pH gradient. The ligand could then be
swept through the stationary zone and the stoichiometric complex, provided its pI value is outside the bounds of the pH gradient created in the capillary, emerge at the detector window, and thus quantified. This concept has been applied to the study of haptoglobin (Hp2)hemoglobin (Hb) complex formation (183).

16.4.2.3 On the Problem of Protein Solubility at their pI One of the most severe shortcomings of all IEF techniques (whether in gel slabs or capillaries, in soluble buffers or IPGs) is protein precipitation at the pI value. This problem is aggravated by increasing sample concentrations (overloading is often necessary in order to reveal minor components) and by decreasing the ionic strength (I) of the background electrolyte. In this last case, it has been calculated that a 1% CA solution, once focused, would exhibit a remarkably low I value (50), of the order of 0.5 milli-equiv/L. As demonstrated by Grönnwall (184), the solubility of an isionic protein, plotted against pH near the isionic point, is a parabola, with a fairly narrow minimum at relatively high I, but with progressively wider minima, on the pH axis, at decreasing I values. This means that, in unfavorable conditions, protein precipitation will not simply occur at a precise point of the pH scale (the pI), but it will occur in the form of smears covering as much as 0.5 pH units.

In the past we have used, with some success, glycerol, ethylene, and propylene glycols (185) when purifying substantial amounts of proteins by preparative IPGs. However, we recently found a number of proteins completely insensitive to these solubilizers. As non-denaturing solubilizers, for protein recovery in a native form, zwitterions can be adopted in cIEF, such as non-detergent sulfobetaines (NDSB) or 1 M bicine (i.e., one of the Good’s buffers) or taurine. Other mixtures of neutral additives are 20% sucrose, but also sorbitol and, to a lesser extent, sorbose (186). It is noteworthy that the use of zwitterions was advocated long ago by Alper and colleagues (187). Although this use had fallen into oblivion, recent reports by Vuillard and colleagues (188–190) suggest that this was indeed an avenue worth exploring, as their results with this novel class of zwitterions, synthesized by them (called non-detergent sulphobetaines, NDSB), have been encouraging not only in focusing mildly hydrophobic membrane proteins, but also in improving protein crystal growth.

It should finally be noted that protein precipitation and denaturation could also be induced by the presence of high salt levels in the sample, as typically occurs in biological fluids (e.g., urine, cerebrospinal fluid (CSF)). Thus, desalting prior to the IEF step is often a must. Manabe and colleagues (191) reported a method for microdialysis of CSF, able to process a volume of as little as 20–30 μL, coupled to a conductivity device. With this pre-cIEF step, these authors were able to successfully fractionate CSF and resolve as many as 70 peaks.

16.4.2.4 Assessment of pH Gradients and pI Values in cIEF In conventional IEF in gel slabs, protein pI markers are commonly offered by a number of suppliers (e.g., GE Healthcare, Bio-Rad, Serva). In the simplest approach, unknown pI values can also be assessed in cIEF by plotting the pI values of a set of markers, cofocused with the proteins under investigation, versus their relative mobility upon elution. In the vacuum method proposed by Chen and
Wiktorowicz (192) this plot is linear, so a high precision (± 0.1 pH unit) is obtained. In another, more intriguing approach, in the focusing of transferrin, Kilár (193) has proposed a novel method for pI assessments: monitoring the current in the mobilization step. If one records simultaneously the peaks of the mobilized stack of proteins and the rising current due to passage of the salt wave in the capillary, one can correlate a given pI value (which should be known from the literature a priori) with a given current associated with the transit of a peak at the detector port; this appears to be a precise method, because the error is given as only about ± 0.03 pH units.

The use of protein markers has problems, however, because not only are they difficult to obtain as single pI components (they are often a family of closely related species), but they are also subject to ageing, due to hydrolysis of side chain amides in Asn and Gln residues. A report by Shimura and colleagues (194) offers an interesting solution to all these problems. These authors have synthesized a set of 16 peptides (trimers to examers), which cover the range quite evenly from pI 3.38 up to pI 10.17. Each peptide contains one Trp residue (for on-line detection at 280 nm) and other amino acids with ionizable side chains, responsible for a good titration curve around their pI values. The sharp focusing, stability, high purity, and high solubility of these synthetic pI markers should facilitate the profiling of a pH gradient in cIEF and thus the determination of protein pI values.

16.5 SEPARATION OF PEPTIDES AND PROTEINS BY CZE IN ISOELECTRIC BUFFERS

This is an interesting development of cIEF, whereby zone electrophoresis can be performed in isoelectric, very low conductivity buffers, allowing the highest possible voltage gradients and thus much improved resolution of peptides and proteins due to reduced diffusion in short analysis times. Although originally described in alkaline pH values (notably in Lys, pI = 9.87) and at neutral pH (notably His buffers, pH = pI = 7.6) we have found that acidic zwitterions offer an extra bonus: they allow the use of uncoated capillaries, due to protonation of silanols at the prevailing pH of the background electrolyte.

Although not strictly related to IEF per se, the use of isoelectric buffers stems from IEF know-how and is having a unique impact in CZE, so we feel it is appropriate to end this review with a glimpse at this field. Stoyanov and colleagues (195) introduced a new parameter for evaluating the performance of amphoteric buffers: the $\beta/\lambda$ ratio, that is, the ratio between the molar buffering power and their conductivity. Ideal buffers are those with the highest possible $\beta/\lambda$ ratio (for nonzero $\lambda$ values), because they allow the delivery of very high voltage gradients with minimal Joule effects. In the field of proteins, and other small $M_s$ compounds, Hjertén and colleagues (196) have explored a number of different amphoteric compounds and given proper guidelines for their use. These authors reported separations at voltage gradients as high as 2000 V/cm. Righetti and Nembrì (197) have generated peptide maps in isoelectric aspartic acid and shown that such maps could be developed in only 8–10 min, as opposed to 70–80 min in the standard pH 2.0 phosphate buffer, with much superior resolution. Isoelectric Asp, at 50 mM concentration, produces a pH in solution almost coincident with its pI value (pI = 2.77 at 25°C). At this pH value, some of the large peptides (tryptic digests of casein) analyzed were strongly adsorbed by the uncoated capillary wall. A buffer mixture comprising 50 mM Asp, pH 2.77, 0.5% HEC (for dynamic coating of the silanols), and 5% 2,2,2-trifluoroethanol (TFE, for modulating peptide mobility) was found to solve this problem. We have now applied this system to the routine analysis of tryptic digests of α and β globin chains, so as to identify point mutations producing amino-acid substitutions (198). However, this system was able to resolve only 11 out of 13 fragments present in the β-chain digest. In attempts at ameliorating the system, we have finally adopted the following buffer mixture: 30 mM Asp, pH 2.97, 0.5% HEC, 10% TFE, and 50 mM NDSB-195. This buffer mixture performed extremely well, fully resolving 13 out of 13 peptides, in a total time window of 15–16 min in a 75 μm i.d. capillary at 600 V/cm.

Another amphoteric buffer, imino diacetic acid (IDA), with a pI of 2.23 (at 100 mM concentration), has also been reported. IDA is compatible with most hydro-organic solvents, including trifluoroethanol (TFE), up to at least 40% (v/v), typically used for modulating peptide mobility. In naked capillaries, a buffer comprising 50 mM IDA, 10% TFE, and 0.5% HEC allows the generation of peptide maps with high resolution, reduced transit times and no interaction with the wall. IDA thus appears to be another valid isoelectric buffer system, operating in a different pH window (pH 2.33 in 50 mM IDA) compared to the other amphotere previously adopted (50 mM Asp, pH 2.77) for the same kind of analysis (199). Soon after IDA, a novel amphoteric, isoelectric, acidic buffer was reported for separation of oligo- and polypeptides by capillary zone electrophoresis: cysteic acid (Cys-A). Cys-A, at 200 mM concentration, exhibited a pI of 1.80; with a ΔpK of 0.6. At 100 mM concentration, this buffer provided an extraordinary buffering power: $140 \times 10^{-3}$ equiv/L/pH. In the presence of 30% (v/v) hexafluoro-2-propanol (HFP), this buffer did not change its apparent pI value, but drastically reduced its conductivity. In Cys-A/HFP buffer, small peptides exhibited a mobility closely following the Offord equation, that is, proportional to the ratio $M^2/Z$ (200). In the last paper of this series, the properties of four acidic, isoelectric buffers were summarized: cysteic acid (Cys-A, pI 1.85), imino diacetic acid (IDA,
pl 2.23), aspartic acid (Asp, pl 2.77), and glutamic acid (Glu, pl 3.22). These four buffers allow the exploration of an acidic portion of the titration curves of macroions, covering \( \sim 1.6 \) pH units (from pH 1.85 to \( \sim 3.45 \)), thus permitting resolution of compounds having coincident titration curves at a given pH value (201).

### 16.6 CONCLUSIONS

As we hope we have demonstrated in this Chapter, modern IEF techniques, both in soluble and immobilized buffers, have much to offer to users. We feel that adequate solutions now exist to the two most noxious impediments to a well functioning technique, namely lack of flexibility in modulating the slope of the pH gradient and protein precipitation at (and in proximity of) the pl value. The solutions we have discussed (use of spacers and novel mixtures of solubilizers, comprising sugars and high molarities of zwitterions) seem to be working quite satisfactorily. In addition, an important spin-off of the IEF know-how seems to be gaining importance in zone electrophoretic separations: the use of isoelectric buffers. Such buffers allow delivery of extremely high voltage gradients, permitting separations of the order of a few minutes, thus favoring very high resolution due to minimum, diffusion-driven, peak spreading. As an extra bonus, by properly modulating the molarity of the isoelectric buffer in solution, it is possible to move along the pH scale by as much as 0.3–0.4 pH units, thus optimizing the pH window for separation.

The new rising star is cIEF, which has a lot to offer to users. Particularly appealing is the fact that cIEF provides a fully instrumental approach to electrophoresis, thus lessening dramatically the experimental burden and the labor-intensive approach of gel-slab operations. Although early capillary electrophoresis equipment was available mainly as single-channel units, the new generation of equipment offers multi-channel capabilities, in batteries from 20 to 96 capillary arrays (but several hundreds have also been envisioned). Rapid growth is expected in this field. Last, but not least, we have to mention here the latest evolution of CZE, that is, integrated, chip-based capillary electrophoresis (ICCE). ICCE is emerging as a new analytical tool allowing fast, automated, miniaturized, and multiplexed assays, thus meeting the needs of the pharmaceutical industry in drug development. It already allows pre- and post-column derivatization, DNA sequencing, on-line PCR analysis, on-chip enzymatic sample digestion, fraction isolation, and immunoasays, all of that in picoliter sample volumes injected and electrophoretic timescales of the order of a few to a few hundred seconds (202).

A few articles are worth quoting here as stop-press contributions. In one, Mack and colleagues (203) report a systematic study in cIEF, defining and optimizing the experimental parameters leading to method reproducibility and robustness, capable of meeting the rigorous demands of therapeutic protein analysis. Among the improvements are the addition of 40 mM L-arginine as a cathodic spacer, which was found to be crucial for proper execution of cIEF, especially in the basic pH range. In another, Ramsay and colleagues (204) report a novel method for prelabeling proteins with fluorophores prior to cIEF, an aspect that had found no solution to date. They use ChromoP540, a fluorescent reagent that converts cationic lysine to cationic fluorescent products. The reaction products are excited in the green, which reduces the background signal produced by CAs. Additionally, CAs are photobleached with high power photodiodes, further reducing the background noise. By this technique, they achieve detection limits of 270 ± 25 fM and mass detection limits of 150 ± 15 zmol from ChromoP540 labeled β-lactoglobulin. In a third paper, Sommer and colleagues (205) present, for the first time, an adaptation of IPGs to chips, via the creation of a pH gradient by diffusion along a 6-mm channel segment. Precise gradient formation could be obtained and IEF established in <20 min, in contrast to the overnight runs in conventional IPG strips.

### 16.7 ACKNOWLEDGMENTS

This work was supported in part by a grant from MIUR (PRIN 2009) and by the Bilateral Project “Novel Methods for Top-Down Analysis of Macromolecular and Nanosized Samples of Biotechnological and Environmental Interest” within the VIII Executive Programme of Scientific and Technological Co-operation between Italy and Korea for 2007–2009.

### 16.8 REFERENCES

TWO-DIMENSIONAL ELECTROPHORESIS IN PROTEOMICS

REINER WESTERMEIER
SERVA Electrophoresis GmbH, Carl-Benz-Strasse 7, D-69115 Heidelberg, Germany

ANGELIKA GÖRG
Department of Proteomics, Technische Universität München, D-85350 Freising-Weihenstephan, Germany

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INTRODUCTION

One-dimensional separations have limited resolving power. When two different polyacrylamide gel electrophoresis techniques are combined, resolution can be enhanced by at least two orders of magnitude. Protein mapping is mostly applied for resolving complex samples with more than 100 proteins. Margolis and Kenrick (1) introduced the first two-dimensional electrophoresis method: disk electrophoresis followed by porosity gradient electrophoresis. Macko and Stegemann (2) were the first to combine isoelectric focusing (IEF) and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis.

The highest resolution and reproducibility is achievable when the protein sample is completely denatured with high molar urea, first separated by denaturing IEF according to their isoelectric points, and subsequently run in a SDS polyacrylamide gel for fractionation according to their sizes. This mapping procedure has been introduced by O’Farrell (3) and is known as “high-resolution two-dimensional electrophoresis.” When the gels are large enough, several thousands of proteins can be resolved. It is the by far most frequently applied protein mapping method, and mostly just called 2D PAGE. Other two-dimensional combinations like native IEF followed by native polyacrylamide electrophoresis according to Klose (4) or cationic detergent 16-BAC acidic electrophoresis followed by SDS electrophoresis according to MacFarlane (5) exhibit a much lower resolution. Those techniques are applied for studies of exclusively hydrophilic or very hydrophobic proteins, respectively.

High-resolution two-dimensional electrophoresis (2D PAGE), especially in its present form using immobilized pH gradients, was introduced by Görg and colleagues (6), and is the basic separation technique for the novel concept of proteome analysis (7) (also called “proteomics”), which can be defined as “the effort to establish the identities, quantities, structures, and biochemical and cellular functions of all proteins in an organism, organ, or organelle, and how these properties vary in space, time, and physiological state.” The introduction of the proteomics approach means a paradigm shift in biology, biochemistry, and related research from single protein analysis to a high throughput parallel analysis of complex protein mixtures. It is estimated that ~20,000 different proteins are expressed in a human cell. As many as possible of these proteins should be monitored in a proteomics experiment. 2D PAGE is not the only separation method used in proteomics; however, it has the highest resolving power available. Furthermore, it delivers a map of intact proteins, which indicates changes in a proteome as changes of spot positions and intensities in a spot pattern image.

The latest development in the 2D gel electrophoresis-based proteomics workflow is shown in Figure 17.1 and can be described briefly as follows. After sample preparation the proteins of different samples are labeled with fluorescent dyes, mixed, and co-run in 2D gel electrophoresis. The gel is scanned, and the protein spots with up- or down-regulated expression levels are detected with image analysis software. These selected proteins are picked from the gel and digested inside the gel plug with trypsin. The eluted peptide mixtures are spotted on matrix-assisted laser desorption ionization (MALDI) targets for identification of the proteins with peptide mass fingerprinting with MALDI time of flight (ToF) combined with protein and peptide database searches. The spot handling steps and MALDI ToF analysis, including database searching, can be automated for high throughput. The peptide mixtures of nonidentified proteins are further analyzed with liquid chromatography/electrospray tandem mass spectrometry (MS) for amino-acid sequence determination followed by database searches with different algorithms. Tandem mass spectrometry, for instance, with an ion trap, is also employed for the study of post-translational modifications such as phosphorylations, acylation, and glycosylations.

With 2D PAGE, intact proteins are separated and displayed, but, in contrast, in the so-called “shot-gun proteomics” approach the complete cell lysate is digested before separation. Very complex peptide mixtures are obtained, which are separated and analyzed by 2D chromatography/MS.

Unfortunately, it is not always possible to get all proteins of biological samples into solution, and to find all of them in the 2D map. Should it be essential to qualitatively find all proteins contained in a cell or a tissue, it is necessary to...
apply some complementary techniques additionally to 2D PAGE, such as 2D peptide chromatography followed by tandem mass spectrometry (8).

The proteins separated with 2D PAGE can either be stained in the gel after separation or labeled with fluorescence dyes prior to separation. Because there are fluorescence dyes with different excitation and emission wavelengths available, the prelabeling approach allows sample multiplexing, leading to a simplified and more reliable pattern evaluation (9). Differential analysis of the complex protein mixtures with 2D PAGE can reveal up- and down-regulations of proteins in biological experiments and diseased versus control samples. Furthermore, newly expressed proteins and post-translational modifications can be displayed. It is possible to detect some post-translational modifications, like phosphorylations and glycosylations, using specific stains in the gel or on blotting membranes. For identification and further characterization, the protein spots of interest are picked from the gel for further analysis. Because it is very difficult, if not impossible, to elute intact proteins from the gel medium, the proteins are digested with trypsin inside the gel plugs. The peptide mixtures are then eluted and submitted to MS. It is not necessary to perform all the steps immediately. The separated proteins can be stored in the gels for many months without degradation.

In the following chapter, the methodology of protein mapping by high-resolution 2D electrophoresis is described, with particular focus on its use in proteome analysis.

17.2 BRIEF TECHNOLOGY REVIEW OF 2D ELECTROPHORESIS

17.2.1 Denaturing Conditions

There are a number of reasons for using completely denaturing conditions in the first dimension:

- Each protein appears only in a single conformation, resulting in a single spot.
- Different oxidation steps are avoided.

Figure 17.1 Workflow of 2D gel electrophoresis-based proteomics.
Hydrophobic proteins go into and stay in solution. The formation of aggregates and complexes between proteins is reduced. Protein–protein interactions are inhibited. All buffering groups are exposed to the medium. Under this condition the pl position in the gel can be matched with the calculated pl derived from in silico translation of the genomic sequence within the open reading frame. The reason for the extraordinary high resolution of 2D PAGE of several thousand protein spots is the separation according to two different, independent physicochemical parameters: the charge in the first dimension and the molecular mass in the second dimension.

17.2.2 Isoelectric Focusing Methods
In the original method IEF was performed in thin polyacrylamide gel cylinders, which are polymerized into glass or plastic tubes. The pH gradient was generated by carrier ampholytes (CAs). After IEF the gel rod was removed from the tube, shortly equilibrated in SDS sample buffer, and transferred to the second dimension gel. SDS electrophoresis was run in a discontinuous porosity gradient polyacrylamide gel. Figure 17.2 shows the principle of high-resolution 2D electrophoresis as described by O’Farrell, and as applied by many followers. However, due to the complex multistep procedure, not every laboratory succeeded in achieving a good quality spot pattern, and unfortunately the results could often be correlated to the skill of the operator. Furthermore, there were a lot of problems with the reproducibility of the first dimension, because the pH gradient changed its profile with different batches of CAs, separation time, composition of the protein mixture, and the kind and concentration of other sample components.

O’Farrell and colleagues (10) soon recognized that, during focusing, the gradient was drifting towards the cathode, resulting in a loss of all basic proteins. Therefore they introduced an alternative separation concept for the analysis of basic proteins: nonequilibrium pH gradient electrophoresis (NEpHGE). The set-up is the same as for IEF/SDS PAGE; however, the polarity of the first dimension is reversed, and the sample is loaded at the anodal end of the gel rod. In the electric field the proteins start to separate electrophoretically and become stacked between the CAs while they are migrating towards the cathode. After a defined time interval (approximately one-third of the regular focusing time) the migration is stopped, the gel rod is treated as usual, and transferred to the second dimension gel. With NEpHGE the basic proteins are included in the gel. However, the proteins are not focused, the resolution is dependent on the number of different CA homologs present, and the results are time-dependent with poor reproducibility.

All attempts to solve the problems of reproducibility by standardization of running conditions and equipment for multiple runs failed due to the limited properties of the CA-generated pH gradients. Facing these limitations, the concept of immobilized pH gradients (IPG) was introduced in 1982 (11) and put into practice by Görg and colleagues (6) by developing narrow film-supported polyacrylamide IPG strips. The pH gradient can no longer drift, basic proteins are well separated and displayed, the profile of the gradient is independent of the sample composition, the film-supported gel strips are mechanically stable, and the results are highly reproducible, independent of the operator. This “IPG-DALT” method (IEF in immobilized pH gradients followed by SDS electrophoresis) can be easily established in every protein laboratory. Figure 17.3 presents the concept of IPG-DALT.
17.2.3 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The second dimension could be simplified and therefore become more reproducible. In vertical systems no stacking gels are needed; correctly polymerized homogeneous gels have almost the same resolving power as porosity gradient gels. Porosity gradient gels can particularly improve the resolution of highly glycosylated proteins, which have a tendency to smear.

When the IPG strips with their flat gel surface are used, the second dimension can also be run on horizontal gels on a flatbed system (Fig. 17.3). This method greatly simplifies the handling and increases the staining sensitivity for scarce samples. The separation patterns of horizontal and vertical gels are very similar (Fig. 17.13). Görg and colleagues showed that increased resolution can be obtained in certain gel areas within the thin-layer horizontal gels (12).

17.3 SAMPLE PREPARATION

It is very important that the protein composition and properties in the sample do not become modified during cell lysis and tissue extraction. For a number of samples it is important to remove some contaminants that disturb the IEF procedure, but without removing or modifying proteins. Various solubilization mixtures and clean-up procedures are employed for different sample types. The applied sample preparation and clean-up method has a very high impact on the results of 2D PAGE.

Unfortunately it is impossible to solubilize all proteins existing in a biological sample. Whatever composition of chaotropes, reductants, and detergents is used, there will always be a number of very hydrophobic proteins that resist going into solution. The challenge remains to develop novel reagents for the solubilization of complete proteomes.

The major goals of sample preparation are as follows:

- solubilization of as many proteins as possible, even the very hydrophobic ones.
- disaggregation of complexes and complete unfolding of the polypeptide chains
- removal of disturbing contaminants such as lipids, polysaccharides, nucleic acids, salts, buffers, polyphenols, and insoluble material
- prevention of protein modifications by other sample components or the sample preparation method itself.
17.3.1 Solubilization

The solubilization process frequently requires the assistance of mechanical treatment, including ultrasonic devices. Tissue samples are frozen with liquid nitrogen and ground in a ceramic mortar with a pestle. There are also disposable grinding kits available using an abrasive resin in a reaction tube and a plastic pestle. Tight cell walls are opened with “French pressure cells” or by grinding with glass beads. When a sonicator is used, the sample should be kept on ice, and the time periods of bursts must be limited, and alternate with resting of the sample, in order to avoid heating of the sample.

The quality of the reagents is very critical. If urea or thiourea contains degradation products such as isocyanate, the conductivity in the IEF gel will rise, and proteins will become carbamylated, resulting in artifactual spots. Also, heating of urea solution and urea-containing samples must be avoided, because carbamylation of proteins will occur. Many 2D laboratories routinely treat the chaotrope solution with mixed-bed ion-exchanger medium to remove any charged components.

Furthermore, the introduction of keratins with the reagents must be avoided, because keratin peptides will show up in the mass spectromogram and complicate protein identification or make identification impossible. There are reagents on the market that contain keratin, for instance 2-mercaptoethanol from certain sources (13).

The following solubilizing mixture has been optimized by Görg and colleagues (14), this cocktail works for many different sample types:

- 9 mol/L urea
- 4% (w/v) CHAPS
- 65 mmol/L DTT
- 0.002% (w/v) bromophenol blue
- 0.8% (w/v) CAs pH 3–10
- 1% (v/v) protease inhibitor cocktail

The solubilization mixture is sometimes called lysis buffer. The functions of the different additives are explained below. Table 17.1 shows an overview of sample amounts for sample preparation.

The protein concentration should be 5–10 mg/mL. The samples must be centrifuged for 30–60 min at 42,000g at 15°C to remove solid components or insoluble proteins completely.

17.3.1.1 TCA–Acetone Extraction

The trichloroacetic acid (TCA)–acetone extraction method according to Damerval and colleagues (15) was developed for plant protein, but can be employed for many other sample types including the extraction of basic proteins (16). The sample is suspended in 20% (w/v) TCA plus in 80% (v/v) acetone plus 0.07% (v/v) 2-mercaptoethanol (or 0.2% (w/v) dithiothreitol, DTT) at −20°C, and kept in this solution at −18°C overnight for complete precipitation. Following centrifugation and removal of the supernatant the pellet is washed several times with 90% (v/v) acetone plus 0.2% DTT for removal of contaminants. The sample is centrifuged again, the supernatant removed, and the pellet dried in vacuum.

17.3.1.2 Additives

Some laboratories remove nucleic acids by adding a protease-free DNAse/RNAse mixture. Unfortunately, these enzymes show up as additional spots in the protein map. There are some alternatives, including pipetting the sample solutions repeatedly through a narrow pipette tip to break the nucleic acid molecules into smaller pieces by shearing forces, or using sonicator treatment to break nucleic acids into small molecules, which do not disturb the 2D gel.

In general, the zwitterionic detergent 3-(3-cholamidopropyl)dimethylammonio-1-propane sulfonate (CHAPS) has replaced nonionic detergents Nonidet NP-40 and Triton X-100, because the latter give very problematic ghost peaks in MS analysis. CHAPS is at present the most useful detergent. SDS would get more proteins into solution, but it has to be removed before or during IEF, because it builds negatively charged micelles with proteins, migrating into the anode. Alternative detergents and nondetergent sulfobetaines have been tried, which move some additional proteins into solution, but at the cost of losing a number of other proteins.

Very hydrophobic proteins, such as membrane proteins, go into solution better with 7 mol/L urea plus 2 mol/L thiourea instead of using urea alone (17). This modification is widely used, but there is a danger of creating artifacts in some samples.

TABLE 17.1 Examples for Sample Preparation

<table>
<thead>
<tr>
<th>Source</th>
<th>Quantity</th>
<th>Treatment</th>
<th>Lysis Buffer</th>
<th>Sonication on Ice Bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human or animal cells</td>
<td>5 × 10⁶ cells</td>
<td>Macerate</td>
<td>1 mL</td>
<td>3 × 10 sec</td>
</tr>
<tr>
<td>Ground plant seeds</td>
<td>20–100 mg</td>
<td>Crush with hammer</td>
<td>1 mL</td>
<td>3 × 10 sec</td>
</tr>
<tr>
<td>Yeast</td>
<td>120 mg wet cells</td>
<td>Homogenize in ice-cold mortar</td>
<td>1 mL</td>
<td>3 × 10 sec</td>
</tr>
<tr>
<td>Human or animal tissue</td>
<td>50–100 mg</td>
<td>TCA-acetone extraction (see Section 17.3.1.1)</td>
<td>1 mL</td>
<td>3 × 10 sec</td>
</tr>
<tr>
<td>Plant tissue</td>
<td>1–2 g</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
DTT or dithioerythreitol (DTE) are both useful reductants. 2-Mercaptoethanol is very rarely used, because it is volatile and causes streaking between pH 8 and 9, as it has high buffering power in this pH interval. DTT and DTE can also create problems in the basic part of the pH gradient; they are weak acids and become deprotonated when they are exposed to an alkaline environment over a certain time. They become negatively charged and start to migrate away from the cysteines. This phenomenon results in horizontal streaking and the appearance of artificial spots, because the unprotected cysteines can react with urea, proteins fold back and build aggregates via interpolypeptide disulfide bonds. Using alternative reductants causes other problems, which are not discussed here. The problem is solved by replacing the reductant in the gel by 100 mmol/L of the oxidant hydroxyethylidisulfide (HED) (18) to the rehydration solution. The cysteines will become oxidized to mixed disulfides in the gel, which protects them from side reactions and stabilizes the unfolded status of the polypeptides. However, the proteins need first to be reduced before they are applied on a gel containing HED.

Bromophenol blue is a useful tracking dye also for IEF. It serves as a control during pipetting of the sample and will migrate to the anode during IEF. CAs are very helpful to cause proteins to go into solution and to keep them there, because the buffering groups of these amphoteric buffers are charged while they are in a mixture. Often a base like Tris or spermine is added to improve protein extraction, precipitate nucleic acids, and inhibit protease activity.

For prelabeling samples with fluorescent CyDyes for 2D gel electrophoresis the lysis buffer needs to be modified as described in Section 17.3.4. Protease inhibitor cocktails are often added to the sample to prevent protein digestion. It is still necessary to work fast because not all proteases are inhibited. Some protease inhibitors are toxic, or can modify the charges of some of the proteins.

Plant proteins are very hard to solubilize. A modified lysis buffer for the solubilization of more plant proteins has been published by Méchin and colleagues (19):

- 5 mol/L urea
- 2 mol/L thiourea
- 2% (w/v) CHAPS
- 2% (w/v) SB-13
- 20 mmol/L DTT
- 5 mmol/L tris(2-carboxyethyl) phosphine (TCEP)
- 0.5% (w/v) CAs pH 4–6.5
- 0.25% (w/v) CAs 3–10

17.3.2 Sample Clean-Up

For a number of sample types it is sufficient to remove salt ions. Chromatographic desalting can be problematic, because proteins can stick to the gel filtration material and get lost. A very effective method is microdialysis in small reaction tubes, which contain a small dialysis membrane built into the open cap.

Protein precipitation removes several contaminants, dissolves complexes, and inhibits proteases irreversibly. Precipitation of the proteins is therefore a very powerful clean-up measure. Before IEF the proteins must be redissolved in solubilization solution (lysis buffer).

Not all precipitation methods can be recommended. Salting out with ammonium sulfate must not be used, because the salt will stick to the pellet and will heavily disturb IEF. Precipitation with trichloroacetic acid (TCA) can be irreversible for some proteins and they are lost. Organic solvents like ethanol or acetone will precipitate only a part of the proteins, also leading to loss of proteins.

A very efficient method had been introduced by Wessel and Flugge (20), in which the sample is mixed with methanol, chloroform, and water with centrifugal steps between. After removal of the upper phase, methanol is again added to the remaining lower phases. After centrifugation the protein pellet is removed from the supernatant, which, after drying, can be stored until use.

Another frequently applied precipitation method is based on TCA–acetone extraction according to Damerval and colleagues (15). The proteins solution is precipitated with 10% (v/v) TCA plus (v/v) 80% acetone at room temperature, because the sample contains urea, which would immediately crystallize at cold temperatures. The pellet is washed several times with 90% (v/v) acetone plus water for removal of contaminants. However, with this method some acidic proteins can unfortunately get lost.

There is a 2D clean-up kit commercially available that uses TCA–detergent precipitation followed by washing with organic solvents. The advantages of this chemistry are speed (finished in 90 min), convenience, the almost quantitative precipitation, and low loss of proteins. Very frequently, a higher number of proteins are found in the 2D gel after this clean-up procedure than with crude samples, because the very obstinate protein complexes become disrupted (21).
with copper solution and measuring with a color reagent the amount of copper not bound to protein. In this way the protein content can be calculated. The result is much more accurate than the results obtained with standard assays (21).

17.3.4 Modified Procedures for Difference Gel Electrophoresis

When protein samples are labeled with CyeDye™ fluorophors for “Difference Gel Electrophoresis” (DIGE), the pH value of the lysis buffer must be higher than 8. Preferably it should have a pH of 8.5. Usually 10–30 mmol/L Tris-base is added to the solubilization mixture. During labeling the sample solution must not contain any CAs and reductants. These reagents are added later after the labeling has been completed. The exact protocol including an instruction on how to adjust the pH value of the sample solution prior to labeling can be found in the Ettan™ DIGE user manual (22). Figure 17.4 shows the concept of 2D difference gel electrophoresis.

Two different types of labeling are possible: lysine and cysteine labeling.

17.3.4.1 Lysine Labeling (Minimum Labeling) With minimum labeling the ε-amino group of the lysine is labeled with a modified CyDye (8). Three different dyes are available. Only 3–5% of the total proteins will receive a label. In this way only singly labeled proteins will be detected, and multiply labeled proteins will statistically remain below the detection limit. The 2D pattern will be the same as for proteins stained after separation, because the dyes are matched for charge and mass, and thus comigrate to the same isoelectric point and molecular size.

Before the samples are labeled, an aliquot of each sample is set aside and mixed to a pooled standard. When this pooled standard mixture is labeled with one of the fluorescent dyes and coseparated in each gel, it serves as an internal standard for all proteins, which makes the differential evaluation and relative quantification of proteins very reliable.

It is very important to know the protein content of each sample. An amount of 50 μg of protein is labeled with 200–400 pmol/L CyDye, depending on the contamination of the sample with nonprotein compounds. The labeling efficiency increases substantially, when contaminating compounds have been removed with a 2D clean-up procedure.

The labeling reaction takes 30 min on ice. Subsequently the reaction is stopped by adding 1 μL of 1 mmol/L lysine solution to each sample. The differently labeled samples are then mixed with the (also labeled) pooled standard and applied on an IPG strip.

![Figure 17.4](image_url) Schematic representation of 2D difference gel electrophoresis in one gel. The example is for two samples. When more samples are to be compared, more gels are run, each including the internal standard.
17.3.5.3 Selective Removal of Abundant Proteins

The sensitivity of detection will increase considerably when all proteins become labeled. This can be achieved with labeling of the cysteines. In this case 5 µg sample protein or less is applied. All available cysteines are labeled, which means that multiply labeled proteins will also occur, resulting in a different spot pattern than those obtained with poststained gels or gels containing minimum labeled proteins (23). However, for a differential approach this does not matter, because identical proteins of different samples will be labeled in the same way, and they will comigrate to the same spot position. The sample proteins are reduced with TCEP before adding the dye. TCEP is about four times more reactive than DTT. The amount of TCEP and dye is determined by a simple titration procedure before starting the experiment. Labeling occurs at pH 8.0 with the temperature set to 37°C for 30 min. The exact labeling protocol can be found in a special instruction “CyDye DIGE fluor labelling kit for scarce samples” (24). In general, the cysteine labeling method is more difficult to carry out than the lysine labeling.

17.3.5 Prefractionation

There are a number of cases where the result of 2D electrophoresis can be considerably improved by prefractionation of the sample. The analysis of smaller subsets of protein mixtures frequently provides an improved pattern and allows the detection of proteins that otherwise do not enter the gel. Preferably, the fractionation parameter should deliver physicochemical or biological information on the protein subset to be separated.

17.3.5.1 Differential Solubility

When proteins are fractionated by sequential extraction with increasingly strong solubilizing agents, very frequently, wide overlaps of proteins are observed, and many problems with reproducibility have been reported. Furthermore, the hydrophobicity of a protein is usually not linked to its function.

17.3.5.2 Subcellular Components

Detergent fractionation can be employed for fractionating cells in cytosol, membrane-organelle, nuclear membrane, and cytoskeletal-matrix proteins (25). This concept has recently been further developed and turned into a commercial kit. The benefits of such a fractionation are the reduction of complexity for the 2D separation as well as the information on protein function, which is closely linked to the subcellular topology (26). In many laboratories subcellular fractionation is still performed with ultracentrifugation (27). Zischka and colleagues (28) have used free flow electrophoresis for the purification of Saccharomyces cerevisiae mitochondria and observed much less protein degradation than with centrifugation.

17.3.5.3 Selective Removal of Abundant Proteins

The most interesting sample for clinical proteomics is the most complicated to analyze: human plasma. It should contain any disease marker expressed in the total human proteome, which would provide easier and more reliable diagnosis. However, the concentrations of proteins exist in an extremely high dynamic range of more than 10 orders of magnitude (29). The most interesting proteins like tissue leakage proteins and interleukins are expected in concentrations of less than a few picograms down to femtograms per milliliter. More than 60% of the protein content is albumin, which serves as a transport protein. This means that with the removal of albumin some other proteins sticking to it will also be removed. The most efficient fractionation is achieved with immuno affinity techniques. There are affinity columns commercially available that remove albumin and some other highly abundant proteins such as the immunoglobulins, transferrin, fibrinogen, and a few more. In order not to lose the proteins sticking to albumin, the removed fraction should be further analyzed instead of being discarded. Removal of abundant proteins is also important for the analysis of plant proteins, for instance to get rid of Rubisco.

17.3.5.4 Blue Native Electrophoresis

Schügger and von Jagow (30) have developed blue native PAGE for the isolation of enzymatically active membrane proteins. Coomassie Blue G-250 is added to the sample and to the cathodal buffer of a native electrophoresis set-up. During the run it competes for binding sites on the protein surface with the nonionic detergents used for solubilization of membrane proteins and complexes. Proteins and complexes bind the dye, become negatively charged (analogous to SDS) and stay soluble in detergent-free solution. All these visible protein–dye complexes migrate towards the anode, as well as those with basic isoelectric points. Werhahn and Braun (31) proposed a so-called “three-dimensional electrophoresis,” with the first-dimension separation of protein complexes by blue native PAGE. The visible bands are eluted electrophoretically from the gel, destained, and further separated by standard denaturing 2D electrophoresis. This method also reveals proteins with high hydrophobicity, most probably due to a reduction in the complexity of the protein mixture.

17.3.5.5 Fractionation According to Charges

With the first dimension of 2D PAGE, and IEF is performed in narrow interval pH gradients, a very high resolution and high protein loading capacity is obtained. The best results are achieved when the protein mixture is prefractionated according to the isoelectric points. Several techniques are possible:

- Free Flow Isoelectric Focusing. Separation occurs in a CA-containing liquid stream in a free-flow electrophoresis apparatus, which can also be used for a fractionation of cell organelles (28).
- Isoelectric Membranes. The purification of protein mixtures in a multicompartment apparatus divided by
amphoteric buffering glass fiber–polyacrylamide membranes with defined isoelectric points has been developed by Wenger and colleagues (32). These membranes are prepared by soaking glass fiber membranes with acrylamide monomer solution containing basic and acidic Immobelines (buffering acrylamide derivatives) to achieve a defined pH value. When the monomers have polymerized, the membranes have a high buffering power at the intended isoelectric point. The divided separation chamber is filled with water, and the protein mixture applied into one of the compartments. When an electric field is applied, the proteins start to migrate through the membranes and will be trapped between the two membranes with the bordering pH values. Herbert and Righetti (33) as well as Speicher and colleagues (34) have applied this concept to the prefractiation of complex protein mixtures according to the isoelectric point.

Carrier Ampholyte Isoelectric Focusing in Sephadex. Preseparation of complex protein mixtures by CA IEF in a granulated gel bed under denaturing conditions, introduced by Görg and colleagues (35), is a very quick, reproducible, and efficient prefractiation method. The procedure is performed on a Multiphor II flatbed electrophoresis equipment at 20°C. The technique is simple: the sample is mixed with Sephadex G-200 superfine, which has been reswollen with rehydration solution containing urea, CHAPS, DTT and CAs. This slurry is pipetted into a trough and covered with a thin layer of silicone oil. After 4 h IEF, the pH fractions are removed with a spatula and directly applied onto pre-rehydrated IPG strips. In the electric field the proteins migrate out from the granulated gel into the polyacrylamide gel of the IPG strip. The fractions can also be stored in a freezer at −70°C. Subsequent separations of the pH fractions in the respective narrow pH interval IPG strips show a considerable increase in spot number. Many more of the lower expressed proteins can be detected in this way.

In Figure 17.5, the effect of prefractiation according to charge is shown for a 2D electrophoresis of mouse liver proteins in a narrow pH interval, 6–7. The total amount of proteins was 250 µg in both cases. In case A most of the proteins accumulate at the electrodes, because they have lower, respectively higher isoelectric points. In case Figure 17.5a, 250 µg of proteins with isoelectric points between 6 and 7 were applied after prefractiation by IEF in Sephadex.

17.4 ISOELECTRIC FOCUSING

The principle of IEF and its features are described in Chapter 16. However, special features are required when IEF is used as the first dimension in high-resolution 2D electrophoresis:

- Under denaturing conditions IEF takes much longer than in native gels, because, due to the presence of urea and the other additives, the viscosity of the medium is very high, and the proteins are unfolded and thus more bulky. Long separation distances and/or narrow range pH gradients are used to achieve maximum resolution. For the resulting long separation times the pH gradient must not drift or change its profile.
- The pore size of the gels should be as large as possible to also include high molecular proteins in the map. These gels exhibit low mechanical stability, and therefore should be supported by some rigid material like a plastic foil.
Each sample has to be run in an individual gel in order to have full quantitative control over the sample to be analyzed.

A high reproducibility of the pH gradient profile and the gel length (= separation distance) is required to allow intergel comparisons, independent of the operator and the laboratory.

These demands cannot be satisfied with CA-generated pH gradients. A multilaboratory approach could show that the IPG concept meets these requirements (36).

### 17.4.1 Immobilized pH Gradients

The basics of IEF in IPG are described in Chapter 16. Here are a few facts on properties of IPG gels that are important for their use in 2D PAGE. In a conventional electrophoresis or IEF experiment, the conductivity of the buffer or of the free CAs is high enough to transport the ionic polymerization catalysts \( N,N',N''-\text{tetramethylethylenediamine} \) (TEMED) and ammonium persulfate out of the gel. However, Immobiline gels exhibit a very low conductivity, because the buffering groups are fixed to the matrix. Therefore the unbound compounds are washed out from Immobiline gels with double distilled water after polymerization. As immobilized pH gradients are pre-established in the matrix, care must be taken to always place them into the IEF apparatus in the correct way: acidic end connected to the anode and basic end connected to the cathode.

Polyacrylamide gels swell during washing, and would shrink in all directions during drying. Therefore Immobiline gels are polymerized as 0.5-mm thin-layer gels on polyester film supports. Several attempts to use gel rods with IPG have had limited success because of the necessity of washing the gels (37, 38).

For the method of running IEF in individual IPG strips according to Görg and colleagues (6), the washed and dried Immobiline gel is cut into 3-mm-wide strips. IPG strips are commercially available in many different lengths from 7 to 24 cm, with a broad selection of wide and narrow pH, linear and nonlinear gradients. The technology of IPG strip preparation is described in detail in several publications (39, 40). As these strips are cast on plastic backings, the gels cannot stretch or shrink; they are much easier to handle than gel rods. There are no edge effects: the iso-pH lines are absolutely straight and fixed to the matrix. The acidic and basic sides are either indicated by cutting the support film pointed on one end or printing the electrode signs on the back of the strip. The width of the strips should not exceed 3 mm; this minimizes the electroendosmotic effects between the first and second dimensions (6, 41), and it reduces the amount of detergent transferred from the first to the second dimension to a minimum (6, 42). The loading capacity depends on the length of the strip and the pH gradient; the longer the strip and the narrower the pH interval, the higher the protein loading capacity. For example, on a 7-cm gel with a wide pH gradient of 3 to 11, \( \sim 20-50 \mu g \) protein can be applied; 24-cm strips with a 1 pH unit interval can tolerate several mg of protein. As already mentioned, the best results are achieved with prefractinated protein mixtures. Commercial IPG strips contain barcode labeling and can thus easily be linked to the applied sample.

### 17.4.2 IEF in IPG Strips

#### 17.4.2.1 Rehydration of IPG Strips

The strips are rehydrated for a minimum of 6 h or overnight at room temperature before use at their original thickness of 0.5 mm. This step can be combined with sample application by rehydrating the strip with the sample mixed with rehydration solution (43). In this case, more than 10 h of rehydration is required, because it takes much longer for the high molecular weight proteins to diffuse into the matrix.

**Prerehydration without Sample** There are two possibilities. First, the strips are inserted into a rehydration cassette filled with rehydration solution as shown in Figure 17.6a. The thickness of the gasket defines the final thickness of the strips. With this procedure, the most uniformly rehydrated strips are obtained. However, due to the high detergent and urea content the liquid starts to leak after a few hours, and a large volume of rehydration solution is needed (15 mL for one cassette).

In the second option, the strips are rehydrated individually. Different systems are available. The strips are rehydrated in a multiple plastic reswelling tray as shown in Figure 17.6b. It can be used for all strips lengths. The volume of rehydration solution for each strip defines the thickness. The volume of applied rehydration solution must be exactly measured, because underswelling causes pore sizes that are too small, and overswelling leads to liquid exudation during IEF at high voltage. A 24-cm-long strip with 0.5-mm thickness and 3-mm width is rehydrated with 450 \( \mu L \) solution; a

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**Figure 17.6** Different ways of rehydrating an IPG strip.
7-cm strip needs 125 μL. As already mentioned in Section 17.3.2.2, care must be taken to reswell the strips evenly. It is therefore necessary to apply the rehydration solution as a streak into the groove of the reswelling tray or strip holder (Fig. 17.6c). For a 7-cm strip the streak must be shorter than 7 cm, for a 11-cm strip shorter than 11 cm, and so on. After the strips have been placed with the dried gel side downwards on the liquid streaks, they are covered by pipetting 1 mL of paraffin oil into each groove, and the tray closed with a sliding lid. Air bubbles must be avoided. Only a maximum of 5.4 mL rehydration solution (for 12 strips of 24-cm length × 450 μL) is needed, and leakage cannot occur.

Usually, the ceramic individual strip holders are only used for rehydration loading. They are equipped with platinum electrodes, and placed on the cooled electrode plates of the IEF apparatus IPGphor (Fig. 17.7). There are different strip holders available for the different strip lengths. Of course, they can also be used for prerehydration of strips without sample. The procedure is the same as for the reswelling tray described above. Figure 17.6c shows how an IPG strip is placed into an individual strip holder. In the strip holder, the IPG strip is covered with paraffin oil.

The rehydration solution should contain the additives in lower concentrations than the lysis buffer, because crystallization of the chaotropes must be avoided, the conductivity in the gel should be kept to a minimum, and micelle effects between the zwitterionic detergent and SDS should be prevented. The reagents have to be of the highest purity, and contamination with keratin must be avoided.

The composition of a solution for prerehydration of IPG strips, which works for most samples, is as follows:

- 7 mol/L urea
- 2 mol/L thiourea
- 0.5% (w/v) CHAPS
- 0.002% (w/v) bromophenol blue
- 100 mmol/L HED (DeStreak™ reagent)
- 0.5% (w/v) CAs pH 3–10

When the rehydration solution contains HED, the sample must be applied at the anodal end of the strip. In most cases, anodal sample application is preferred anyhow. If cathodal sample application would be required, HED needs to be replaced by 20 mmol/L DTT.

Rehydration with Sample Here, the sample is mixed with rehydration solution and applied into the groove of the reswelling tray or the strip holder as shown in Figure 17.6b,c. For rehydration loading DeStreak cannot be used, because it would be reduced to 2-mercaptoethanol by DTT or any other reductant in the sample. The rehydrated strips containing the sample proteins can be focused directly in the strip holders in an IPGphor apparatus, or they are transferred to a Multiphor Immobiline DryStrip tray, an IPGphor cup-loading strip holder, or an IPGphor Manifold. Further information is available in the following section.

17.4.2.2 Loading of the Sample

Rehydration Loading of the Sample The advantages of rehydration loading include the following:

- The sample proteins are distributed evenly over the gradient. This means that proteins will not aggregate and precipitate at the application point.
- A large volume of sample can be applied in this way.
- Several steps can be combined into one step: rehydration of the IPG strip, sample application, and IEF (Fig. 17.7). This reduces the number of manual steps, and can therefore improve the reproducibility.
- When rehydration loading is performed in strip holders on the IPGphor, the rehydration can be carried out under...
voltage, which allows a higher number of high molecular weight proteins to enter the gel than passive rehydration (44).

The disadvantages of rehydration loading are as follows:

- It does not work for basic proteins, here, proteins can get lost.
- The DeStreak approach for stabilizing cysteines cannot be employed.
- Some samples show more protein spots when they are separated after cup- or paper-bridge loading (see paragraph Paper Bridge Loading in this section).

Figure 17.7 shows the IPGphor apparatus with a strip holder for combining rehydration loading and IEF in one step. In this case the IPG strips are run with the surface down. The instrument is programmed either to apply an electric field after a rehydration time of 10–12 h, or to apply a low voltage during the rehydration phase.

**Cup Loading of the Sample** In the original procedure according to Görg and colleagues (6), the sample is applied at a defined point of the pH gradient, in most cases at the anodal end. Figure 17.8 presents a drawing of a laboratory-made IPG strip with a so-called sample holder, which is a little frame cut from a 2-mm-thick silicone rubber plate. These strips are run directly on the cooling plate of a Multiphor chamber, and cannot be covered with paraffin oil.

This concept has been further developed into sample cups for loading higher volumes of sample solution up to 150 μL. Furthermore, running IPG strips in trays allows covering them with paraffin oil to prevent drying of the strips, crystallization of the urea, and air contact. In this way, neither oxygen nor carbon dioxide can diffuse into the matrix. As shown in Figure 17.9, there are several possibilities:

- The Immobiline DryStrip kit for the Multiphor chamber is a tray, which can accommodate up to 12 IPG strips (Fig. 17.9a). The loading cups are held by a bar, which is attached to the lateral walls of the tray. About 100 mL of paraffin oil is required in order to cover strips and cups completely.
- Up to nine ceramic cup-loading strip holders (Fig. 17.9b) can be placed on the IPGphor cooled electrodes. Before the cups are inserted, the IPG strip, electrode filter paper pads, and electrodes are placed into the tray, and 4 mL of paraffin oil is pipetted over the strip. The sample is underlayered below the oil.
- The ceramic manifold (Fig. 17.9c) for the IPGphor can accommodate up to 12 IPG strips.
- The volume should not be less than 20 μL: with lower volumes the sample entrance would be problematic, because of protein aggregation in highly concentrated solutions. A rule of thumb says that the more diluted the sample is applied, the better.

**Paper Bridge Loading** There are cases where rehydration loading cannot be used, but more than 150 μL sample solution has to be applied, such as when a large amount of protein has to be separated in a basic pH gradient. In this case a thick filter paper strip, the paper bridge, is soaked with sample solution and inserted between the electrode filter paper pad and the anodal end of the IPG strip. With this method, 500–800 μL of sample solution can be applied (45). Figure 17.10 shows how paper bridge loading is carried out in the different tray systems. The electrode assembly of the IPGphor manifold is designed to enable paper bridge loading. Also, the side opposite the electrodes contains teeth to hold the paper bridge down on the gel (Fig. 17.9). In this case the electrodes are placed into the manifold with the electrodes at the outer sides.

**Protein Amount** It has already been mentioned that the maximum amount of protein that can be loaded depends on the length of the strip as well as the slope of the gradient. However, the sample amount is also dependent on the detection technique used. For proteome analysis, 18- to 24-cm gels are usually used, because shorter gels do not exhibit sufficient resolution and have a small loading capacity. It is possible to achieve protein identification from silver stained spots with tandem MS (46). Thus, it has become very questionable whether 2D gels can be distinguished by the terms analytical or preparative. Nevertheless, so-called “analytical gels” contain a protein load below 500 μg, and “preparative gels” have protein loads above 500 μg. Up to 10 mg of crude protein preparations have been applied onto IPG strips for 2D PAGE (47).

In a typical proteomics workflow, as shown in Figure 17.1, the proteins of interest are first analyzed with a MALDI ToF for high throughput identification by peptide mass fingerprinting, as extensively described in Reference 48. For this type of MS, it is preferable to load more protein on a

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**Figure 17.8** IEF in individual IPG strips according to Görg and colleagues (6). Drawing of a laboratory-made IPG strip with a sample holder frame cut from a silicone rubber plate.
gel to be on the safe side, to get a signal. This means that a sample load of several 100 μg protein is optimal. For difference gel electrophoresis, for example, 150 μg of each of two samples and the internal standard are labeled with the selected CyeDyes and mixed together to result in a sample load of 450 μg applied on an 18- or 24-cm-long IPG strip.

17.4.2.3 IEF Temperature It is has been shown by Görg et al. (49), that spot positions vary along the pH axis with different applied temperatures. It is thus very important to run the separations at an actively controlled temperature, where 20°C proved to provide the optimal conditions. Furthermore, it is necessary to remove local heat development very efficiently in order to avoid sparking or local overheating. Because of its high temperature conductivity ceramics is the preferred material for separation trays and strip holders, particularly when high voltages and high protein loads are applied.

17.4.2.4 General Comments on Electric Field Conditions A current setting of 50 μA per strip is usually not exceeded. In this way the initial voltage applied on each strip is automatically kept low, because, due to salts and not-migrated CAs, the conductivity is rather high. The salt ions and CAs will start to migrate, resulting in a decrease in conductivity. The conditions are different for strips with protein mixtures distributed all over the strip length compared to cup-loading or paper bridge loading, where the proteins enter the strip on a small area on the surface. In the latter case the voltage must be kept low for a longer time period than for rehydration-loaded proteins, in order to avoid aggregation and precipitation of proteins in the protein loading area.

Because different samples contain different protein compositions and salt contents, the conductivity also varies between different strips. The time for conductivity decrease will therefore vary as well. To achieve comparable and reproducible loads of electrical field strengths it is very useful to
measure the applied volt-hour integrals rather than measuring the maximum voltage applied over a certain time period.

Because of the IPG, focusing has to be carried out at very high voltages: at least 3000 V is essential, even for short strips. It is very important to use a programmable power supply with volt-hour measurement, in order to apply a dedicated voltage program in a reproducible way. Practice has shown that, for certain sample types, loading principles, and gradient types there should be a choice between stepwise or gradual voltage changes.

The longer the strip and the shorter the gradient interval, the higher the volt-hour load required to achieve sharp bands. When a protein mixture contains a high portion of high molecular weight proteins or when preparative sample loads are analyzed, it might be necessary to extend the suggested volt-hour load by 10%. On the other hand, when basic pH gradients are used, or when a gradient reaches pH values above 8, it is important to avoid overfocusing, because some proteins may become unstable at their isoelectric points and degrade or fragment. Therefore, in some cases, focusing conditions need to be optimized according to particular protein sample compositions. Usually, separations in long IPG strips are carried out overnight. However, basic strips should be run during the work day after prerehydration during the previous night.

17.4.2.5 Electric Field Conditions for Rehydration-Loaded Proteins Proposals of electric conditions depending on length and gradient are printed in the instructions delivered with purchased IPG strips. Table 17.2 shows an example of power supply settings for strips with rehydration-loaded proteins in different apparatus types for an IPG strip type used very frequently in proteomics: 18 cm long, pH 3–10 nonlinear. The first few low voltage steps are a safety measure for samples with relatively high salt contents. It is recommended to apply them in every case, as they have no negative effect on samples with low salt contents.

In contrast to the modular Multiphor, the IPGphor can be run with up to 8000 V, because it is a closed system containing Peltier cooling, an internal power supply, and an electrode chamber. As already mentioned above, it can be advantageous to apply a low voltage during rehydration (44). This is only possible when it is carried out in dedicated strip holders on the IPGphor. In this case an additional voltage step is programmed in at the beginning: 12 h at 50 V. For high protein loading above 1 mg the strips should preferably be run with the surface up, because highly abundant proteins form high ridges on the gel surface, which could cause horizontal smearing problems when they are in contact with the tray bottom.

17.4.2.6 Electric Field Conditions for Cup-Loaded Proteins For cup- and paper bridge loading it is also recommended to check the instructions in the strip package for running conditions. As an example, Table 17.3 presents conditions for the very frequently used 18-cm strip pH 3–10 nonlinear. Here, the first few low voltage steps are a safety measure for samples with high salt contents and would not have any negative impact on samples with low salt contents.

Figure 17.11 shows a 2D electrophoresis result for a mouse liver extract focused in a 24-cm-long IPG strip containing the widest commercially available gradient, pH 3–11. The sample was applied by cup loading.

17.4.2.7 Electric Field Conditions for Basic IPG Strips The analysis of basic proteins is very important in

<table>
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<tr>
<th>TABLE 17.2 Electric Settings and Running Conditions for 18-cm IPG Strips with pH 3–10 Nonlinear with Rehydration-Loaded Proteins</th>
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<tbody>
<tr>
<td>Apparatus</td>
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<td>Current per strip</td>
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Voltage Program

| Step and hold | 30 min/150 V | 30 min/300 V |
| Step and hold | 1 h/600 V | 1 h/1500 V |
| Step and hold | 5 h 40 min/3500 V | 2 h 30 min/8000 V_{\text{max}} (20,000 volt-hour) |
| (20,000 volt-hour) | | |

Total time 7 h 40 min 4 h 30 min |
proteomics, because most of the regulatory proteins and transcription factors have an isoelectric point above pH 7. When very basic strips such as pH 6–11 or 7–11 are used, it is mandatory to apply the sample at the anode (cup-loading) (16) and use 8000 V for focusing. Table 17.4 presents the running conditions in the IPGphor. Overfocusing should be avoided, because horizontal streaking at the basic end of the gradient can develop (50). As already mentioned in the discussion of sample preparation, cysteines should be stabilized as mixed disulfides by using DeStreak™ reagent in the rehydration solution for the IPG strips instead of a reductant. The final focusing step is controlled with the volt-hour setting, because different sample types can exhibit very different conductivities in these gels.

Figure 17.12 shows the low molecular weight section of the separation of very basic proteins of *Lactococcus lactis* in an 18-cm IPG strip containing a pH gradient 9–12.

### 17.4.2.8 End of the Separation

When a run has ended before the work day has begun, proteins start to diffuse. In general, the proteins should be refocused at their isoelectric point. Table 17.4 presents the running conditions in the IPGphor. Overfocusing should be avoided, because horizontal streaking at the basic end of the gradient can develop (50). As already mentioned in the discussion of sample preparation, cysteines should be stabilized as mixed disulfides by using DeStreak™ reagent in the rehydration solution for the IPG strips instead of a reductant. The final focusing step is controlled with the volt-hour setting, because different sample types can exhibit very different conductivities in these gels.

Figure 17.12 shows the low molecular weight section of the separation of very basic proteins of *Lactococcus lactis* in an 18-cm IPG strip containing a pH gradient 9–12.

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When a run has ended before the work day has begun, proteins start to diffuse. In general, the proteins should be refocused at their isoelectric 

### Table 17.3 Electric Settings and Running Conditions for 18-cm IPG Strips with pH 3–10 Nonlinear for Cup-Loading of Proteins

<table>
<thead>
<tr>
<th>Apparatus</th>
<th>Multiphor II</th>
<th>IPGphor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accessory type</td>
<td>DryStrip Kit (tray)</td>
<td>Cup-loading strip holder</td>
</tr>
<tr>
<td>Gel orientation</td>
<td>Gel surface up</td>
<td>Gel surface up</td>
</tr>
<tr>
<td>Temperature</td>
<td>20°C</td>
<td>20°C</td>
</tr>
<tr>
<td>Power</td>
<td>5 W</td>
<td>5 W</td>
</tr>
<tr>
<td>Current per strip</td>
<td>50 μA</td>
<td>50 μA</td>
</tr>
</tbody>
</table>

#### Voltage Program

<table>
<thead>
<tr>
<th>Step and hold</th>
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<th>30 min/150 V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step and hold</td>
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<td>30 min/300 V</td>
</tr>
<tr>
<td>Step and hold</td>
<td>1 h/600 V</td>
<td>1 h/600 V</td>
</tr>
<tr>
<td>Step and hold</td>
<td>1 h/1500 V</td>
<td>1 h/1500 V</td>
</tr>
<tr>
<td>Gradient</td>
<td>2 h/8000 V</td>
<td>6 h/8000 V</td>
</tr>
<tr>
<td>Gradient</td>
<td>1 h/3500 V</td>
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</tr>
<tr>
<td>Step and hold</td>
<td>6 h/3500 V</td>
<td>2 h 40 min/8000 V&lt;sub&gt;max&lt;/sub&gt; (21,000 volt-hour)</td>
</tr>
<tr>
<td></td>
<td>(21,000 volt-hour)</td>
<td></td>
</tr>
<tr>
<td>Total time</td>
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<td>13 h 40 min</td>
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### Table 17.4 Electric Settings and Running Conditions for Basic 18-cm IPG Strips with pH Gradients in the Region 6–12 (Cup-Loading of Proteins)

<table>
<thead>
<tr>
<th>Apparatus</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Accessory type</td>
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</tr>
<tr>
<td>Gel orientation</td>
<td>Gel surface up</td>
</tr>
<tr>
<td>Temperature</td>
<td>20°C</td>
</tr>
<tr>
<td>Power</td>
<td>5 W</td>
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<tr>
<td>Current per strip</td>
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</table>

#### Voltage Program

<table>
<thead>
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<th>1 h/150 V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step and hold</td>
<td>1 h/300 V</td>
<td>1 h/300 V</td>
</tr>
<tr>
<td>Step and hold</td>
<td>1 h/600 V</td>
<td>1 h/600 V</td>
</tr>
<tr>
<td>Gradient</td>
<td>1 h/8000 V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>8000 V&lt;sub&gt;max&lt;/sub&gt;/Volt-hour controlled:</td>
</tr>
<tr>
<td>Step and hold</td>
<td>30,000 volt-hour</td>
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</tr>
<tr>
<td>Total time</td>
<td>6–8 h</td>
<td>6–8 h</td>
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</table>

### Table 17.3 Electric Settings and Running Conditions for 18-cm IPG Strips with pH 3–10 Nonlinear for Cup-Loading of Proteins

<table>
<thead>
<tr>
<th>Apparatus</th>
<th>Multiphor II</th>
<th>IPGphor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accessory type</td>
<td>DryStrip Kit (tray)</td>
<td>Cup-loading strip holder</td>
</tr>
<tr>
<td>Gel orientation</td>
<td>Gel surface up</td>
<td>Gel surface up</td>
</tr>
<tr>
<td>Temperature</td>
<td>20°C</td>
<td>20°C</td>
</tr>
<tr>
<td>Power</td>
<td>5 W</td>
<td>5 W</td>
</tr>
<tr>
<td>Current per strip</td>
<td>50 μA</td>
<td>50 μA</td>
</tr>
</tbody>
</table>

#### Voltage Program

<table>
<thead>
<tr>
<th>Step and hold</th>
<th>30 min/150 V</th>
<th>30 min/150 V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step and hold</td>
<td>30 min/300 V</td>
<td>30 min/300 V</td>
</tr>
<tr>
<td>Step and hold</td>
<td>1 h/600 V</td>
<td>1 h/600 V</td>
</tr>
<tr>
<td>Step and hold</td>
<td>1 h/1500 V</td>
<td>1 h/1500 V</td>
</tr>
<tr>
<td>Gradient</td>
<td>2 h/8000 V</td>
<td>6 h/8000 V</td>
</tr>
<tr>
<td>Gradient</td>
<td>1 h/3500 V</td>
<td>2 h/8000 V&lt;sub&gt;max&lt;/sub&gt;</td>
</tr>
<tr>
<td>Step and hold</td>
<td>6 h/3500 V</td>
<td>2 h 40 min/8000 V&lt;sub&gt;max&lt;/sub&gt; (21,000 volt-hour)</td>
</tr>
<tr>
<td></td>
<td>(21,000 volt-hour)</td>
<td></td>
</tr>
<tr>
<td>Total time</td>
<td>12 h</td>
<td>13 h 40 min</td>
</tr>
</tbody>
</table>

### Table 17.4 Electric Settings and Running Conditions for Basic 18-cm IPG Strips with pH Gradients in the Region 6–12 (Cup-Loading of Proteins)

<table>
<thead>
<tr>
<th>Apparatus</th>
<th>IPGphor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accessory type</td>
<td>Cup-loading strip holder</td>
</tr>
<tr>
<td>Gel orientation</td>
<td>Gel surface up</td>
</tr>
<tr>
<td>Temperature</td>
<td>20°C</td>
</tr>
<tr>
<td>Power</td>
<td>5 W</td>
</tr>
<tr>
<td>Current per strip</td>
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</tr>
</tbody>
</table>

#### Voltage Program

<table>
<thead>
<tr>
<th>Step and hold</th>
<th>1 h/150 V</th>
<th>1 h/150 V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step and hold</td>
<td>1 h/300 V</td>
<td>1 h/300 V</td>
</tr>
<tr>
<td>Step and hold</td>
<td>1 h/600 V</td>
<td>1 h/600 V</td>
</tr>
<tr>
<td>Gradient</td>
<td>1 h/8000 V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>8000 V&lt;sub&gt;max&lt;/sub&gt;/Volt-hour controlled:</td>
</tr>
<tr>
<td>Step and hold</td>
<td>30,000 volt-hour</td>
<td></td>
</tr>
<tr>
<td>Total time</td>
<td>6–8 h</td>
<td>6–8 h</td>
</tr>
</tbody>
</table>

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**Figure 17.11** 2D electrophoresis of a TCA–acetone extract of mouse liver proteins, separated by IEF in a 24-cm-long IPG strip containing a nonlinear pH gradient 3–11, followed by SDS PAGE in a vertical 12.5% gel. Protein detection with silver staining.
points for 15 min at 3500 or 8000 V, respectively, short before the strips are further handled.

Sometimes it is very useful to check whether the protein mixture has properly separated and focused before the strips are run in the second dimension. The best staining protocol for these strips in the presence of detergents and chaotropes is the Acid Violet 17 staining according to Patelos and colleagues (52), which takes only ≏40 min. This stained strip can, of course, no longer be applied to the second dimension, because the proteins are tightly fixed into the matrix.

When the result is found to be adequate, the remaining strips can be refocused for 15 min and then run on the second dimension.

If the second dimension cannot be performed directly after IEF, the strips should immediately be frozen at −70°C and stored at this temperature (6).

17.5 SDS ELECTROPHORESIS

The second dimension SDS PAGE is usually performed in the discontinuous Tris-chloride/glycine buffer system in the presence of 0.1% SDS according to Laemmli (53). For readymade gels the buffer system needs to be modified, because the high pH 8.8 of the Laemmli gels causes loss of the sieving properties due to alkaline hydrolysis of the polyacrylamide matrix within a few weeks. This does, however, not influence the composition of the equilibration buffer.

17.5.1 Equilibration of the Strips

Equilibration in SDS buffer is performed immediately prior to the second-dimension run. The protocol according to Görg and colleagues (54) has become the standard procedure: an equilibration stock solution is prepared that is composed of 2% (w/v) SDS, 50 mmol/L Tris HCl pH 8.8, 6 mol/L urea, 30% glycerol, 0.01% Bromophenol Blue, and water.

Each gel is equilibrated in a tube on a shaker:

- 15 min in 10 mL equilibration stock solution plus 100 mg DTT
- 15 min in 10 mL equilibration stock solution plus 250 mg iodoacetamide.

Glycerol and urea are hygroscopic and reduce the electro-osmotic water flow caused by negatively charged fixed carboxylic groups of the immobilized pH gradient exposed to the electric field. The treatment with iodoacetamide scavenges the excess reductant to prevent vertical point streaking. Furthermore, it completely alkylates all cysteines. Acrylamide would partly alkylate the polypeptides, which would cause problems for protein identification with peptide mass fingerprinting and other MS analyses. When the proteins have been separated in an IPG strip containing DeStreak reagent, either the procedure described above is carried out without any modification, or, alternatively, the gel is equilibrated only once in the equilibration stock solution containing 100 mmol/L of the DeStreak reagent (HED) for 15 min. Because there is no alkylation step involved, the mass of the DeStreak reagent has to be taken into account during database searching after MS analysis.

When the IPG strips have been run in the Multiphor tray or the IPGphor manifold, they can be equilibrated together while clamped to the tray with the electrodes. The solution is discarded and the gels are placed on the second-dimension gel immediately.

17.5.2 SDS Polyacrylamide Gels

The second dimension can be run on vertical or horizontal flatbed SDS polyacrylamide gels. In a vertical electrophoresis tank up to 12 gels can be run in parallel; flatbed systems require a cooling plate for each gel. In Figure 17.13 a vertical system for running up to six gels, and a flatbed system for running up to four gels is shown.

As already mentioned in the introduction and shown in Figure 17.14, the results are very similar. A 0.5-mm horizontal gel, however, produces sharper spots than a 1-mm-thick vertical gel. Table 17.5 shows an overview of the different types of second-dimension gels used for 2D electrophoresis.
17.5.2.1 Laboratory-Made Gels Comprehensive protocols for the preparation of horizontal gels are found in References 39, 40, and 55, for vertical gels in References 39, 40, and 48, as well as the instruction manuals of the respective instruments. Here are a few general hints to achieve good quality air bubble-free gels:

- Even when it is stated that it is not necessary, add 0.1% SDS to the monomer solution. The addition of the detergent supports deaeration of the solution during mixing; in this way the polymerization effectiveness is considerably improved.
- Add the TEMED to the solution from the beginning (0.5 µL 100% TEMED/mL solution). The solution will not start to polymerize without the ammonium persulfate.
- Cool the freshly prepared monomer solution in a refrigerator while you assemble the caster to avoid a premature onset of polymerization, and to obtain the same gel properties independent from season (note mixing).

![Figure 17.13](image1.png) 2D electrophoresis systems for large format gels. (a) Ettan DALTsix chamber for running six vertical gels in cassettes. Courtesy of GE Healthcare, Little Chalfont, UK. (b) FlatTop Tower for running four horizontal gels on retractable cooling plates. Courtesy of ETC, Kirchentellinsfurt, Germany.

![Figure 17.14](image2.png) 2D electrophoresis of mouse liver proteins. Comparison of a 2D map obtained with a vertical and a horizontal flatbed SDS gel in the second dimension. First dimension: 18-cm IPG strip, pH 4–9. Second dimension: SDS polyacrylamide gels 12% T, 1-mm-thick vertical gel, 0.5-mm-thin horizontal gel. From Reference 12, with permission.
that polymerization effectiveness is dependent on temperature).

- Add the freshly prepared 10% (w/v) ammonium persulfate solution (4 mL/mL solution) short before casting and mix well.
- Pour the monomer solution into the gel cassette or casting box as fast as possible, and also when you prepare gradient gels.
- When an automatic spot picker for subsequent MS analysis of selected proteins is planned to be used, one of the glass plates is treated with Bind-silane to prevent the gel from changing its shape and size between scanning and picking the spots.

Figure 17.16 shows the casting procedure for gels in horizontal and vertical systems. Whereas gels for horizontal systems can be cast individually in a cassette with a U-shaped gasket, gels for vertical systems need to be prepared in a casting box. The stacking gel area for the horizontal gel, indicated in Figure 17.15a, is created by first pipetting a 6% monomer solution containing 25% glycerol into the cassette. Because of its higher density it will not mix with the resolving gel solution, which is poured directly on top of it. The stacking gel area contains the same buffer as the resolving gel. The pouring of large monomer solution volumes into multiple vertical gel casting boxes is considerably accelerated when the solution is poured directly onto the cassette edges instead of using the built-in channels, which have been intended for gel casting by the designer. Monomer solutions of all gel types are overlaid with water-saturated butanol or 70% v/v isopropanol to achieve a straight edge and complete polymerization without oxygen influence. In particular for vertical gels, a straight upper edge is very important, because the IPG strip is cast on a rigid film and cannot bend sideways.

### 17.5.2.2 Film-Supported Ready-Made Gels

One of the greatest handling problems with large SDS polyacrylamide gel is a lack of mechanical stability. Unfortunately, the gels swell and shrink during staining and can easily break. The addition of gel strengtheners, which are available on the market, often leads to reduced resolution and spot sharpness, in some cases even giving double spots. A superior solution to the problem is to attach the gels tightly to a plastic film during polymerization. For the application of the DIGE

![Figure 17.15](image_url)
system and for fluorescence staining it is important that the film support is nonfluorescent.

For electrophoresis in flatbed systems cassettes are not needed. The precast SDS gel is just unpacked and placed on the cooling plate with the gel surface upwards, and the stacking gel area oriented towards the cathodal side. Narrow electrode wicks soaked in buffer or ready-made polyacrylamide strips are laid onto the edges, and the equilibrated IPG strip is laid on the gel surface close to the cathodal buffer wick or strip as shown in Figure 17.3. Newly developed precast gels, prepared from a modified polyacrylamide matrix, contain a slot for the accommodation of the IPG strip in order to increase the efficiency of the protein transfer.

Film-supported gels for vertical systems are placed into a special designed cassette as shown in Figure 17.16. The small volume of water along the spacer has two functions: it allows the gel edge to slide easily up or down until the lower gel/film edge is flush with the glass plate; and it causes a slight swelling of the gel edge along the closing side for proper sealing of the cassette. Excess water and air bubbles are squeezed out with a roller to prevent surface effects. The cassette is tightly closed and kept in the rack with the upper edge down until all gels are mounted into the cassettes to prevent drying of the edge.

17.5.3 Setting up the Equipment and Application of the IPG Strips

17.5.3.1 Flatbed Systems The gel is placed on the cooling plate with the gel surface upwards, and the stacking gel area oriented towards the cathodal side. When ready-made gels are used, the respective electrode wicks or polyacrylamide strips are laid on the edges. Laboratory-made gels are run with liquid buffer, which is poured into the two buffer tanks. The edges of the gels are connected to the electrode tanks with paper wicks. The equilibrated IPG strip is laid on the gel surface close to the cathodal strip or wick as shown in Figures 17.3 and 17.17, respectively. Molecular weight standards are applied by placing filter pieces next to the ends of the IPG strip. There is no embedding with agarose.

Figure 17.16 Placing the film-supported gel into the cassette for vertical SDS electrophoresis.

Figure 17.17 Running the second dimension SDS electrophoresis in lab-made gels on a flatbed system according to Reference 6.
After 1 h of transferring the proteins from the IPG strip into the SDS gel under low voltage conditions the run is interrupted. First, the IPG strip and the filters are removed from the gel surface, and then the cathodal buffer strip or the cathodal wick, respectively, is moved and placed over the area of the previous IPG strip position (Fig. 17.17). This measure is necessary to prevent electroendosmosis effects, which are caused by negatively charged carboxylic groups of the immobilized pH gradient. The run is then continued with higher voltage settings.

The temperature of the thermostatic circulator is set to 15°C. The running conditions for large horizontal gels (Table 17.6) show that the Tris-acetate/tricine buffer system of the ready-made gels allows a much faster separation than the Lämmli buffer in laboratory-made gels.

### Table 17.6 Running Conditions for Large Flatbed SDS Gels 19 × 25 cm

<table>
<thead>
<tr>
<th>Power Supply Settings</th>
<th>Lab-Made Horizontal Gels</th>
<th>ExcelGel XL 12-14</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 V, 20 mA, 30 W</td>
<td>70 min 40 min</td>
<td></td>
</tr>
<tr>
<td>600 V, 40 mA, 40 W</td>
<td>3 h 30 min 2 h 40 min</td>
<td></td>
</tr>
</tbody>
</table>

17.5.3.2 **Vertical Systems**

The IPG strip is first placed on the protruding edge of the glass plate with the film side touching the glass plate, and then pushed down on the upper edge with a thin ruler as shown in Figure 17.18. One or two filter pieces soaked with molecular weight marker solution are applied at the two lateral sides of the gel edge, more than 1 cm apart from the spacer. It is not necessary and not recommended to embed the strip with hot agarose. If one wants to be absolutely on the safe side, it is sufficient to glue the strip to the SDS gel at the two ends. This prevents the IPG strip from swimming up or moving when buffer is filled. In this way there is no problem with capturing air bubbles between the IPG strip and SDS gel.

As shown in Figure 17.19, the gel cassette orientation can differ depending on the apparatus design. Figure 17.19a shows the concept of the DALT tank for running multiple gels according to Anderson and Anderson (56), in which the protein migrates from left to right. When it is necessary to use an anodal buffer with different composition than the cathodal buffer, as for Ettan DALT gels, the two electrode buffer tanks must be separate as in the classical vertical chamber design shown in Figure 17.19b. The latter design is the basis for the Ettan DALT chambers for 12 and 6 gels (see also Fig. 17.13). A further advantage of design concept (b) is the

![Figure 17.18](image1)

**Figure 17.18** Application of an IPG strip on the vertical gel and gluing it to the SDS polyacrylamide gel with hot agarose (step 3 is optional).

![Figure 17.19](image2)

**Figure 17.19** Different designs for vertical SDS electrophoresis.
much lower buffer consumption, which is about half the volume of concept (a) for the same number and size of gels.

Vertical gels also have to be run at low voltage for the first 1 h for a smooth transfer of the proteins from the IPG strips to the SDS gels, because the electroosmotic water flow, induced by the negatively charged carboxylic groups of the immobilized pH gradient, would carry a large amount of proteins against the electrophoretic migration towards the cathode buffer tank.

Both Ettan DALT instruments (for 6 and 12 gels) have a very efficient temperature dissipation system built in: a strong pump vigorously agitates the anodal buffer between the gel cassettes and over a heat exchanger. This design allows running the gels much faster than in a conventional electrophoresis tank, without creating a “smiling effect” of the running front. Ideally, the gels are run with constant power instead of constant current or voltage, because in the discontinuous buffer systems used (Table 17.5) the conductivity is changing during the run. When the power supply allows limiting of the maximum power, a high current is first applied at a low voltage, continuously changing to high voltage and low current. These two features, heat dissipation and power control, make it possible to achieve fast separations without overheating, for example, running 12 gels within 4 h and 30 min (Table 17.7). Note, that a temperature of 30°C is proposed for overnight runs: this is necessary because of the high temperature dissipation effectiveness. At lower temperature the pK value of the buffer would shift leading to incomplete binding of SDS to the proteins.

Both ways of running the gels have their benefits for certain situations. During fast runs there is lower diffusion of proteins, resulting in the following:

- higher spatial resolution of protein spots
- higher sensitivity of detection
- higher yield of tryptic peptides because of the more favorable relation of protein concentration to gel volume.

When gels contain fluorescence labeled proteins, they should be scanned as early as possible after the run has finished. As a working day does not usually extend too far into the evening, it makes more sense to run the gels overnight and scan them in the morning.

### TABLE 17.7 Settings for Fast and Overnight Runs for EttanDALT Systems

<table>
<thead>
<tr>
<th></th>
<th>Fast Run</th>
<th>Overnight Run</th>
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</tr>
<tr>
<td>Current set to</td>
<td>maximum</td>
<td>Maximum</td>
</tr>
<tr>
<td>Protein transfer</td>
<td>2 h: 0.5 W per gel</td>
<td>1 h: 0.5 W per gel</td>
</tr>
<tr>
<td>Separation</td>
<td>3 h 50 min: 15 W per gel</td>
<td>16 h: 2 W per gel</td>
</tr>
</tbody>
</table>

#### 17.6 PROTEIN DETECTION

The method for protein detection should be as sensitive as possible, and have a wide dynamic range, as well as a wide linear relationship between the quantity of protein and the staining intensity. It should be easy to carry out, nontoxic, environmentally friendly, MS-compatible, and available at low costs. Unfortunately there is no protein detection technique that fulfills all these demands completely. It might therefore be necessary to have more than one of the techniques available in a proteomics laboratory.

When the spots are analyzed inside the gel, the proteins are in most cases fixed in a 30% ethanol/10% acetic acid for 30 min to 1 h, depending on the gel thickness (57). For an efficient fixation of low molecular weight peptides (<10 kDa), the proteins are crosslinked in the gel with 0.2% glutaraldehyde in the presence of 0.2 mol/L sodium acetate and 30% ethanol (58).

### 17.6.1 Fluorescence Labeled Proteins (DIGE)

When low fluorescence glass cassettes are used, the fluorescence labeled proteins can be scanned without taking the gel out of the cassette. Sensitivity of detection and quantitative results are better when no fixing procedure has been carried out. Very good resolution and reliable quantitative results are obtained with a fluorescence scanner with confocal optics, because the excitation and emission light can be focused to a very small area. The different cyanine dyes Cy2, Cy3, and Cy5 have absorption maxima at 491, 553, and 645 nm, and they are scanned sequentially with different laser light sources at blue, green, and red light. In order to prevent crosstalk between the different channels during measurement of the emitted light, narrow bandpass filters are inserted. When minimum labeling is applied, only 3–5% of each of the proteins are labeled and contribute to the signal. The sensitivity of detection is close to the sensitivity obtained with the most sensitive protein staining technique: silver staining. The dynamic range is very wide: four orders of magnitude. Unfortunately there is no low fluorescent plastic film available at present: gels have to be run in cassettes without support or have to be covalently bound to one of the glass plates with Bind-silane.

Because the light emission levels are different for the three dyes, the measured values have to be normalized before the real image analysis is performed.

### 17.6.2 Staining Methods

#### 17.6.2.1 Visual Staining

Visual staining techniques are relatively inexpensive, because the consumables do not cost very much, and the images can be acquired with a modified desktop scanner.
• **Zinc Imidazol Negative Staining.** Hardy and colleagues (59) have introduced a sensitive staining technique that does not stain the proteins, but the gel. Protein of only 20 ng can be detected against the white background. It is the preferred technique for protein chemists, because it does not modify the proteins. Its usefulness for quantification is limited, because protein–dye complexes cannot be measured.

• **Coomassie Brilliant Blue Staining Methods.** Among the different Coomassie Brilliant Blue staining protocols, only those that work in the absence of alcohol are useful for quantitative analysis: colloidal staining according to Neuhoff and colleagues (60) and hot Coomassie staining, both described in References 48 and 55. When alcohol is present during destaining, it competes with proteins for the dye, and some proteins lose the dye faster than the background. The limit of detection is only ~100 ng of protein and the dynamic range is rather poor, but it is still the most frequently used staining procedure for 2D gels when MS analysis of spots is performed: the presence of a Coomassie-detected spot indicates that there is enough protein in the spot for peptide mass fingerprinting.

• **Silver Staining Methods.** Since the introduction of silver staining for polyacrylamide gels by Merril and colleagues (61), many different modifications have been published and declared as the most sensitive detection method. Some protocols have a limit of detection of as low as 500 pg per protein, like the methods according to Heukeshoven and Demick (57) and to Blum and colleagues (62). The 2D separations in Figures 17.5, 17.11, 17.12, and 17.14 have been stained with silver staining. Silver staining is a multistep procedure, has a very limited dynamic range, and cannot be recommended for quantitative analysis of protein expression levels. Silver staining occurs mainly on the gel surface; the thinner the gel the higher is the sensitivity. Sometimes proteins show negative staining, and high abundant protein spots often look like a crater of a volcano, with a dark border and a yellow center. Staining of film-supported gels requires an optimization of the durations of the different steps. The best results can be achieved in a staining automate, because every step is performed reproducibly with the precision of a machine rather than a human individual.

When glutaraldehyde is omitted from the sensitizer and formaldehyde from the silver nitrate solution, the proteins will not be crosslinked into the matrix and can be further analyzed by MS (63). This modification reduces the sensitivity of detection by a factor of five.

17.6.2.2 **Fluorescence Staining** Fluorescence staining requires a higher investment for a dedicated scanner, and the dyes are more expensive than the reagents for visual staining, but they offer very high sensitivity and excellent quantification features because of the wide linear dynamic range. These dyes are MS-compatible. The film support or glass plates of the gels must be nonfluorescent.

• **General Staining.** A novel developed natural fluorescent dye Deep Purple™, also called LavaPurple, or “Lightning Fast” in the literature (64), is about one order of magnitude more sensitive than SYPRO® Ruby in its modified formulation (65). Because the “Purple” dye becomes fluorescent only when attached to a protein, there are no speckles in the background and it easy to dispose of, because the formulation is not based on heavy metals. Figure 17.20 shows a 2D gel stained with Deep Purple™.

• **Specific Staining.** New fluorescent dyes have lately been introduced for specific staining of phosphoproteins (Pro-Q® Diamond) and glycoproteins (Pro-Q® Emerald) with high sensitivity. In this way it is possible to first analyze the pattern for the respective post-translational modifications and then stain the gel again with a general staining procedure.

17.6.2.3 **Double Staining** It has been shown by Görög and colleagues (14) for SYPRO® Ruby and silver staining that the

**Figure 17.20** Deep Purple™ stained 2D electrophoresis gel. Proteins of HBL breast cell line and BT 474 breast cell carcinoma separated on a 24-cm IPG strip, pH 3 – 10 nonlinear. The area of proteins with isoelectric points between 3 and 8 is shown. Second dimension: 12.5% SDS polyacrylamide gel.
staining results are not identical for different staining techniques. An easy way to avoid negative staining and missing spots is double staining by fixing the proteins with hot Coomassie Brilliant Blue/acetic acid, destaining the background with 10% (v/v) acetic acid, and subsequent silver staining (48).

17.6.3 Autoradiography and Fluorography

**Autoradiography** is highly sensitive, and as little as 0.1 pg of protein is detected per spot (3). The proteins can be labeled by growing a cell or tissue culture in the presence of $^{35}$S]-methionine or $^{14}$C]-amino acids or sugars, by labeling specific groups of proteins like $^{32}$PO$_4$ for phosphoproteins, and sugar precursors for glycoproteins. Gels can be stained with $^{59}$Fe. The gels are dried and then exposed to X-ray film for an appropriate time (one or several days). Horizontal 0.5-mm-thick gels on carrier films are easily dried and thus very practical for this detection method.

**Fluorography** is a scintillation autoradiography for low-energy $\beta$-particles like $^3$H (66): the gel is first impregnated with dimethyl sulfoxide (DMSO) and then with the organic scintillator 2,5-phenyloxazole (PPO). After this, the gel is dried and exposed to a blue-sensitive X-ray film at $-70^\circ$ C. Because DMSO and PPO are toxic and expensive, they have been replaced by the water-soluble fluor sodium salicylate (67). The sensitivity of fluorography is at least one order of magnitude higher than autoradiography, and can be further increased by prolonged exposure.

Autoradiography and fluorography methods are increasingly being replaced by nonradioactive labeling techniques because of environmental issues.

17.6.4 Blotting

Blotting techniques are used for protein identification after 2D electrophoresis with immunological or lectin-glyco-protein affinity methods. Details on blotting methods are found in Chapter 18 of this book. An example for detecting allergens on a blot of 2D separated wheat grain proteins is shown in Figure 18.10 of that chapter.

Meanwhile MALDI and electrospray MS analyses of tryptic peptide mixtures from in-gel digests of protein spots have completely replaced protein identification methods like amino-acid composition analysis and N-terminal sequencing of protein spots on blotting membranes.

17.7 IMAGE ANALYSIS

17.7.1 Measurement of Isoelectric Points and Molecular Weights

There are two ways to measure the isoelectric points and molecular weights and calibrate the images: 1D and 2D calibration.

**1D Calibration** The isoelectric points are determined by using the graphs of pH gradients of IPG strips available in the GE Healthcare Life Sciences Data file (68). These graphs represent the pH gradients calculated at 20°C running temperature in the presence of 8 mol/L urea. The molecular weights are interpolated between the bands of coseparated molecular weight standards.

**2D Calibration** The isoelectric points and molecular weights of an interesting protein can be determined by interpolation between spots of identified sample proteins with known pI and $M_r$. Prominent spots are analyzed for identification and amino-acid sequence information. The theoretical pIs and $M_r$ values are used as keystones for interpolating the values of the other proteins. This procedure is based on the paper by Bjellqvist and colleagues (69), who correlated the theoretical pIs derived from the amino-acid sequences of proteins with their locations in immobilized pH gradients. Because the masses of the proteins are based on MS measurements, this 2D calibration is more accurate than 1D calibration.

17.7.2 Pattern Evaluation

The complex 2D electrophoresis spot patterns are analyzed with dedicated image analysis and database software. Image analysis results can only become reproducible when image analysis becomes independent of the operator, in other words, when the possibilities of human interference are as low as possible. The major tools of such 2D electrophoresis software include the organization of experiments, gels, and reports; detection and quantification of spots; pI and $M_r$ calibration; different statistical tools; differential analysis to detect up- and down-regulated, newly appearing and disappearing proteins, and post-translational modifications; preparing spot picking lists; annotating MS results, linking to internal and web databases; database search engines.

The ways of spot detection, quantification, and statistical analysis are different, depending on whether the samples are run in individual gels or performing multiplex analysis of fluorescence labeled proteins in difference gel electrophoresis.

17.7.2.1 Multiplex 2D Separations in DIGE Gels

With the dedicated software called DeCyder™, the comigrated protein spots of different sample origin are codetected. This makes quantitative comparison very easy and reliable, because for each protein the strongest spot is selected and its spot boundary area and shape applied on the lower expressed spots of the same protein.

One channel can be reserved for the internal standard, which is created by pooling aliquots of each sample of an experiment. When several gels are compared, the expression
levels of a protein in the different gels can be normalized to
the level of the respective spot representing the internal stan-
dard. In this way, gel-to-gel variations are compensated.
Relatively small changes of protein expression levels can thus be quantified with high statistical confidence.

17.7.2.2 Individual 2D Separations In this case the spot
detection is performed in each gel individually. The reliability
of quantitative results is highly dependent on the type of
protein detection technique. The parameters for spot detec-
tion have to be set in a way to keep manual editing of the
spots to a minimum. For a series of 2D experiments a refer-
ence gel is created, and the spot positions of the different
gels are matched to those in the reference gel. Feature-
based matching on the basis of typical pattern characteristics
like spot shapes and spot clusters is a more robust approach
than pixel-based matches. Because an internal standard
cannot be used in this approach, average gels of replicate
results have to be created and compared in order to increase
the statistical confidence level.

17.8 STRATEGY FOR PROTEIN MAPPING
IN PROTEOMICS

Unfortunately these highly complex protein mixtures can
produce variances in the spot patterns. When differences
between samples are to be verified, it is very important to
first check the reproducibility of the pattern, and replicate
runs of the same sample should be analyzed for differences.
If patterns of the same sample vary too much, the sample
preparation protocol and the way of sample application on
the IPG strip should be reviewed. In a non-DIGE approach,
replicate gels have to be run to increase the statistical confi-
dence level of the result.

When a protein spot contains more than one protein, it is
impossible to obtain information on protein quantities. The
only way to solve this problem is to increase the spatial resol-
ution. Handling of gels larger than 25 × 20 cm is quite com-
licated. The increase of spatial resolution is more easily
achieved by performing IEF in narrow interval pH gradients.
An example for narrow interval separation in an acidic gradi-
ent is shown in Figure 17.5, and one for basic yeast gels in

![Figure 17.21](image)

Figure 17.21 2D reference map of yeast strain FY1679 separated in a 24-cm-long IPG strip, pH 6–12, with annotations of identified proteins. From Reference 16, with permission.
the form of a reference map with annotated protein spot in Figure 17.21.

Post-translational modifications of proteins can be indicated by changes of spot positions, but the conclusion of whether there is a post-translational modification or an amino-acid sequence polymorphism cannot be drawn from the 2D pattern alone. For further analysis as well as for the identification of proteins, MS has to be used. MS analysis of protein spots is described in a very condensed form in the following section.

17.9 PROTEIN IDENTIFICATION AND CHARACTERIZATION

17.9.1 Spot Picking and Digestion

17.9.1.1 Automated Spot Picking
In principle, the protein spots of interest could be cut out of the 2D gel manually. However, there are a number of reasons for using a spot-picking robot:

- to avoid contamination with keratin
- to prevent errors and to achieve traceability of the MS samples
- to obtain high accuracy of picking
- to achieve high throughput
- to pick invisible spots, which are stained or labeled with a fluorescent dye, or radioactive labeled.

For automated picking with high precision the gel must not change its shape and size between scanning and the picking process. There are two possibilities to achieve this:

- The gel is covalently bound to a film support or a glass plate.
- A nonbacked gel is placed on a dry cellophane film, which is clamped into a set of plastic frames as shown in Figure 17.21 (Burghardt Scheibe, personal communication). The procedure works also for fluorescent dyes, because Cellophane does not cause any fluorescent background.

Two self-adhesive fluorescence labeled internal reference markers are stuck on the film support, the glass plate, or the cellophane film, as shown in Figure 17.22. They are scanned together with the spot pattern and have a characteristic shape. After image analysis a picking list is established containing the x- and y-coordinates of the selected spots as well as of the two reference markers. The picking list is imported to the spot-picking computer. The spot-picker is equipped with a camera, which automatically searches for the two reference markers. The software is programmed so that these markers are recognized, and the pixel coordinates of the spots are automatically converted into the machine coordinates of the spot-picking robot.

The gel plugs containing the spots are cut out with a round-shaped picking head and transferred to microtiter plate wells. The well positions of the respective protein plugs are automatically added to the picking list.

17.9.1.2 Automated Protein Digestion
Intact proteins do not easily leave the polyacrylamide gel. They are therefore digested into peptides. The preferred endoprotease is trypsin, because it specifically cleaves the polypeptides at the C-terminus of the basic amino acids arginine and lysine.

Digestion is a multistep procedure, because the plugs have to be destained, washed, and dried before the enzyme is added. For high throughput a robot can be very useful. The best yields of tryptic peptides can be obtained with a spot-handling workstation, which integrates automated spot-picking, digestion, and spotting the peptide mixtures automatically on MALDI target slides.
17.9.2 MS Analysis

The following is a very short overview, and more comprehensive descriptions are found in References 48, 70, and 71.

17.9.2.1 Mass Spectrometers A mass spectrometer consists of three major parts: a device for the ionization of the sample components, a mass separator, and a detector. For protein and peptide analysis two different ionization principles are used: matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). Generally it can be stated, that MALDI instruments are easier to operate than ESI mass spectrometers.

**MALDI** MALDI is performed in an off-line system. The peptide mixture is mixed with an organic compound, the matrix, and pipetted and dried as a spot on a metal plate, the target. A pulsed laser beam is fired into the sample spot crystals while the target plate is connected to a high voltage potential. The matrix is selected to absorb the energy of the laser light at the specific wavelength and convert it into kinetic energy. The matrix molecules and the peptides are sublimated, and the charged matrix molecules transfer one proton to each peptide. In MALDI, singly charged peptide molecules are mainly produced, which are then analyzed in vacuum in an ion separator.

**Electrospray Ionization** ESI mass spectrometers are usually coupled to liquid chromatography systems. The mobile phase containing the peptide fractions are squeezed through a tight needle with high pressure, which is connected to a high voltage potential. The droplets of the resulting spray become reduced in size by a drying gas. Shortly the mutual repulses of the charged peptide molecules exceed the surface forces of the droplets; this causes the peptide ions to leave the droplets. These are predominantly multiply charged ions, which are introduced through an orifice into a vacuum for separation.

**Ion Separation** The behavior of charged peptide gas molecules in a vacuum is highly dependent on their molecular weight. For instance, they fly with different speed through a time of flight tube (ToF), travel through a channel between metal rods connected to specific radio frequencies (quadrupole), or they are expelled under defined electrokinetic conditions from an ion trap.

**Detection** The detectors are highly sensitive and register arriving ions at the attomole or even ceptomole level. In a mass spectrometer the molecular weights of the peptides can be measured with very high precision and accuracy.

17.9.2.2 Peptide Mass Fingerprinting Because with MALDI predominantly singly charged ions are produced, each peak in the mass spectrum represents one peptide. This is the ideal feature for peptide mass fingerprinting, where the composition and masses of the measured peptides are compared to known peptide compositions, or to theoretical peptide compositions derived from known protein sequences in databases. The size of tryptic peptides can be predicted due to the specific cleavage feature of trypsin. The precalculation of these peptide mixtures is often called “in silico translation.”

MALDI instruments can be easily used for high throughput analyses: a few hundred analyses per day can be run in a fully automated mode.

Peptide mass fingerprinting does not in all cases deliver an unambiguous identification; on average, only 60–80% of proteins can be identified with an adequate confidence level. This limitation arises because of a number of reasons, for instance, if more than one protein is present in a spot, not enough peptides have been ionized, post-translational modifications have occurred, or for other reasons not discussed here.

17.9.2.3 Sequencing Tandem MS Amino-acid sequence information is highly significant for the identification of a protein, even when it is available only for a relatively short portion of the polypeptide. Sequence information is obtained by fragmenting selected peptides in the mass spectrometer and measuring the differences of the molecular weights of the fragment ion series in the spectrum. Usually, fragmentation is initiated by applying high energy to a collision chamber, which contains an inert gas like helium. This kind of analysis is called tandem MS, because a peptide spectrum is first obtained, followed by a second measurement of the daughter ions of a selected peptide ion. Doubly charged ions are preferably selected, which are produced with ESI. The mass differences of the N-terminal fragment peaks and of the C-terminal fragment peaks represent the masses of the respective amino acids.

**Chemically Assisted Fragmentation (CAF) for MALDI MS** After digestion, the tryptic peptides can be chemically derivatized by attaching a strong acid to their N-termini. This negative charge on the molecule introduces a second proton to the peptide ion during MALDI conditions, leading to a strong enhancement of peptide fragmentation in the MALDI mass spectrometer (72). CAF MALDI allows the acquisition of peptide sequence information in a simplified way: only the positively charged C-terminal fragments are seen as peaks in the mass spectrum, the derivatized N-terminal fragments are neutral. In this way the amino-acid sequence can easily be read in the spectrum from the C-terminal ion series.

17.9.2.4 Further Characterization of Proteins MS allows further characterization of proteins with higher throughput and sensitivity than many of the chemical methods. The analysis of post-translational modifications
like phosphorylation and glycosylation requires peptide fragment analysis and therefore tandem MS, in some cases with additional fragmentation steps down to several levels.

17.9.3 Bioinformatics

A growing demand for proteomics analysis is the development of new informatics tools to cope with the huge amount of generated data. The entire proteomics workflow can be monitored and controlled by laboratory workflow system software in order to enable good laboratory practice (GLP) and to detect analytical errors.

The linkage of 2D electrophoresis, chromatography, MS, and biological data reveals additional information on protein structure and function, and it enhances the confidence level for protein identification. Some post-translational modifications can be identified without further MS or chemical analysis, just by further evaluating results and database data.

17.10 REFERENCES

PROTEIN ELUTION AND BLOTTING TECHNIQUES

REINER WESTERMEIER
SERVA Electrophoresis GmbH, Carl-Benz-Strasse 7, D-69115 Heidelberg, Germany

18.1 INTRODUCTION
After electrophoretic separation, further characterization of the protein bands or spots can be performed outside the gel matrix. Either the desired fraction is eluted directly into a liquid phase, or the complete separation pattern is transferred onto immobilizing membranes. The latter method is called blotting.

Direct elution needs a previous detection of the fraction, or a series of gel segments have to be eluted in parallel. Because the protein is often diluted during the procedure, it is generally followed by a concentrating step.

Blotting techniques are increasingly preferred to direct elution, as many new and sophisticated analytical micro methods have become available. Gentle detection methods can be performed on the blotting membrane, and no concentrating steps are necessary.

18.2 PROTEIN ELUTION
When a protein is to be purified by an electrophoretic method and is needed in the liquid phase, the sample is preferably separated under native conditions: by native basic or acidic electrophoresis, or by isoelectric focusing (IEF).

18.2.1 Elution by Diffusion
Elution by diffusion is only recommended for agarose and dextran gels, because polyacrylamide gels are very restrictive. Because isoelectric focusing has a high loading capacity and an in-built concentration effect, it is the most suitable electrophoretic method for preparative purification of proteins (1). Furthermore, this method can be performed in a granulated gel, such as in Sephadex, as it does not need the sieving properties of a gel. The pH gradient can be measured directly...
on the flatbed surface, or paper prints can be taken from the surface to detect the positions of the desired fractions (2).

With a fractionating grid and a spatula, the fractions of interest are removed from the flatbed, placed in a column, and eluted with buffer. It is not always necessary to separate the carrier ampholytes (CAs, Ampholine or Pharmalyte) and protein before performing further steps.

18.2.2 Electrophoretic Elution

Elution from polyacrylamide has to be performed in an electric field. Nguyen and colleagues (3) used isotachophoresis (displacement electrophoresis) to move the proteins out of a gel slice in a compact zone, and collected them inside a dialysis membrane. Because of the danger of adsorption of protein to the membrane, Öfverstedt and colleagues (4) electrophoretically drive the proteins into Sephadex G-25, from where further elution can be performed in an easy way.

Very effective methods for electro-elution from polyacrylamide gels combine the electrophoretic transport with a concentrating step. This can be done by using a discontinuous conductivity gradient (5, 6). Figure 18.1a shows the principle of this technique: A polyacrylamide supporting gel is polymerized into a glass tube. This gel contains 10% T and 25 mmol/L Tris/75 mmol/L glycine pH 8.8. The gel slices to be eluted are imbedded in 1.5% agarose, containing this buffer. The recovery solution contains this buffer and 40% (w/v) glycerol. The tube is carefully filled with 2 mol/L sodium chloride. The running buffer consists of 50 mmol/L Tris/150 mmol/L glycine pH 8.8. The eluted sample collects in the interface between the liquid buffer and the salt layer, due to the steep conductivity step. This principle is also used in the construction of a widely used elution device (7), where the salt layer is introduced in a V-boring in the divider of a horizontal buffer chamber (Fig. 18.1b).

18.3 PROTEIN BLOTTING

18.3.1 Principle

Blotting is the transfer of large molecules onto the surface of an immobilizing membrane. The proteins adsorbed on the membrane surface are freely available for macromolecular ligands, for example antigens, antibodies, lectins, or nucleic acids. Before specific detection, the free binding sites must be blocked with substrates that do not take part in the ensuing reaction (Fig. 18.2). Several detection steps can be applied one after the other, because the proteins are fixed on the membrane surface. In addition, blotting is an intermediate step in protein sequencing and an elution method for subsequent amino-acid analysis. A very comprehensive review on protein blotting can be found in a review by Beisiegel (8).

Generally, the blotting concept was introduced in 1975 by Ed Southern (9) for hybridization of electrophoretically separated DNA restriction fragments with radioactive labeled DNA molecules. The method became known as “Southern blotting.” The procedure for transferring RNA molecules onto a membrane (10) is called “northern blotting.” In both procedures the molecules are transferred with capillary or vacuum forces. The electrophoretic transfer of proteins from polyacrylamide gels to blotting membranes with immunological detection was developed by Towbin and colleagues (11), and is called “western blotting.” In western blotting, either a number of different samples are separated and the entire membrane probed with one antibody-containing solution, or one sample is applied across the entire gel width and the membrane is cut into narrow strips for probing with different antibody-containing solutions, like human sera.
The main features of protein blotting are as follows:

- accessibility for specific detection methods, such as antigen–antibody reactions, glycoprotein–lectin affinity, protein–protein interaction
- immunological detection with monoclonal antibodies, which do not precipitate
- highly sensitive nonradioactive detection methods (e.g., enhanced chemiluminescence)
- low consumption of antibodies compared to immunofixation and immuno-electrophoresis.
- multiple reprobing for different antigens on one membrane
- short incubation times
- native fixing of proteins, which do not lose their biological activity
- easy long-term storage of proteins (up to 1 yr)
- purification method for monospecific antibodies.

18.3.2 Transfer Methods for Proteins

18.3.2.1 Diffusion Blotting The blotting membrane is laid on the gel surface. The proteins are transferred by diffusion. It is mainly used after IEF in gels with large pores, such as agarose (12).

18.3.2.2 Pressure Blotting For polyacrylamide IEF it is recommended to place a stack of filter paper on top of the blot sandwich, followed by a glass plate, and by a weight of several kilograms. In this way, a high portion of the proteins (up to 90%) is pressed out of the gel onto the surface of the membrane. Towbin and colleagues (13) have published a very useful and sensitive protocol for blotting of proteins separated with IEF.

18.3.2.3 Capillary Blotting Braun and Abraham (14) have introduced a simple, but effective technique for transferring proteins out of PhastGel media gradient 10–15 (Fig. 18.3). The gel is laid on the blotting membrane, which is laid on a strip of filter paper soaked with phosphate buffered saline pH 7.2. The sandwich is clamped between two small glass plates. One end of the filter paper strip is immersed in a tray with buffer, and the long end hangs down freely, preferably over the edge of a table. Alternatively (for thermolabile proteins) the set-up is placed in a refrigerator. The constant flow of the buffer below the membrane causes a more efficient transfer of proteins than diffusion alone. Although the gels are not removed from the plastic support, an almost complete protein elution can be achieved within 2 h.

18.3.2.4 Vacuum Blotting This technique is applied only for agarose gels, because polyacrylamide gels can stick forever to the membrane after this procedure.

18.3.2.5 Electrophoretic Blotting Electrophoretic transfers are the most effective for proteins after electrophoresis in polyacrylamide (11, 15).

Originally, vertical buffer tanks with platinum wire electrodes on two sidewalls were used. For this technique, the gel and blotting membrane were clamped in grids between filter papers and sponge pads, and suspended in the tank filled with cooled buffer (Fig. 18.4). The blotting stack is set up under buffer to prevent the inclusion of air bubbles. The transfers are usually run overnight.

18.3.2.6 Semidry Blotting Semidry blotting between two horizontal graphite plate electrodes is simpler, cheaper, and
faster, and a discontinuous buffer system can be used (16, 17). Graphite is the best material for electrodes in semidry blotting, because it conducts well, does not have to be cooled, and does not catalyze reactions with anodal oxidation products. The applied current should not exceed 0.8 A per cm² of blotting area. If higher currents are used, the gel can heat up, resulting in precipitation of some proteins.

The transfer time is approximately one hour and depends on the thickness and concentration of the gel. When longer transfer times are required, as for thick (>1 mm) or highly concentrated gels, a weight is placed on the upper plate so that the electrolyte gas is expelled out of the sides.

Figure 18.5 shows a diagram of a semidry blot. The stacks of filter paper are soaked in the respective buffer, before they are laid on one another on the graphite blotter.

When gels supported by films, such as ready-made horizontal gels, need to be blotted electrophoretically, the film has to be removed without damage to the gel. Figure 18.6 shows an apparatus for pulling a taut thin stainless steel wire between gel and film support.

Figure 18.7 shows the graphite electrode system NovaBlot, which is placed into the electrophoresis chamber instead of the cooling plate. A detailed methodological description of the practical steps and a troubleshooting guide can be found in Reference 18.

It is also possible to perform electrophoretic transfers on two membranes simultaneously in a process termed “double replica blotting” (19). An alternating electric field is applied on a sandwich with a membrane on each side of the gel, with increasing pulse time, resulting in two symmetrical blots.

### 18.3.2.7 Semiwet Blotting

When a specially designed insertion module is used, semidry blotting can also be performed in a vertical electrophoresis chamber (Fig. 18.8). This module is watertight, and continuous as well as discontinuous buffers can be used. The vertical buffer tank can be filled with water, which acts as a heat sink.
Nitrocellulose is the most commonly used membrane. It is available in pore sizes from 0.05 μm to 0.45 μm. The pore size is a measure of the specific surface: the smaller the pores, the higher the binding capacity. Its disadvantages include a limited binding capacity and poor mechanical stability. Mechanically stronger membranes with supporting web are available, but the choice of detection techniques is limited for these mixed ester nitrocellulose membranes. A better adsorption of glycoproteins, lipids and carbohydrates is obtained by ligand precoating of nitrocellulose (20).

Polyvinylidenedifluoride (PVDF) membranes on a Teflon base have a high binding capacity and high mechanical stability, like nylon membranes. PVDF membranes can also be used for direct protein sequencing (21, 22).

Polyamide (nylon) membranes have a high mechanical stability and a high binding capacity, usually due to electrostatic interactions. This means that staining can be a problem because small molecules are also strongly bound. Positively and negatively charged, but also neutral nylon membranes exist. Fixing with glutardialdehyde after transfer is recommended to increase the binding of low molecular weight peptides to nylon membranes (23).

Ion-exchange membranes, diethylaminoethyl (DEAE) or carboxymethyl (CM), are used for preparative purposes because of the reversibility of the ionic bonds. Activated glass fiber membranes are used when blotted proteins are directly sequenced. Several methods to activate the surface exist: for example, bromocyanide treatment, derivatization with positively charged silanes (24) or hydrophobization by siliconizing (25).

During electroblotting, a portion of the proteins (mostly smaller individuals) migrate through the film and get lost, while larger proteins do not completely leave the gel. To trap low molecular weight proteins, a second blotting membrane is sometimes placed behind. It would be desirable to obtain as regular transfers as possible. Unfortunately a blotting membrane that binds 100% of molecules does not yet exist.

Thus, blotting conditions have to be optimized for a simultaneous transfer of high and low molecular weight proteins. When SDS electrophoresis is applied for the separation, a porosity gradient gel should be used in order to provide high retardation for small proteins and low retardation for high molecular weight proteins. Also, the correct choice of buffer is important for an optimal transfer.

18.3.4 Buffers for Electrophoretic Transfers

A continuous Tris–glycine–SDS buffer is often recommended for semidy blotting. Yet experience has shown that in general a discontinuous buffer system is preferable as it yields sharper bands and more regular and efficient transfers.

The continuous buffer (e.g., for double replica electroblotting this consists of 48 mmol/L Tris, 39 mmol/L glycine, 0.0375% (w/v) SDS, 20% methanol.

The discontinuous buffer system (according to Ref. 13) consists of the following:

- Anode I: 0.3 mol/L Tris, 20% methanol
- Anode II: 25 mmol/L Tris, 20% methanol
- Cathode: 40 mmol/L 6-aminohexanoic acid (same as ε-aminocaproic acid), 20% methanol, 0.01% SDS.

This buffer system can be used for SDS as well as for native and IEF gels. Methanol serves two functions. First, it avoids swelling of gel during the transfer. Second, it improves the binding capacity of nitrocellulose, which can be necessary, particularly in the presence of SDS.

If the transfer efficiency of high molecular weight proteins (>80 kDa) is not satisfactory, the gel can be equilibrated in the cathode buffer for 5–10 min before blotting. For enzyme detection the buffer must not contain methanol,
otherwise biological activity is lost. Brief contact with a small amount of SDS does not denature the proteins.

Figure 18.9 shows a stained nitrocellulose membrane, after semidy blotting with the discontinuous buffer system after SDS electrophoresis in a ready-made porosity gradient gel (an ExcelGel SDS gradient 8–18).

18.3.5 General Staining

Proteins adsorbed on nitrocellulose can be reversibly stained so that the total protein can be estimated before specific detection (26). Besides staining with Amido Black or Coomassie Brilliant Blue, mild staining methods such as the very sensitive Indian Ink method (27) exist, as well as reversible ones with Ponceau S (26) or Fast Green FCF (Fig. 18.9). The sensitivity of Indian Ink staining and the antibody reactivity of the proteins can be enhanced by alkaline treatment of the blotting membrane (28). Unfortunately, Indian Ink is no longer produced. Some laboratories have reported good results with standard script-ink diluted with TBS-Tween instead of PBS-Tween. The composition of TBS-Tween is 10 mmol/L NaCl, 10 mmol/L Tris-base, 0.01% (v/v) Tween-20.

Recently, a naturally occurring fluorophor of the fungus *Epicoccum nigrum* has been detected that can be applied as a highly sensitive, reversible stain for proteins (29) in gels and particularly also on blotting membranes. It is sold under the name “Deep Purple.” The dye molecule binds to the primary amines of proteins at low pH (pH 2.4), and can be tracelessly removed by washing the membrane at a higher or lower pH. Standard fluorescence scanners or charge coupled device (CCD) camera systems are employed for detection.

Proteins that have been tagged with fluorescent tags prior to separation with minimal labeling in the DIGE procedure (see Chapter 17) can be detected directly on the blotting membrane. Only about 3% of the proteins carry a fluorescent tag, so specific detection with antibodies is no problem. Blotting is often applied, because very sensitive general detection methods are possible on a membrane: general
immunostain (30) colloidal gold (31), fluorography (11), and chemiluminescence (32) with the highest sensitivity currently available.

Nylon membranes bind anionic dyes very strongly, so normal staining is not possible. However, nylon membranes can be stained with caccodylate iron colloid (FerriDye) (33).

18.3.6 Blocking

Macromolecular substances that do not take part in the visualization reaction are used to block the free binding sites on the membrane. A number of possibilities exist:

- 2–10% bovine albumin (11)
- skim milk or 5% skim milk powder (34)
- 3% fish gelatine
- 0.05% Tween 20
- casein preparations with a wide spectrum of different molecular sizes block membranes very effectively

18.3.7 Specific Detection

18.3.7.1 Enzyme Blotting

The transfer of native separated enzymes onto blotting membranes has the advantage that the proteins are fixed without denaturation and thus do not diffuse during slow enzyme–substrate reactions and coupled staining reactions (35).

18.3.7.2 Western Blotting (Immunoblotting)

Specific binding of immunoglobulins (IgG) or monoclonal antibodies are used to probe for individual protein zones after blocking. An additional labelled protein is then used to visualize the zones. Several possibilities exist, as described in the following:

Radioiodinated Protein A The use of radioactive protein A, which attaches itself to specific binding antibodies, enables high detection sensitivities (36). But, $^{125}$I–protein A only binds to particular IgG subclasses. In addition, radioactive isotopes are avoided as much as possible in the laboratory. In Figure 18.10, a blot of a 2D separation of IgE binding proteins (allergens) of wheat grain extracts is shown, where visualization was performed with $^{125}$I-labeled antihuman IgE followed by autoradiography (2 weeks exposure time).

Secondary Antibodies with an Enzyme Label An antibody to the specific binding antibody is used, which is conjugated to an enzyme. Peroxidase (37) or alkaline phosphatase (38) are usually employed as the conjugated reagent. The ensuing enzyme–substrate reactions have a high sensitivity. Figure 18.11 shows wheat gliadins separated by SDS electrophoresis and detected by rabbit anti-gliadin horse radish peroxidase conjugate on a PVDF membrane (37).

Gold Coupled Secondary Antibody Detection by coupling the antibody to colloidal gold is very sensitive (39). In addition, the sensitivity can be increased by subsequent

Figure 18.10 Two-dimensional separation of specific IgE binding proteins (allergens) of wheat grains; visualization with $^{125}$I-labeled antihuman IgE followed by autoradiography (2 weeks exposure time). Provided by W. Weiss, Technische Universität München, Weihenstephan (37), with permission.
silver enhancement, and the lower limit of detection lies at \( \sim 100 \) pg (40).

**Avidin Biotin System** Higher sensitivity is achieved with an amplifying enzyme detection system, with peroxidase complexes (41) or complexes with alkaline phosphatase.

**Chemiluminescence** The highest sensitivity without using radioactivity can be achieved with enhanced chemiluminescent detection (32). Reagents exist with different sensitivities. A schematic drawing of the principle of the most sensitive detection method (ECL Advance) is shown in Figure 18.12. For this detection kit, horse radish peroxidase is used as the substrate, and light with a wavelength of 440 nm is emitted for 4–6 h. Different reagents also exhibit different emission times. Those with short emission times are particularly useful for multiple reprobing for different antigens without removing the reagents of the previous detection.

**Secondary Antibodies with Different Fluorophor Labels** By using two fluorescent dye labels (ECL Plex goat-\( \alpha \)-mouse IgG-Cy3 with \( \lambda_{\text{max}} = 550 \) nm and ECL Plex goat-\( \alpha \)-rabbit IgG-Cy5 with \( \lambda_{\text{max}} = 649 \) nm), multiple detection protein analysis on blots can be carried out without stripping and reprobing. A low fluorescent membrane has to be chosen for this detection method. Standard fluorescence scanners or CCD camera systems can be used for detection. With direct fluorescence detection (without enzyme–substrate amplification), this detection system provides increased reproducibility and accuracy for the quantitative assessment of protein expression levels. The procedure is based on the CyDye technology used for difference gel electrophoresis (DIGE, see

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**Figure 18.11** Wheat gliadins separated by SDS electrophoresis and detected by rabbit antigliadin horse radish peroxidase conjugate on a PVDF membrane (37). Provided by W. Weiss, Technische Universität München, Weihenstephan, with permission.

**Figure 18.12** Schematic drawing of the principle of the most sensitive enhanced chemiluminescent detection.

**Figure 18.13** \( \beta \)-Galactosidase detected on blotting membranes with rabbit anti-\( \beta \)-galactosidase and antirabbit secondary antibody. Doubling dilution series starting at 10 ng. Detection with Hyperfilm ECL (5 min exposure). (a) ECL Advance, primary antibody 1:50,000, secondary antibody 1:500,000. (b) ECL Plus, primary antibody 1:5000, secondary antibody 1:100,000. (c) ECL, primary antibody 1:2000, secondary antibody 1:10,000. From GE Healthcare, Little Chalfont, UK, with permission.
Chapter 17). Both procedures, DIGE and ECL Plex, can easily be combined.

The signal is detected by either placing the membrane on a very sensitive film, or by exposing the membrane in the dark to a CCD camera for an extended time. The light signal will be accumulated. With ECL Advance, as little as $10^{-16}$ g of antigen can be detected. In order to achieve this very high sensitivity, care must be taken to use the correct blocking antigen can be detected. In order to achieve this very high sensitivity, care must be taken to use the correct blocking and sensitivity. This workflow is called “proteomics” and is a big step forward for protein chemistry and molecular biology.

18.3.8 Protein Sequencing

The use of blotting for direct protein sequencing (21, 24, 25, 44, 45) and amino-acid composition analysis (46) has been a big step forward for protein chemistry and molecular biology. One-dimensional SDS gels or 2D gels are usually blotted. If the proteins are separated by isoelectric focusing, an immobilized pH gradient should be used, because CAs would interfere with the sequencing signals (47). References 48 and 49 present comprehensive reviews of different methods. The different membranes on the market have been checked for their sequencing properties in Reference 50. The techniques are comprehensively described in Reference 51.

Meanwhile, the technology for identification of proteins has been developed much further, and proteins are digested inside the gel matrix with trypsin. These peptide mixtures are eluted and analyzed with MS with very high throughput and sensitivity. This workflow is called “proteomics” and is described in Chapter 17.

18.4 REFERENCES


Far-Western Blotting  The method of probing a membrane containing transferred protein with a nonantibody protein to detect specific protein–protein interactions is called “far-western blotting” (42). The probing protein can then be tagged with a labeled antibody for detection.

18.3.7.3 Lectin Blotting  The detection of glycoproteins and specific carbohydrate moieties is performed with lectins. The visualization methods are carried out by aldehyde detection or, in analogy to immunoblotting, for example, with the avidin–biotin method (43).
19

CAPILLARY ELECTROPHORETIC SEPARATIONS

WOLFGANG THORMANN

Department of Clinical Pharmacology, University of Bern, Murtenstrasse 35, CH-3010 Bern, Switzerland

19.1 INTRODUCTION

Electrophoresis is the premier analytical separation method for biological macromolecules. Electrophoretic separations and analyses in the capillary format, generally referred to as capillary electrophoresis (CE), have matured during the last three decades and steadily gained importance for analysis of polynucleotides, proteins, polysaccharides, small molecules, and ions (1–9). CE methods should be regarded as complementary or as attractive alternatives to other separation techniques, including high performance liquid chromatography (HPLC) and gel electrophoresis. The advantages of CE are high resolution, efficiency, mass sensitivity and speed, full automation, minute sample size, rapid method development, the use of small amounts of inexpensive and nonpolluting chemicals, as well as simple adaptation for micropreparative work. More importantly, CE techniques can exploit numerous separation principles, making them flexible and easily applicable to a variety of separation problems. For example, CE has been used to separate a broad spectrum of species and compounds ranging from small molecules (inorganic and organic ions) to large molecules and particles (proteins, oligonucleotides, and cells). On the other hand, the concentration sensitivity is somewhat lower than in many other techniques, including HPLC, this often calling for effective on-line or off-line preconcentration of analytes prior to analysis. Fortunately, electrophoretic techniques feature unique concentration effects (which are inherent to electrophoretic mass transport and very rarely seen in other separation techniques) providing compensation for the low concentration sensitivity (10, 11).

The various CE techniques can be categorized according to their mode of operation (Fig. 19.1). In the conventional configuration the electric field is applied parallel to the capillary or column axis (Fig. 19.1a). In terms of nomenclature, it is important to note that the expressions capillary and column...
are used interchangeably. Electrophoretic separations of this nature have successfully been conducted in quiescent or flowing solutions with narrow bore plastic tubes, glass or fused-silica capillaries, in rectangular troughs and in microchannels of chips and microstructures that are formed in various materials. The specific techniques comprising this group are capillary zone electrophoresis (CZE), capillary isotachophoresis (CITP), capillary isoelectric focusing (CIEF), and a range of electrokinetic capillary chromatography techniques, including micellar electrokinetic capillary chromatography (MEKC). Furthermore, sieving according to molecular size is effected using capillaries or microchannels filled with gels or entangled polymers. These approaches are typically referred to as capillary gel electrophoresis (CGE). Capillary electrochromatography (CEC) encompasses electrophoretic separations in a capillary or microchannel that contains a chromatographic stationary phase and is essentially driven by electroosmosis.

There is a fundamental unity that underlies all electrophoretic processes. A single mathematical model can describe the characteristic behavior of all basic modes of electrophoresis (12, 13). The superimposition of additional constraints, such as specific affinities, molecular sieving, cross- or counterflow, magnetic fields, fixed charges, and so on, yields all the electrophoretic methods in use. The simulation data presented in Figure 19.2 depict sample zone dynamics in CZE, CITP, and CIEF. CZE is conducted in a continuous buffer where the samples are the only discontinuities present. Under the influence of the electric field, sample zones migrate without exhibiting any steady-state behavior and thus their shape and position continuously change with time (Fig. 19.2, left panel). In this technique, separation is based upon differences in net mobility. Conversely, CITP (1–3) is performed in a discontinuous buffer system, the so-called leading and terminating electrolytes. The sample components are introduced in small quantities at the interface between the two electrolytes. The establishment of isotachophoretic zones requires the net mobility of samples to be intermediate to the mobilities of the buffers. Under the influence of an applied electric field, the sample components separate according to their net mobilities by forming a pattern of consecutive zones between the leader and the terminator (Fig. 19.2, center panel). The system attains a migrating steady-state in which all components have the same velocity (hence the prefix isotacho). Ideally, enough sample is applied to produce zones with constant composition, the lengths of which are proportional to the amount present. In CIEF, sample components are sorted according to their isoelectric point in an equilibrium gradient. In its most common form, ampholytes are focused in a pH gradient. Typically, a mixture of carrier ampholytes (CAs), with different pI values, is exposed to an electric field in a convection free medium. Eventually, a pH gradient is formed where the most acidic component has condensed in the most anodal position of the capillary, the most basic at the cathode, and constituents of intermediate pIs in between. Proteins and other amphoteric compounds can be separated in this gradient provided their isoelectric points are sufficiently different (Fig. 19.2, right panel). In
the absence of any flow along the column, a stationary steady-state is eventually attained. Many other electrophoretic processes can be classified as combinations of these methods. For example, discontinuous zone electrophoresis (14) is conceptually a composite of CZE and CITP. As in CITP, the sample is introduced at the interface between two buffers. However, the terminating constituent’s mobility is slightly larger than the mobilities of the sample components. It therefore penetrates the sample zones from the rear side upon current flow. After an initial sharpening at the buffer transition the sample components separate zone electrophoretically within the terminating electrolyte. Similar efficient sample stacking can be achieved by having one or more major components of like charge in the sample. Also, stacking at the initial sample buffer interfaces occurs when the conductivity of the sample solution is lower compared to that of the buffer (10, 11, 14, 15).

MEKC is an electrokinetic method allowing the separation of neutral and charged small molecular mass molecules. In this technique two distinct phases are used, an aqueous and a micellar, or pseudo-stationary, phase. These two phases are established by employing buffers containing surfactants, such as sodium dodecyl sulfate (SDS), which are added above their critical micellar concentration. Electrophoresis takes place in open tubular capillaries in which the two phases are being transported with different velocities. Nonionic solutes partition between the two phases and elute with zone velocities between those of the two phases. Separation is of a chromatographic nature. For ionic solutes that are also differentially distributed between the two.
phases, separation is of a chromatographic and electrophoretic nature (16). Finally, CEC can be considered as a composite of chromatography and electrophoresis in which electroosmosis transports the mobile phase and analytes through a capillary or microchannel with a chromatographic stationary phase. It comprises the following components:

- a column packed with microparticulate sorbents that is referred to as packed bed CEC
- a monolithic block of a chromatographic stationary phase that is formed in a column (continuous-bed CEC)
- a column containing a molecularly imprinted polymer that is prepared through polymerization of assemblies of a template molecule and functional monomers (a special format of continuous-bed CEC)
- a column with a replaceable entangled polymer solution that provides both chromatographic interactions and electroosmotic pumping (e.g., a polyelectrolyte with grafted hydrophobic ligands)
- open-tubular CEC with a thin layer of the chromatographic phase attached to the inner column wall

CEC is driven by electroosmosis (17) and can be operated without or with application of pressurized flow. CEC involves both, partitioning between two phases (chromatography) and electrophoresis, thus uncharged (neutral) and charged solutes can be separated. CEC provides a mean for separation of compounds that have similar electrophoretic mobilities and partition coefficients, that is, molecules that are difficult to separate by CZE or open-tubular liquid chromatography (18–21).

In the second category, the electric field is applied perpendicular to the capillary axis, and the separation of charged solutes is based upon their differential alignment across the mobile phase profile of a field flow fractionation (FFF) device (Fig. 19.1b). Such separations are performed in thin ribbonlike channels (22), in hollow ultrafiltration fibers (23), or in channels of trapezoidal cross-section (24). The techniques representing this class are electrical FFF (EFFF), electropolarization chromatography (EPC) and electrical hyper-layer FFF (EHFFF) or isoelectric focusing FFF. In EFFF and EPC the electric field, applied perpendicular to the flow direction, causes the solute molecules to accumulate in a layer of distinct thickness near one channel wall. The solute is then transported by flow along the channel at a rate determined by the mean thickness of the layer. In EHFFF, separation is based upon partitioning by the concordant presence of a pH equilibrium gradient and an electric field, as in isoelectric focusing (IEF). Solute layers having different depths from the walls and that travel at different velocities along the column are thereby established.

In the third category, the electric field is also oriented perpendicular to the direction of flow, but parallel to the flow profile of a thin fluid film flowing between two parallel plates (Fig. 19.1c). This represents the configuration of continuous flow electrophoresis (CFE), which can be operated in various modes, including zone electrophoresis (ZE), isotope electrophoresis (ITP) and IEF (25, 26), or recycling electrophoresis, in which outlet and inlet ports of the separation cells are connected by closed circuit loops for process fluid recirculation by means of a multichannel peristaltic pump. In CFE the buffers and the sample are continuously introduced at one end of the electrophoresis chamber and are fractionated by an outlet array at the other end (Fig. 19.1c). CFE is considered a CE technique because fluid stabilization is achieved by a small gap (typically 0.5 mm or less) between the two parallel plates. Although CFE is mainly a preparative methodology for simultaneous separation and fractionation under mild operating conditions, it is also used for analytical purposes, particularly sample preparation in proteomics and for the separation and characterization of cells, cell organelles, and cell membrane systems. CFE permits quick determination of electrophoretic mobility distribution of cells or proteins. It can be used as an aid in understanding the phenomena that take place on the cell surface, such as the effect of drugs, antigens, and mitogens.

19.2 CAPILLARY-TYPE ELECTROPHORETIC INSTRUMENTATION

The design variables for electrokinetic capillary analyzers are presented in Table 19.1 and the basic arrangement used in conventional CE is depicted schematically in Figure 19.3. It features a capillary tube mounted between two electrode compartments. Typically, the sample is injected in small quantities into the beginning of the separation column, electrophoresed through the capillary, and then on-column detected towards the capillary end. The power supply and a method for buffer introduction (e.g., with application of pressure or vacuum) complete the basic arrangement. Introduction of the buffers and the sample, data manipulation (i.e., collection, storage, and evaluation), and fraction collection are ideally controlled by a computer. The computer system also allows the establishment of data dictionaries as in other analytical methods. Pulse free counter or co-flow of electrolyte is useful for (i) changing the column volume without changing the physical capillary length in CZE and CITP and thereby manipulating loading capacity and analysis time, (ii) mobilization of the zone spectrum in CIEF, (iii) sample fractionation, and (iv) sample detection outside of the capillary. The last two features may not require a pump for the open tubular configuration with a strong electroosmotic flow (EOF). In CE, a high voltage DC electric field is applied along the column, which not only induces electrophoretic transport and separations of charged compounds, but in the case of a charged inner wall and no fluid entrapment via
use of a gel, also a movement of the entire liquid along the capillary. This latter process is termed electroosmosis. In open tubes having a negative (positive) surface charge, an EOF towards the cathode (anode) is generated. The magnitude of the EOF is dependent on the electric field strength, the zeta potential of the double layer at the capillary wall and \( \frac{1}{\varepsilon \eta} \) where \( \varepsilon \) and \( \eta \) represent the permittivity and viscosity of the solution, respectively (4–7). The magnitude of electroosmosis can also be expressed with its mobility value, that is, \( \mu_{EO} = v_{EO}/E \), where \( v_{EO} \) and \( E \) are the electroosmotic velocity and the electric field strength, respectively.

In contrast to pressure-driven hydrodynamic flow, electroosmosis is characterized by a plug flow profile, which has little impact on zone boundary dispersion (Fig. 19.4a). Thus, high resolution separations can take place in the presence or absence of electroosmosis along the separation axis. The net velocity of a charged compound \( i \) can be expressed as:

\[
v_i = (\mu_i + \mu_{EO}) E \tag{4–7}
\]

where \( \mu_i \) represents the electrophoretic mobility of compound \( i \).

Depending on application and instrumental set-up, capillaries of \( \sim 5–120 \) cm in length are connected to buffer reservoirs containing the driving electrodes. Small amounts of samples (nL volumes or pmol to sub-fmol solvent quantities) are introduced by electrokinetic or hydrodynamic techniques. Upon application of power (\( \sim 2–30 \) kV, 1–150 mA), samples are transported through the capillary by the combined action of electrophoresis and electroosmosis. In principle, many of the detection methods developed for HPLC can also be used for CE (Table 19.2), with spectroscopic techniques (on-column direct and indirect absorbance, direct and indirect fluorescence detection) and MS being the most popular. In addition, unique principles can be used that rely on the electric current flow through the separation capillary, such as the monitoring of potential gradient, conductivity, and temperature. In CE, tiny zone volumes must be measured accurately. In any case, the detection limit is determined both by operational and equipment parameters. The technology for the design of detector cells with picoliter to microliter volumes is now well established. In most arrangements, one or two on-column sensors placed towards the capillary end are used, and monitoring occurs while the electrophoretic zone structures migrate across their location. The advent of versatile and inexpensive microprocessor systems also permits simple control of sensor arrays, thereby providing increased analytical information through the repeated detection of the whole separation pattern along the capillary (imaging detection) or the comparison of electropherograms from at least three completely different locations of the separation trough (27–30).

### Table 19.1 Design Variables for an Electrokinetic Capillary Analyzer

1. **Modes**
   - Zone electrophoresis, discontinuous zone electrophoresis
   - Isotachophoresis
   - Isoelectric focusing
   - Electrophoresis in sieving media
   - Electrophoretic capillary chromatography
   - Electrophotography

2. **Separation Capillary/Microchannel**
   - Number of separation columns
   - Material (fused-silica, glass, plastic)
   - Length (1–200 cm depending on mode, material, and instrument)
   - Cross-section (circular, rectangular, trapezoidal)
   - Cross-sectional area (capillary i.d., aspect ratio)
   - Gel/microparticulate sorbents or monolithic block/free solution
   - Surface modification of column wall (permanent/dynamic coating)
   - Temperature control

3. **Sample Injection**
   - Electrokinetic
   - Hydrodynamic flow (vacuum/pressure/gravity)
   - Syringe/sample splitting/sample mixing

4. **Sample Detection**
   - On-column/end-column/off-column
   - Single/multiple/array/scan sensors
   - Pre-/postcolumn derivatization

5. **PC-Based Instrument Control and Data Analysis**
   - Software for instrument control
   - Software for data collection/evaluation

6. **Peripheral Components**
   - Electrode compartments
   - Power supply (voltage/current/power regulated)
   - Autosampler (optional)
   - Fraction collector (optional)
   - Electrolyte pumps (optional)
   - Sample preparation unit (optional)
The first CE approaches were mainly geared towards CITP and comprised electrophoretic separations in quiescent solutions via use of coated glass capillaries of 0.1–1 mm i.d., narrow bore plastic tubes of 0.2–0.8 mm i.d., or thin ribbon-like channels of rectangular cross-sections (0.1–0.5 mm channel height and 0.5–2 mm channel width) having various plastic and glass walls (for reviews refer to References 2, 3, 8, and 9). With this kind of instrumentation, microliter sample quantities were applied with a conventional microliter syringe. In the 1970s and 1980s, a number of commercial

Figure 19.4  (a) Schematic representation of the negative surface charge with double layer in a buffer-filled fused-silica capillary (left graph), electroosmotic plug flow profile in an open-tubular system with a negative surface charge (solid line, center graph) and parabolic pressure-driven flow profile (broken line, right graph). (b) pH-dependent electroosmotic mobility in a fused-silica capillary determined with 40 mM phosphate buffers (filled triangles) and data fit (solid line) according to \( \mu_{EO} = \mu_0 + \mu_{ads}(1 - \alpha) \) with \( \mu_0 \) and \( \mu_{ads} \) taken as \( 6.3 \times 10^{-3} \text{ m}^2/\text{Vs} \) and \( 1.0 \times 10^{-8} \text{ m}^2/\text{Vs} \), respectively. Reprinted with permission from Reference 31. Copyright, American Chemical Society. (c) CZE electropherogram of a model mixture of cations and anions detected at 220 nm. The dotted line in (b) represents the dissociation curve of silanol with a \( pK_a \) value of 5 and a mobility at full dissociation of \( 6.3 \times 10^{-8} \text{ cm}^2/\text{Vs} \) (first term of fit equation). The CZE analysis of (c) was executed at room temperature in a homebuilt instrument using a 75 \( \mu \text{m} \) i.d. fused-silica capillary of 70 cm total (50 cm effective) length, a pH 7.79 buffer composed of 100 mM ACES and 90 mM NaOH and a constant voltage of 15 kV (current, 80 \( \mu \text{A} \)). The sample comprised tryptamine (Tra), procaine (Pro), L-tyrosyl-1-\( \alpha \)-lysine (Tyr–Lys), tryptophan (Trp), L-tryptophyl–L-glutamic acid (Trp–Glu), salicylurate and salicylate. EO, electroosmotic void peak.
TABLE 19.2 Detection Methods for Electrokinetic Capillary Analyses

<table>
<thead>
<tr>
<th>Detection Method</th>
<th>On-Column</th>
<th>End-Column</th>
<th>Off-Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-VIS absorbance</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Polarimetry-interferometry</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Circular dichroism</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Chemiluminescence</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Temperature</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Conductivity</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Electric field</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Amperometry/voltammetry</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Potentiometry/pH</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Mass spectrometry</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Nuclear magnetic resonance</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Radiometry</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

Instruments equipped with plastic capillaries emerged. These are the Tachophor 2127 (LKB, Bromma, Sweden), the Tachophor Delta (Itaba, Järfalla, Sweden), the IP-1A, IP-2A, IP-3A Analyzers (Shimadzu, Tokyo, Japan), and the ITP Analyzers of VVZ PJT (now Villa Labeco, Spisska Nova Ves, Slovak Republic). The EA 102 and 202 analyzers feature a coupled, two-stage capillary column together with conductivity and UV/VIS detection and are currently available as manually operated or automated instruments (distributor: J&M Analytik, Essingen/Aalen, Germany). With this kind of CE instrumentation, procedures for fractionation using microsyringes or elution onto a strip of cellulose acetate were designed. The second approach was commercialized by LKB, Bromma, Sweden, under the trade name “Tachofrac.”

Electrokinetic capillary instrumentation featuring open-tubular fused-silica capillaries of very small i.d. (10–100 μm) became the focus of active research and routine use during the past 25 and 15 years, respectively. Fused-silica capillaries permit rapid removal of ohmic heat, application of high voltages (300 V/cm) and hence realization of short runs, and are transparent to UV light. Furthermore, they exhibit a pH-dependent, strong EOF towards the cathode if no special wall coatings are applied (Fig. 19.4b). The major contributor to the surface charge is dissociation of silanol groups (expressed via a pKₐ value), which can be described by

\[
\mu_{\text{EO}} = \mu_0 \alpha, \text{ where } \mu_0 \text{ and } \alpha = \left[1 + (10^{pH-pK_a})^2 \right]^{-1}
\]

are the electroosmotic mobility at full dissociation and the dissociation coefficient, respectively (the dotted line in Fig. 19.4b was obtained with a pKₐ value of 5). At low pH, anion adsorption is typically providing some additional surface charge such that the mobility does not vanish at around and below pH 2. The data fit shown as a solid line in Figure 19.4b was obtained by

\[
\mu_{\text{EO}} = \mu_0 \alpha + \mu_{\text{ads}}(1 - \alpha), \text{ where } \mu_{\text{ads}} \text{ represents the electroosmotic mobility at pH 2 (31).}
\]

The EOF at alkaline pH permits the detection of first cations and then anions in the same run. A typical electropherogram obtained with a single UV absorbance detector placed towards the capillary end is presented in Figure 19.4c. The presented example shows the separation of three cations and four anions in a pH 7.79 buffer at an electric field strength of 214.3 V/cm (current, 80 μA; power level, 1.71 W/m). The detection times, velocities, calculated mobilities, and estimated charge values are presented in Table 19.3. In such a configuration, all cations can reach the detector in a rather short time. Furthermore, anions with electrophoretic mobility values smaller than the electroosmotic mobility can be detected as well (example shown in Fig. 19.4c: \(\mu_{\text{EO}} = 6.40 \times 10^{-8} \text{ cm}^2/\text{V}s\); \(\mu_{\text{salicylate}} = -4.15 \times 10^{-8} \text{ cm}^2/\text{V}s\); net mobility of salicylate equals \(2.25 \times 10^{-8} \text{ cm}^2/\text{V}s\)). Modifications of the fused-silica surface properties result in diminished, abolished, or reversed electroosmosis with pH dependent or independent mobility values. Fused-silica capillaries are manufactured

TABLE 19.3 Velocities, Mobilities and Estimated Charge of Small Molecular Mass Compounds at an Electric Field Strength of 214.3 V/cm and pH 7.79

<table>
<thead>
<tr>
<th>Compound</th>
<th>Detection Time, min</th>
<th>Velocity, mm/sec</th>
<th>Net Mobility, (10^{-8} \text{ m}^2/\text{V}s)</th>
<th>Electrophoretic Mobility, (10^{-8} \text{ m}^2/\text{V}s)</th>
<th>Estimated Charge Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>4.13</td>
<td>2.02</td>
<td>9.43</td>
<td>3.03</td>
<td>1.0</td>
</tr>
<tr>
<td>Procaine</td>
<td>4.48</td>
<td>1.86</td>
<td>8.68</td>
<td>2.28</td>
<td>1.0</td>
</tr>
<tr>
<td>Tyr–Lys</td>
<td>5.85</td>
<td>1.43</td>
<td>6.65</td>
<td>0.25</td>
<td>0.1</td>
</tr>
<tr>
<td>Electroosmosis (EO)</td>
<td>6.08</td>
<td>1.37</td>
<td>6.40</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>6.36</td>
<td>1.31</td>
<td>6.12</td>
<td>-0.20</td>
<td>-0.1</td>
</tr>
<tr>
<td>Trp–Glu</td>
<td>11.63</td>
<td>0.72</td>
<td>3.35</td>
<td>-3.05</td>
<td>-1.0</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>13.21</td>
<td>0.63</td>
<td>3.05</td>
<td>-3.45</td>
<td>-1.0</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>17.33</td>
<td>0.48</td>
<td>2.25</td>
<td>-4.15</td>
<td>-1.0</td>
</tr>
</tbody>
</table>

*Experimental conditions: 75 μm i.d. fused-silica capillary at room temperature (air cooling) with a pH 7.79 buffer composed of 100 mM ACES and 90 mM NaOH and a constant voltage of 15 kV (current, 80 μA; power level, 1.71 W/m). For electropherogram see Figure 19.4c.
with a thin outside polymer coating, which makes them extremely flexible, permitting easy manipulation. Using these capillaries makes the customary microliter syringe for sample injection obsolete. The sample is typically introduced either by hydrodynamic flow, that is, a displacement technique based on (i) application of vacuum to the capillary outlet, (ii) application of pressure to the sample vial with an inert gas, (iii) changing the relative heights of the sample and outlet buffer vials (gravity injection), or (iv) a split-flow injection, or an electrokinetic technique. For the latter approach, the electrode compartment at the high voltage end is replaced with the sample vial. Application of high voltage for a short amount of time allows the insertion of a small amount of sample into the capillary (combined action of electrophoresis and electroosmosis). The sample vial is then exchanged by the buffer compartment before further application of current (Fig. 19.3).

Since the first report in 1981 (32), many different instruments featuring fused-silica capillaries of 10–100 μm i.d. have been assembled and employed in researchers’ laboratories. Although the first commercial instrument emerged in 1988, a few years later there were about a dozen companies manufacturing single capillary instrumentation with on-column optical sample detection. Fully automated systems, modularly built instruments, and manually operated set-ups were constructed. Instruments from Beckman (MDQ and PA800), Agilent Technologies (Agilent CE System, formerly HP3D), Prince Technologies (models 650 and 660), and Convergent Bioscience (iCE280) are currently available (Table 19.4). The iCE280 is designed for CIEF only and comprises whole column imaging that permits real-time following of protein focusing along a capillary with a length of 5 cm (Fig. 19.5). The instruments from the other three manufacturers can be directly linked to electrospray ionization mass spectrometry (MS), and the Beckman apparatuses also have laser-induced fluorescence (LIF) detection. The instruments of Agilent and Prince use forced air to control the temperature of the capillary, whereas those of Beckman feature liquid cooling. The latter approach is more effective and may permit the use of higher electric field strengths. Along with the commercialization of fused-silica capillary based instrumentation, capillaries with different surface properties (Table 19.5), buffers and kits containing complete protocols, precut capillaries, conditioning reagents, and

<table>
<thead>
<tr>
<th>Instrument (Supplier)</th>
<th>Sample Detection</th>
<th>Sample Injection</th>
<th>Autosampler</th>
<th>Capillary Temperature Control</th>
<th>System Control</th>
<th>Special Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>P/ACE 5000&lt;sup&gt;a&lt;/sup&gt; (Beckman Coulter)</td>
<td>UV (filter), diode array, LIF</td>
<td>Electrophoretic, pressure</td>
<td>34 positions, temp. control</td>
<td>Liquid</td>
<td>Front panel, PC</td>
<td>MS interface</td>
</tr>
<tr>
<td>P/ACE MDQ or PA800 (Beckman Coulter)</td>
<td>UV (filter), diode array, LIF</td>
<td>Electrophoretic, pressure, vacuum</td>
<td>96-well plates, racks with 2 mL or microvials, temp. control</td>
<td>Liquid</td>
<td>PC</td>
<td>MS interface</td>
</tr>
<tr>
<td>BioFocus 3000&lt;sup&gt;b&lt;/sup&gt; (Bio-Rad Laboratories)</td>
<td>UV-VIS, 190–800 nm, fast scanning, dual LIF</td>
<td>Electrophoretic, pressure</td>
<td>32 positions, temp. control</td>
<td>Liquid</td>
<td>PC</td>
<td>MS interface</td>
</tr>
<tr>
<td>HP&lt;sup&gt;3D&lt;/sup&gt; (Agilent Technologies)</td>
<td>UV-VIS, 190–600 nm, diode array</td>
<td>Electrophoretic, pressure</td>
<td>48 positions, temp. control</td>
<td>Forced air</td>
<td>PC</td>
<td>Fraction collector, bubble detection cell</td>
</tr>
<tr>
<td>iCE280 (Convergent Bioscience)</td>
<td>UV 280 nm (whole column)</td>
<td>Pressure</td>
<td>None; can be combined with modular sampler&lt;sup&gt;c&lt;/sup&gt;</td>
<td>None</td>
<td>PC</td>
<td>CIEF only, whole column imaging along 5-cm focusing space</td>
</tr>
<tr>
<td>PrintCE 650 and 660 (Prince Technologies)</td>
<td>Modular&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Electrophoretic, pressure, vacuum</td>
<td>30 or 48 positions</td>
<td>Forced air</td>
<td>Front panel, PC</td>
<td>Fraction collector</td>
</tr>
</tbody>
</table>

<sup>a</sup>No longer available but still in use.
<sup>b</sup>For these sampling units there is a choice of detectors, including UV-VIS, DAD, fluorescence, conductivity, LIF, and MS. They can also be used for the iCE280 instrument.
<sup>c</sup>Offered with PrintCE or Alcott samplers.
prefabricated running buffers have also become available (Table 19.6). These consumables provide the basis for a widespread applicability of CE technology. Furthermore, set-ups comprising multiple fused-silica capillaries in parallel were designed to increase throughput. Advanced Analytical Technologies (formerly CombiSep, Ames, IA, USA) offers the cePRO 9600, a fully automated, multiplexed 96 capillary instrument with absorbance-based detection for high throughput protein sizing, $pK_a$ and log $P$ determinations, chiral analyses, and many other applications. Multicapillary instruments with fluorescence detection are offered from the same company. In that context, one should also mention the automated, high throughput CZE instruments solely dedicated to serum protein analysis (the Paragon CZE 2000

Clinical Capillary Electrophoresis System of Beckman with seven capillaries and the Capillarys system of Sebia with eight capillaries) and DNA analysis [e.g., systems of Applied Biosystems, GE Healthcare, and Beckman Coulter featuring between 4, 8, 16, 24, 48, 96, 192, or 384 capillaries (34)]. These instruments, together with commercial test kits, are designed for unattended operation and are used in many clinical and forensic laboratories.

CE on miniaturized instrumentation (35–42), that is, CE on microfluidic platforms on which separation channels, sample pretreatment, injection, and detection are combined on an area of a few cm$^2$, also referred to as a miniaturized total chemical analysis systems, represents another development in the manufacturing of CE set-ups. In this approach, fluid flow is driven electrokinetically through a network of intersecting small channels that have been fabricated on planar glass, fused-silica, or plastic substrates by various techniques, including photolithographic masking and chemical etching (Fig. 19.6). The substrates are covered with a plain plate and the microchannels are connected to small vials (for buffer, sample, waste, and so on) that contain the driving electrodes (not shown in Fig. 19.6). Microchannels with a width of 30–70 μm, a height of $\approx 10$ μm, and a length of a few centimeters length are typically used. Microfabricated linear (Fig. 19.6), curved, cyclic, and array structures have been shown to provide rapid separations (as fast as a few seconds and up to the minute time domain) of small and large molecules, compounds that were primarily monitored by on-column LIF detection. Moreover, electrospray ionization interfaces for MS detection have been developed (Fig. 19.6b), and shown to provide an efficient means for solute monitoring and identification. The first commercial chip-based CE instrument, the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany), was introduced in 1999. This lab-on-a-chip instrument comprises assays for automated quality control, sizing, and quantitation of DNA, RNA, and proteins in sieving matrices, and features disposable chips and reagents for sequential analysis of 10–12 samples. All solutes are detected in a single separation channel by LIF. A chip for the analysis of fluorescence parameters of single cells using pressure-driven flow to move the cells in a controlled manner through the microchannels is also available for the 2100 Bioanalyzer. The Experion automated electrophoresis system for protein and RNA analyses (Bio-Rad, Hercules, CA, USA, which features similar chips to the 2100 Bioanalyzer) and the 5100 automated lab-on-a-chip platform for high throughput and high resolution sizing of proteins and DNA (Agilent Technologies) indicate a broader acceptance of (and belief in) chip technology. The same is true for the LabChip 90 system (Caliper Life Sciences, Hopkinton, MA, USA), which performs fast, automated, 1D electrophoretic separations of protein, DNA, and RNA samples directly from a 96- or 384-well plate.

Figure 19.5 Focusing dynamics of four human hemoglobins (HbA, HbF, HbS, HbC, with $pI$ values of 7.0, 7.1, 7.3, and 7.5, respectively) in a pH 3–10 gradient monitored by whole-column imaging at 300 V/cm on the iCE280 instrument. Absorption graphs (280 nm) at the indicated, successive time points in seconds are presented with a y-axis offset of 0.3 AU. Focusing of a hemoglobin control sample was performed in a 100 μm i.d. fluorocarbon coated fused-silica capillary using 3.2% w/v Pharmalyte 3-10 CAs and 0.35% w/v methylcellulose (absence of electroosmosis). From Reference 33, with permission.
<table>
<thead>
<tr>
<th>Product (Supplier)</th>
<th>Capillary Coating</th>
<th>Characteristics</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSP CE capillaries (Polymicro Technologies)</td>
<td>Untreated</td>
<td>Plain capillary</td>
<td>Without detection window ($8–12 per m)</td>
</tr>
<tr>
<td>CE capillaries (Instrument suppliers)</td>
<td>Untreated</td>
<td>Precut capillary</td>
<td>With detection window</td>
</tr>
<tr>
<td>eCAP Neutral (Beckman Coulter)</td>
<td>Linear polyacrylamide</td>
<td>Low surface charge</td>
<td>Protein analysis</td>
</tr>
<tr>
<td>eCAP N-CHO (Beckman Coulter)</td>
<td>Polyvinyl alcohol</td>
<td>Low surface charge</td>
<td>Protein analysis, not recommended with borate buffers</td>
</tr>
<tr>
<td>eCAP Amine (Beckman Coulter)</td>
<td>Dynamic coating with amine polymer</td>
<td>Reversed electroosmosis</td>
<td>Must be used with amine regenerator solution</td>
</tr>
<tr>
<td>BioCAP LPA-coated (Bio-Rad)</td>
<td>Linear polyacrylamide</td>
<td>Low surface charge</td>
<td>General protein analysis, CIEF</td>
</tr>
<tr>
<td>BioCAP XL-coated (Bio-Rad)</td>
<td>N-acryloylaminoethoxyethanol (AAEE)</td>
<td>Low surface charge</td>
<td>General protein analysis, CIEF</td>
</tr>
<tr>
<td>PVA-coated capillary (Agilent Technologies)</td>
<td>Polyvinyl alcohol</td>
<td>No electroosmosis</td>
<td>Protein analysis, CIEF</td>
</tr>
<tr>
<td>Guarant (Alcor BioSeparations)</td>
<td>Polysaccharide</td>
<td>Very low electroosmosis</td>
<td>Stable at pH 2–10, low analyte adsorption, borate buffer compatible</td>
</tr>
<tr>
<td>CelerityCE C8/C18 (MicroSolv Technology)</td>
<td>Bonded C8/C18 phases</td>
<td>Reduced anionic surface charge</td>
<td>Protein analysis</td>
</tr>
<tr>
<td>CelerityCE Diol (MicroSolv Technology)</td>
<td>Diol phase</td>
<td>Reduced anionic surface charge</td>
<td>Protein analysis</td>
</tr>
<tr>
<td>CelerityCE Cholesterol (MicroSolv Technology)</td>
<td>Cholesterol (liquid christol) phase</td>
<td>Reduced anionic surface charge</td>
<td>Protein analysis</td>
</tr>
<tr>
<td>CelerityCE Butyl Phenyl (MicroSolv Technology)</td>
<td>Butyl phenyl phase</td>
<td>Reduced anionic surface charge</td>
<td>Protein analysis</td>
</tr>
<tr>
<td>μSIL-FC and μSIL-DNA (J&amp;W Scientific or Agilent Technologies)</td>
<td>Cross-linked and bonded fluorocarbon (FC) polymer</td>
<td>Hydrophobic surface with low surface charge</td>
<td>CIEF, CGE, protein analysis</td>
</tr>
<tr>
<td>μSIL-WAX (J&amp;W Scientific or Agilent Technologies)</td>
<td>Polyethylene oxide</td>
<td>Hydrophilic surface with low surface charge, high stability</td>
<td>Protein analyses, CE-MS</td>
</tr>
<tr>
<td>μSIL DB-1 (J&amp;W Scientific)</td>
<td>Dimethyl polysiloxane (DB-1)</td>
<td>No electroosmosis</td>
<td>CIEF</td>
</tr>
<tr>
<td>μSIL DB-17 (J&amp;W Scientific)</td>
<td>(50%-phenyl)-methyl polysiloxane (DB-17)</td>
<td>No electroosmosis</td>
<td>Protein analysis, CITP</td>
</tr>
<tr>
<td>EKT NaAMPS (Electro-Kinetic Technologies, Capital-HPLC Limited)</td>
<td>Polymer with sulphonic acid groups</td>
<td>pH independent negatively charged</td>
<td>Cathodic electroosmosis (low, medium, or high)</td>
</tr>
<tr>
<td>EKT Zero Flow (Electro-Kinetic Technologies, Capital-HPLC Limited)</td>
<td>Polyacrylamide</td>
<td>No electroosmosis</td>
<td>CIEF, protein analysis</td>
</tr>
<tr>
<td>EKT Anodic Flow (Electro-Kinetic Technologies, Capital-HPLC Limited)</td>
<td>Polymer with quaternary amine groups</td>
<td>Positively charged</td>
<td>High anodic electroosmosis, protein analysis</td>
</tr>
</tbody>
</table>

*Capillaries can be mounted into most instruments. Bio-Rad also offers preassembled, disposable capillary cartridges that can only be employed with their instruments.

*In combination with dynamically adsorbed surfactants (e.g., Brij-35) as described by Towns and Regnier (71).
TABLE 19.6 Selected Commercial Capillary Electrophoresis Kits for Protein Analysis

<table>
<thead>
<tr>
<th>Product (Supplier)</th>
<th>Capillary Coating</th>
<th>Principle</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE-SDS protein kit</td>
<td>Untreated</td>
<td>Dynamic sieving hydrophilic polymer</td>
<td>Size separations for 14- to 200-kDa proteins</td>
</tr>
<tr>
<td>(Bio-Rad)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eCAP SDS 14-200 kit</td>
<td>Untreated capillary</td>
<td>Dynamic sieving hydrophilic polymer</td>
<td>Size separations for 14- to 200-kDa proteins</td>
</tr>
<tr>
<td>(Beckman Coulter)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eCAP CIEF 3-10 kit</td>
<td>eCAP neutral capillary</td>
<td>No electroosmosis</td>
<td>CIEF</td>
</tr>
<tr>
<td>(Beckman Coulter)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEOFIX CDT kit (Analis)</td>
<td>Ionic polymer-based dynamic double coating</td>
<td>Strong cathodic EO</td>
<td>CDT in serum</td>
</tr>
<tr>
<td>CEOFIX HbA1c kit (Analis)</td>
<td>Ionic polymer-based dynamic double coating</td>
<td>Strong cathodic EO</td>
<td>HbA1c</td>
</tr>
<tr>
<td>CEOFIX HbA2 kit (Analis)</td>
<td>Ionic polymer-based dynamic double coating</td>
<td>Strong cathodic EO</td>
<td>HbA2 and other Hb variants</td>
</tr>
<tr>
<td>CEOFIX HRE kit (Analis)</td>
<td>Ionic polymer-based dynamic double coating</td>
<td>Strong cathodic EO</td>
<td>Serum protein analysis</td>
</tr>
</tbody>
</table>

*Kits typically include complete protocols, buffers, standards, and capillaries. Kits solely dedicated to specific instruments are not included. EO, electroosmosis.

Figure 19.6 Polymeric microfluidic chip fabricated from Zeonor with a straight, open-end separation channel showing (a) the design and dimensions and (b) the overall configuration with the microliquid junction for electrospray ionization MS. Reprinted from Reference 43, with permission. Copyright, American Chemical Society. Chips with on-column LIF detection do not have a separation channel with an open end, but feature a waste reservoir with a driving electrode at its end.
19.3 CAPILLARY ELECTROPHORESIS OF PROTEINS

Proteins are macromolecules composed of a large number of amino acids, and in the case of glycoproteins, carbohydrates attached to selected amino acids of the polypeptide chain. Proteins may possess hundreds of ionizing groups and are amphoteric. Thus, depending on pH, proteins exhibit positive, negative, or no charge. Isoelectric points, titration curves, and electrophoretic mobilities of proteins are most accurately determined experimentally. Reasonable approximations can, however, be obtained by calculation using the Henderson–Hasselbalch equation if the amino-acid composition is known (44). This approach assumes that any specific ionizing group has the same $pK_a$ value everywhere on the molecule. Calculated titration curves of four proteins, cytochrome $c$ (CYTC), ovalbumin (OVA), $\beta$-lactoglobulin B (BLB), and ribonuclease A (RNase), are presented in Figure 19.7. According to these data, it is evident that proteins may have a much higher charge than small molecular mass compounds such as those used to generate the data presented in Figure 19.4c for which, at pH 7.79, the estimated charge values are between $\pm 1$ (Table 19.3). The value for Tyr–Lys could become as high as $\pm 2$ at the two pH extremes, whereas the value for Trp–Glu could reach $-2$ at very alkaline pH. All other compounds cannot have charge numbers $> |\pm 1|$. It is anticipated that the proteins of Figure 19.7 should be easily separable by CIEF and, by proper selection of the pH value of the running buffer, also by CZE and CITP. Furthermore, the diffusion coefficient of a protein is small, which favors the realization of high plate numbers in CE. The highly charged proteins (particularly positively charged proteins in negatively charged fused-silica), however, can unfavorably interact with the capillary walls, a process that causes band broadening or, if strongly adsorbed, prevents their analysis by CE. Moreover, interactions based upon hydrophobicity and hydrophilicity are also possible. Thus, for a successful choice of suitable CE conditions for the sample protein(s), that is, the interplay of capillary size, material, inner capillary wall surface, and electrophoretic buffer, this basic understanding of chemical properties of proteins has always to be kept in mind. Furthermore, the intra-capillary temperature, that is, the temperature of the fluid under current flow, should be kept low enough such that proteins remain intact for separation and detection. Owing to Joule heating, the intracapillary temperature is typically higher than the temperature setting of the instrument. For a given power level $P$ in W/m, the intracapillary temperature $T_i$ can be estimated according to $T_i = T_0 + bP$ where $T_0$ and $b$ are the temperature controlled by the instrument and a coefficient that is dependent on the type of temperature control employed ($b = 14$ Km/W for air cooling and 7 Km/W for liquid cooling) (45). For example, for the data presented in Figure 19.4c, which were generated at room temperature with $P = 1.71$ W/m, the intracapillary temperature $T_i$ is $\sim 24^\circ$ above room temperature. CE of proteins was discussed in the previous editions of this book (8, 9), in a number of review articles (46–51), and a book solely dedicated to CE of proteins and peptides (52). Clinical applications of protein CE, including the analysis of hemoglobin variants and serum proteins, are described in sections of reviews (53, 54) and books (55, 56).

19.3.1 Capillary Electrophoresis in Polymer Capillaries and Microchannels

Compared to fused-silica and glass, polymer or plastic capillaries may offer a somewhat higher hydrophobicity and a lower and different origin of surface charge. Conversely, polymer capillaries suffer from several disadvantages, including lower heat dissipation, decreased optical transparency at low wavelengths, and the difficulty in manufacturing tubes having constant i.d. smaller than $\sim 100 \mu$m. On the other hand, fabrication of plastic microchannels is straightforward and is being explored in many laboratories. With the exception of dynamic coating with surfactants and other reagents, modification of wall surface properties of polymers may be difficult and tedious. It is, however, often required, particularly to provide desired surface properties that avoid or induce special affinities.

Narrow-bore polytetrafluoroethylene (PTFE, Teflon) and fluorinated ethylene-propylene polymer (FEP) tubes of 0.2–0.8 mm i.d. have been employed successfully for high resolution CITP analyses of proteins (2, 3, 57). For CZE (8, 58) and CIEF (8, 59), however, they have been explored much less. Electropherograms obtained with model proteins are presented in Figure 19.8. They were monitored with the Tachophor 2127 capillary analyzer (LKB, Bromma, Sweden), featuring both on-line conductivity and absorbance.

Figure 19.7 Calculated charge versus pH relationships of cytochrome $c$ (CYTC), ovalbumin (OVA), ribonuclease A (RNase), and $\beta$-lactoglobulin B (BLB). The data were generated according to the procedure given in Reference 44.
detection at the end of a 0.5 mm i.d. PTFE column. Electropherograms of a cationic CZE separation of equine CYTC (1), bovine carbonic anhydrase (2), and RNase (3) in 10 mM HCl and tris(hydroxymethyl)aminomethane (Tris) buffer at pH 3.50 are shown in Figure 19.8a. Without the addition of a fluid stabilizing agent, no complete separation of the three proteins is achieved with a column length of 25 cm, whereas the separation is markedly improved in the presence of 1% hydroxypropylmethyl cellulose (HPMC). It is interesting to note that the cationic protein peaks are sharper than those obtained in the same capillary at alkaline pH [anionic separation, data presented elsewhere (8)]. However, plate heights smaller than \(\sim 0.1 \text{ mm} \) are rarely observed. Figure 19.8b depicts electropherograms obtained for the CIEF separation of equine CYTC (1, 0.18 mg/mL), bovine RNase (2, 0.36 mg/mL), and canine CYTC (3, 0.08 mg/mL) in pH 3.5–10 Ampholine (1%) with 8 mM arginine. Focusing (F) was performed at a constant voltage of 2000 V and cathodic mobilization (E) with the catholyte replaced by the anolyte occurred at a constant current of 100 \(\mu\text{A}\). Absorption measurements were made at 277 nm in all cases. The conductivity data (dashed lines for panels a and b) are expressed as increasing resistance R.

Figure 19.8 CE data obtained in a 0.5 mm i.d. Teflon capillary. (a) Cationic CZE separation of CYTC (1), carbonic anhydrase (2), and RNase (3) at pH 3.50. 0.5 \(\mu\text{L}\) sample with protein quantities of 1 \(\mu\text{g}\) (1), 2.5 \(\mu\text{g}\) (2), and 6.5 \(\mu\text{g}\) (3) was injected and the run was executed at a constant 300 \(\mu\text{A}\). (b) CIEF separation of CYTC (1, 0.18 mg/mL), RNase (2, 0.36 mg/mL), and Hb (3, 0.08 mg/mL) in pH 3.5–10 Ampholine (1%) with 8 mM arginine. Focusing (F) was performed at a constant voltage of 2000 V and cathodic mobilization (E) with the catholyte replaced by the anolyte occurred at a constant current of 100 \(\mu\text{A}\). (c) Cationic CITP data showing the steady-state distribution of RNase (1), BLB (3), and OVA (5) in the absence (left panel) and presence (right panel) of tetratpentylammonium (2), \(\gamma\)-aminobutyric acid (4), and Tris (7) as low molecular mass spacers. Zone 6 and Na\(^+\) represent impurities. L and T refer to leader and terminator, respectively. A constant 150 \(\mu\text{A}\) was applied. While recording the CITP data, the current was reduced to 50 \(\mu\text{A}\). Absorption measurements were made at 277 nm in all cases. The conductivity data (dashed lines for panels a and b) are expressed as increasing resistance R.
hemoglobin (Hb) (3.08 mg/mL) in pH 3.5–10 Ampholine (1% w/v) with 8 mM arginine. A 10-cm column was used together with conditions outlined elsewhere (59). Finally, the cationic CITP data of bovine RNase, BLB, and OVA depicted in Figure 19.8c were obtained in a capillary with a length of 28 cm and using 0.01 M potassium acetate and acetic acid (pH 4.75) as the leader and 0.01 M acetic acid as the terminator. The impact of low molecular mass spacers on the separation pattern is also shown. For CITP, no buffer additive was used. For CZE and CIEF, the impact of the addition of other polymers, such as polyvinyl alcohol (PVA), linear (noncrosslinked) polyacrylamide (LPA), and polyethylene glycol (PEG), and solubilizing agents such as urea and CHAPS, to the buffers was also explored. The use of 1% HPMC, however, was found to produce the best results for these proteins. Despite obvious shortcomings, the Tachophor 2127 can effectively be employed for the investigation of buffer systems for preparative electrophoretic separations and for the validation of theoretical predictions (44).

It is evident that the performance of the 0.5 mm i.d. Teflon capillary for CZE and CIEF of proteins is characterized by a rather low efficiency, long analysis times, and by the need for a viscosity enhancing/electroosmosis diminishing agent. Plastic capillaries with smaller i.d. provide increased resolution and separation speed, as was shown for CZE of low molecular weight compounds in PTFE capillaries with 0.2 mm i.d. (60). Much improved efficiency was demonstrated using a 56 μm i.d. (350 μm o.d.) polypropylene capillary produced by meltspinning (61). For chymotrypsinogen A, a plate height of ~5 μm was observed when the hydrophobic capillary was dynamically coated with polyethoxylated (n = 23) lauryl alcohol. This performance is similar to that obtained in well conditioned fused-silica capillaries of comparable physical dimensions (Fig. 19.9) and could be confirmed in other laboratories with polypropylene capillaries modified with polyacrylamide (62), cellulose (63), dextran (64), and polymethylmethacrylate (PMMA, Plexiglas) (65). Bayer and Engelhardt investigated the use of polybutylene terephthalate, ethylene/vinylacetate, PMMA, and nylon capillaries with and without surface modification (66). Chen and Lee further exploited the use of unmodified PMMA hollow fibers for protein analyses (67). Furthermore, there

**Figure 19.9** CZE protein data in fused-silica capillaries. (a) CZE data at pH 7 of CYTC in uncoated (left panel; fourth injection, for injections 1 to 3 no peak could be recorded) and C-18/Brij-35 coated (right panel; first injection) 75 μm i.d. capillary of 85 cm effective length. The applied voltage (current) was 30 kV (30 μA). Reprinted from Reference 71, with permission. Copyright, American Chemical Society. (b) CZE data of model anionic proteins at pH 9.5 using vinyl-bound polyacrylamide coated (left panel, suppressed electroosmosis) and uncoated (right panel, strong electroosmosis towards cathode) 50 μm i.d. capillaries of 45 cm effective length. The applied voltages (currents) were ~20 kV (~15 μA) and 10 kV (7 μA), respectively. Note the reversed order of detection of the proteins. 1, insulin chain A (porcine); 2, bovine serum albumin; 3, OVA; 4, porcine insulin; 5, bovine α-lactalbumin; 6, bovine casein; 7, insulin chain B (porcine). From Reference 68, with permission.
is currently a great deal of interest in the manufacturing of inexpensive plastic chips containing microchannel networks, including those made from PMMA, poly(dimethylsiloxane) (PDMS), Zeonor (Fig. 19.6), flexible polypropylene, and other plastic materials (40, 42).

19.3.2 Capillary Electrophoresis in Fused-Silica and Glass Columns

A very large number of studies have been (and many more still are) conducted in fused-silica capillaries, which permit effective heat removal and fabrication of capillaries with very small inner diameters (10–100 μm). Similarly, microchannels etched into glass and fused-silica are used in many chip designs. In these devices, difficulties may arise when proteins are analyzed, because these macromolecules can interact unfavorably with the surface of the column walls (68–70). The inner walls of fused-silica and glass are typically negatively charged due to the dissociation of silanol groups (Fig. 19.4). Thus, positively charged sites of a protein interact electrostatically with the capillary surface, this causing peak broadening (Fig. 19.9a) and possibly also a complete loss of the polypeptide due to adsorption (71). A number of strategies to minimize or prevent protein–wall interactions have been developed (for reviews refer to Refs 68–70). First, adjusting the pH of the buffer above the isoelectric point of the proteins (where they possess a net negative charge) results in coulombic repulsion. Protein–wall interactions are thereby strongly reduced and efficient separations can be achieved in uncoated capillaries (Fig. 19.9b, right electropherogram, obtained with normal polarity). Also, coulombic attraction between proteins and fused-silica can be reduced by working at very low pH, where silanol groups are undissociated, by having a high concentration of salts in the buffer, and by using various types of buffer additives, which either provide a dynamic coating on the capillary wall, by interacting with charged groups of the proteins or by inducing a conformational change in the protein structure. Examples of additives include nonionic surfactants, denaturants, polymeric polycations, as well as zwitterionic, polycationic, and polyanionic salts. The latter compounds are believed to form ion-pairs with the positively charged silanol groups (Fig. 19.4). Thus, positively charged sites of a protein can interact unfavorably with the surface of the column walls during wash cycles or during the run when added to the buffer (70). Nonionic adsorbed agents mask the surface charges, thereby reducing or eliminating electroosmosis as well as protein–wall interactions. Adsorption of polycations may drastically alter the surface properties and can even reverse the surface charge. Such a procedure was commercialized in a kit format (MicroCoat of Applied Biosystems) but is no longer available. Effective ionic polymer reagents for that task are offered by Analis (Namur, Belgium) under the trade name C Eföix, MicroSolv (Eatontown, NJ, USA) under CElixir and Target Discovery (Palo Alto, CA, USA) under EOTrol and UltraTrol. Alternatively, ionic polymer-based dynamic multicoatings have been described. In this approach, the fused-silica is first rinsed with an initiator buffer containing a polymeric polycation that is adsorbed to the wall surface followed by introduction of a buffer containing a polymeric polyanion that is forming a second layer and thus providing the negative charge for a strong EOF towards the cathode. The bilayers formed by noncovalent adsorption of ionic polymers were produced from Polybrene/dextran sulfate (73, 74), poly( diallyldimethylammonium)/poly (styrene sulfonate) (75) and Polybrene/poly(vinylsulfonate) (76). The EOF in these systems was determined to be essentially independent of pH and highly reproducible. Stable cationic capillary coatings with successive multiple ionic polymer layers can also be formed (74, 75). Reagents for a double coating are commercially available from Analis (Table 19.6), for high resolution analysis of transferrin isoforms in human serum at alkaline pH and thus the determination of carbohydrate-deficient transferrin (CDT) as marker for excessive, chronic alcohol intake (CEofix-CDT reagents) (77, 78), for analysis of hemoglobin variants (CEofix-HbA2 and -HbA1c kits, for an example refer to Reference 54), and for analysis of serum proteins (CEofix-HRE kit). Typical data obtained for CDT analysis are presented in Figure 19.10. For that analysis, a small amount (~10 nL) of an equivolume mixture of human serum and an Fe(III) solution (typically 60 μL each) is injected and electrophoresed for ~12 min. Transferrin isoforms differing in isoelectric points between 5.9 (asialo-transferrin) and 5.2 (hexasialo-transferrin) are thereby quantitatively determined without elaborate sample pretreatment. The double coating was identified to be crucial for the robustness and precision of the assay (77). Relative standard deviation (RSD) values for detection times of transferrin isoforms were found to be much smaller than 1%. Corresponding data for peak areas are peak size dependent, the RSD values being <3.0% and <1.5% for area values of 1.0% and 4.5% of total transferrin, respectively (78). The excellent performance of this assay permits its use in clinical and forensic applications in a routine environment. Dynamic capillary coating is a simple approach with the advantage that the capillary surface is regenerated for each run. Also, using PVA, it has been shown that for protein analysis dynamic coatings can be as effective as permanent capillary coatings (79).

An enormous amount of work has been devoted to developing many different coatings formed by polymers covalently attached to the silanol groups of fused-silica...
Hydrophilic polymers, including those based upon polyacrylamide (Fig. 19.9b, left electropherogram, obtained with reversed polarity in a capillary with suppressed electroosmosis), polyoxyethylene, PVA, and cellulose derivatives, have mainly been used. Compared to the use of an untreated fused-silica capillary and otherwise identical conditions, the use of the polyacrylamide coating results in higher resolution of the seven proteins (Fig. 19.9b). In addition, the use of dynamic coatings on chemically modified capillary walls (particularly on C18 hydrophobically coated capillaries, Fig. 19.9a) was reported to be effective and versatile (71). The advantage of all these approaches is that a large variety of surface properties can be achieved. This is important, because proteins are a heterogeneous class of compounds with widely differing characteristics. Unfortunately, many of the coatings developed thus far are not sufficiently stable over a wide pH range and are difficult to prepare with a high batch-to-batch reproducibility, this hampering somewhat the widespread use of coated capillaries. Nevertheless, a number of companies are offering coated capillaries for protein analysis (Table 19.5). Furthermore, fluid-impervious poly(styrene-divinylbenzene) tubing inside a fused-silica capillary (80) and a Plexiglas capillary coating bonded on a fused-silica capillary (81) have been reported to provide stable systems for protein analyses in which the CE medium is kept away from the fused-silica wall, approaches that have great similarity to the use of polymeric capillaries (see Section 19.3.1). Buffer selection can also be crucial for protein separation. Systems with improved resolution are obtained with buffers of low conductivity. CZE of proteins in isoelectric buffers of very low conductivity, such as those produced by aspartic or imino diacetic acid, have been shown to permit rapid, high resolution separations of peptides and proteins in uncoated fused-silica capillaries. This topic is discussed in Chapter 16 (82).

19.3.3 Capillary Electrophoresis in the Presence of Sieving Media

One of the main analytical applications of slab gel electrophoresis is the determination of the molecular mass of proteins using SDS and a gel formed by crosslinked polyacrylamide (SDS-PAGE). In this process proteins form complexes with dodecyl sulfate to yield molecules of constant surface charge density and thus equal electrophoretic mobility. Separation is based on size or molecular mass differences via sieving through the gel matrix. CGE has been introduced by direct adaptation of SDS-PAGE to the capillary format, using crosslinked polyacrylamide matrices covalently attached to the capillary inner wall. Excellent resolution has been achieved (83). However, the gel matrix prevents protein detection at low wavelengths. Furthermore, the production of the gel-filled capillaries turned out to be difficult and their lifetime was limited to a few experiments owing to bubble formation and deterioration of the gel structure, particularly at high electric field strengths. Therefore, capillaries filled with crosslinked polyacrylamide are rarely used for protein analysis.

Alternate processes were studied, employing replaceable (84, 85) or in situ formed (86) gels of LPA, which is not
bonded to the capillary walls. The advantage of introducing the sieving matrix after its formation is that the capillary can easily be emptied and refilled, this leading to flexible and simple operation. Although this is possible using LPA, its relatively low UV transparency prevents sensitive protein detection at low wavelengths. Thus, Ganzler and colleagues explored the use of dextran and PEG as replaceable sieving matrices and reported successful molecular mass sieving of dodecyl sulfate–protein complexes and detection at 214 nm in these media (85, 86). Subsequently, several companies developed CE kits containing hydrophilic polymers to permit dynamic sieving in which dodecyl sulfate–protein complexes separate in a size-dependent fashion with a linear correlation over the entire range (Fig. 19.11; Table 19.6). Molecular masses of proteins of pharmaceutical interest determined by replaceable SDS polymer-filled CE were found to be close to those obtained by conventional SDS-PAGE (87).

Protein sizing on a microchip using sieving matrices was described by Yao (88), Bousse (89) and colleagues. The chip used in the latter work was developed for a commercial instrument, the Agilent 2100 Bioanalyzer, and features a single separation channel and 11 sample wells for fast

Figure 19.11  Dynamic sieving of SDS protein complexes in the presence of a replaceable hydrophilic polymer (CE-SDS Protein Kit on BioFocus 3000 with 50 μm i.d. capillary of 24 cm length and detection effected at 220 nm), showing (a) the separation of protein standards, (b) the corresponding molecular weight calibration graph, and (c) the response linearity for carbonic anhydrase. Courtesy of Bio-Rad, Hercules, CA, with permission.
sequential analysis (Fig. 19.12a). In this protocol, a staining dye is added directly to the sieving matrix and SDS–protein complexes are labeled on-chip in less than 100 ms after entry into the separation column. To minimize background fluorescence, a post-column electrokinetic SDS dilution step is included on-chip (Fig. 19.12b). The side channels are filled with sieving matrix, but do not contain dye or SDS. Continuous electrokinetic addition of buffer from these channels results in the effluent from the separation column being focused into a thin stream down the middle of the main channel, and SDS diffuses from the middle to the sides and thereby becomes diluted. An optimum value for the dilution ratio was found to be ~9 (Fig. 19.12c). Sizing accuracy is claimed to be better than 5%. Agilent currently offers two protein LabChip Kits for the 2100 Bioanalyzer and its biosizing software, one for protein analysis in the size range of 5 and 50 kDa and one for proteins up to 200 kDa. Automated protein analysis is thereby accomplished easily. Chips are rapidly prepared, the electrophoretic run time per sample is less than 1 min (Fig. 19.12c), and the results are quickly evaluated. Only 4 µL of material is required and the total time interval for analysis of 11 samples is about 30 min. A similar

![Figure 19.12](image)

(a) Chip design for protein sizing, with wells shown in light gray. Well D4 is filled with buffer not containing any SDS or label. It is connected by two channels to the three-pronged Y-shaped dilution intersection. Wells A4 and C4 contain separation buffer, and B4 and D3 are used as load wells. The rest of the reservoirs are available for samples. (b) Images of the SDS dilution process at the three-pronged Y-shaped dilution intersection. The left image shows a dilution ratio of 4, and the right a ratio of 12. The SDS is diluted to below its critical micelle concentration in the right image. (c) Electropherograms for a series of separations of a Bio-Rad protein ladder at dilution ratios of 1–14. Total protein concentration: 0.4 mg/mL for all eight fragments. Reprinted from Reference 89, with permission. Copyright, American Chemical Society.
approach for 10–260 kDa proteins is offered by Bio-Rad on
the Experion system (Experion Pro260 chip). The use of this
kind of instrumentation has become quite popular in recent
years. This is demonstrated with the monitoring of the pro-
duct quality attributes of monoclonal antibodies using the
LabChip 90 platform, which features a separation channel
with a length of 1.4 cm and a width of 31 μm, filled with a
polydimethylmethacrylate polymer solution in a Tris–
Tricine buffer containing a noncovalent staining dye and
SDS (90). Under reducing conditions, this microchip
CE-SDS separation was found to be similar to that of con-
ventional CE-SDS, providing reasonable resolution of the
nonglycosylated and glycosylated heavy chains. On-chip flu-
orescence detection with noncovalent fluorescent labeling
was about as sensitive as the 220 nm UV absorbance detec-
tion in a conventional CE apparatus. Analyses were ~70
times faster using the microchip format.

19.3.4 Capillary Isoelectric Focusing

Because of the extremely high resolving power (ΔpI of 0.005
pH units), IEF is a popular method for protein characteriza-
tion. It is typically conducted in slab gels, an approach that
allows the processing of 10–20 samples in parallel lanes and
which requires protein fixation and visualization pro-
cedures after focusing (see Chapter 16). In the past two de-
ades, attention has been focused on developing IEF into a
more instrumental format by using gel-free capillaries as
focusing columns. Focusing can be conducted in stationary
or flowing solution. CIEF in quiescent solution requires cap-
illaries that do not exhibit any electroosmosis under current
flow. For solute detection, either an array or scan detector
has to be employed (30, 91) (for an example see Fig. 19.5),
else the sample pattern must be mobilized during or fol-
lowing focusing (92–95) (for an example see Fig. 19.8b).
Mobilization is achieved by pumping the entire IEF zone
structure past a stationary monitor with the voltage applied
to avoid a decay of the pattern (mobilization by pressure,
vacuum, or gravity) or by electrophoretic means. Electrophor-
etic mobilization towards the anode is accomplished by repla-
cing the acid at the anode by a base or by putting salt into the
acidic electrode buffer and reapplication of power. Conver-
sely, mobilization towards the cathode is effected by repla-
cing the base at the cathode by an acid or a solution of the
base containing salt (Fig. 19.8b). Focusing followed by elec-
trophoretic mobilization can also be achieved in a one-
step process via direct use of a catholyte containing a salt of
an acid (cathodic displacement) (95) or an anolyte contain-
ing the salt of a base (anodic displacement). In all these CIEF
approaches, proteins are detected as they pass the on-column
optical (absorbance or fluorescence) detector, which is
mounted towards one column end, or as they are swept into
a detector placed at the capillary end (MS detection). CIEF
protocols comprising mobilization by application of flow
have been commercialized by ABI and Beckman Coulter,
whereas electrophoretic (or chemical) mobilization was
promoted by Bio-Rad using their LPA and N-acryloylami-
noethoxyethanol (AAEE) coated capillaries (Table 19.5).
Recently, Mack and colleagues identified the critical pa-
rameters of two-step CIEF in a systematic study and came
up with an optimized, comprehensive protocol that provides
reproducibility and robustness (96). The method comprises
a neutral, LPA-coated fused-silica capillary, the use of imino-
diacetic acid as anodic and L-arginine as cathodic spacer com-
pound, urea in a gel composed of a low percentage mixture of
ethylene glycol and polyethylene oxide in water to optimize
protein solubility, 200 mM phosphoric acid and 300 mM
NaOH as anolyte and catholyte, respectively, and electro-
phoretic mobilization towards the cathode using 350 mM
acetic acid as catholyte. This approach was shown to provide
better resolution compared to pressure mobilization and was
used to characterize the charge heterogeneity of monoclonal
antibodies.

In CIEF performed in the presence of an EOF (97, 98),
mobilization of focused zones is unnecessary because these
zones are displaced towards and across the point of detection
by the EOF. Using untreated fused-silica capillaries and par-
tial filling of the capillary with sample (98), a typical exper-
iment proceeds as follows. First, the entire capillary is filled
with the catholyte containing a small amount of a neutral
polymer, for example, HPMC, as capillary conditioner.
Sample composed of CAs and test compounds is introduced
at the anodic end and occupies ~35–50% of the effective
capillary length. After power application, the formation of
the pH gradient, separation of the sample compounds, and
displacement of the entire ampholyte pattern towards the
cathode occur simultaneously. Basic ampholytes and test
substances reach the point of detection before neutral and
acidic ones. This technique was found to be an attractive
and simple method for analysis of normal and pathological
hemoglobin (99). Examples are presented in Figure 19.13.
In the process described by Mazzeo and Krull (97), the
entire capillary is filled with the sample.

To prevent the focusing of basic proteins between detector
and cathodic capillary end, a fair amount of a strong base
(such as TEMED) has to be added to the sample. This
approach has the advantage of producing a shallower pH gra-
dient and thereby provides better resolution. Electroosmo-
tic zone displacement for acidic proteins may be insufficient.
This is particularly the case for analyte detection at the capi-
llary end, for example, for hyphenation of CIEF with MS.
Computer simulations revealed that EOF decreases with
time in columns with a pH-dependent surface charge and
that the entire IEF process is characterized by the asymptotic
formation of a stationary steady-state zone configuration in
which electrophoretic transport and electroosmotic zone
displacement are opposite and of equal magnitude (100).
Additional mobilization for the detection of the entire pH
gradient at the capillary end is required. Using concomitant electrophoretic mobilization with an acid as coanion in the catholyte could be shown to provide sufficient additional cathodic transport for that purpose (100). Furthermore, it should be stated that all CIEF approaches with electrosmotic zone displacement are less reproducible than those based upon mobilization with imposed flow or electrophoretic mobilization. Protein adsorption onto the capillary wall is believed to cause changes in surface charge and thus electroosmosis.

Applications of CIEF range from proteins with clinical relevance to recombinant proteins (101). CIEF has successfully been coupled to MS (102), and researchers are currently trying to implement it on chips. It is not only a high resolution technique employed in research laboratories. In routine settings, it is used for hemoglobin analysis (103, 104) and for protein characterization using commercial CIEF kits. The analysis of charge heterogeneities in monoclonal antibodies is currently the most important application. This is performed in the biotechnology and pharmaceutical industry using the iCE280 instrument, which features whole-column imaging (105) or the PA800 with an optimized two-step CIEF procedure featuring electrophoretic mobilization (96).

19.3.5 Capillary Isotachophoresis

In ITP of proteins, zones of rather high concentration (typically of the order of 10–20 mg/mL provided that there is
enough material present) (106) are formed and proteins are vulnerable to interactions with the column walls. Not surprisingly, experiments with proteins in untreated fused-silica capillaries were found to be largely unreproducible, whereas much improved data were obtained in capillaries coated with LPA (14, 106, 107) or with a bonded and crosslinked film of DB-17 (108). Dynamic coatings with additives comprising neutral macromolecules, such as HPMC, were demonstrated to largely reduce but not completely abolish protein interactions with fused-silica (109). Such an effect is clearly demonstrated with the example shown in Figure 19.14, in which the analysis of the same sample in a Teflon capillary (Tachophor, Fig. 19.14a) and an untreated fused-silica capillary (laboratory-made instrument, Fig. 19.14b) is depicted. For these experiments, the same cationic electrolyte system as for Figure 19.8c was employed. The sample was composed of two proteins, lysozyme (LYSO) and conalbumin (CAL), and creatinine (CREAT) as spacer. In the Tachophor, analysis of a 1 µL aliquot provided plateau-shaped zones for both proteins (upper graph in Fig. 19.14a) and the spacer. In the fused-silica capillary with HPMC (0.3%) conditioning (dynamic coating) LYSO and CREAT could be nicely detected, whereas CAL did not produce the expected ITP zone between CREAT and the terminator (marked with an arrow in the lower graph of Fig. 19.14b), nor could it be monitored as a migrating zone within the leader or terminator. However, much higher concentrations of CAL have been shown to produce proper ITP zones (109). Thus the small amount of CAL in the investigated sample is presumably adsorbed onto the capillary wall and lost for the analysis. LYSO appears to have a much lower or no affinity to the column material and can nicely be analyzed under the investigated conditions. Application of this technique, however, appears to be protein-specific, a restriction that has to be kept in mind. Nevertheless, LPA-coated (107) and untreated capillaries dynamically coated with HPMC (110) have been successfully employed to fractionate serum proteins by ITP via use of spacers.

Figure 19.14  Cationic CITP data of a model mixture comprising two proteins (LYSO and CAL, about 1 mg/mL each) and a low molecular mass spacer (CREAT) obtained (a) in a 0.5 mm i.d. Teflon capillary of 28 cm length (Tachophor; applied constant current, 150 µA) and (b) in a 75 µm i.d. fused-silica capillary (70 cm effective length) with on-column multiwavelength detection. In panel (a), the UV absorption data (277 nm, upper graph) and the conductivity data (lower graph) are presented. Panel (b) comprises UV absorbance data from 195 to 320 nm (5 nm interval, upper graph) and for 280 nm (lower graph) at different time and absorbance scales. Sample injection was effected by gravity (height differential, 34 cm; time interval, 60 sec), the applied voltage was a constant 20 kV, and the current decreased from 12 to 4 µA within the first 16 min.
CITP of proteins in Teflon capillaries has been used in many laboratories for some time (57). The same technique in fused-silica capillary and chip instrumentation has not been studied much. On the other hand, CITP is used to pre-concentrate (sharpen) protein zones prior to their separation by CZE or CE–MS (15, 111, 112).

### 19.3.6 Affinity Capillary Electrophoresis and Immunoassays

Affinity capillary electrophoresis (ACE) represents a combination of affinity (immuno) chemistry and CE. The chemical basis underlying ACE is a biospecific interaction between an affinity probe (ligand) and an analyte, including an antibody or a FAB’ fragment of an antibody reacting with an antigen or a hapten, a lectin reacting with a glycoprotein or an oligosaccharide, and an enzyme interacting with a substrate. Traditionally, affinity electrophoresis was defined as electrophoresis of molecules interacting during the electrophoretic process. Typically, this was achieved by incorporating one of the interacting components into a medium through which the other interacting compound was electrophoresed. Here, ACE is seen in a broader sense, also including assays in which an incubation of the reactants occurs prior to CE, such as in immunoassays (see below). ACE is mainly used as a microscale analytical procedure or for the study of complex formation between analyte and affinity probe. In the latter case, a series of experiments is performed in which one of the reactants is applied as a small sample plug and the other is supplied in different quantities as additive to the running buffer. Sample retardation as a function of the reactant present in the buffer is employed to characterize complexation of the two reactants. ACE is used to estimate quantitative binding data (binding constants, binding stoichiometries, and rate constants), which are important for the understanding of biology. A more detailed description of CE with such molecular interactions is provided in a number of key review articles (113–118).

For the analysis of peptides and proteins, different strategies for performing immunoassays have been developed. They all relate to the well known reaction schemes between antigens and antibodies or fragments of antibodies and different labeling strategies. The CE basis for immunoassays is the work of Nielsen and colleagues, in which the CZE separation of human growth hormone (hGH), anti-hGH, and complexes of hGH and anti-hGH was demonstrated (119). Four early examples are given here. In affinity probe CE, described by Shimura and Karger (120), a fluorescently labeled affinity probe (tagged FAB’ fragment) is used to detect the analyte as a complex after the separation of the excess of free probe by CIEF with LIF detection (Fig. 19.15a). With that non-competitive reaction approach, recombinant hGH could be successfully determined down to detection levels of \( \sim 5 \times 10^{-12} \) M and separated from its mono- and di-deamidated variants. Kennedy and colleagues described a competitive immunoassay for human insulin using fluorescently labeled insulin as antigen (121). In that approach, the free tracer and the tracer–FAB’ complexes were separated by CZE and detected by LIF. With a detection limit of 3 nM, this assay was successfully used to determine the insulin content and insulin secretion from single rat islets of Langerhans (122). Using the same CE conditions, this group also described a non-competitive immunoassay for anti-insulin FAB’ fragments (121). Reif and colleagues described a non-competitive immunoassay for IgG in human serum, employing fluorescently labeled protein G as affinity ligand (123). The FITC–protein G–IgG complex was quantified by CZE down to the nM concentration level. The data obtained were found to correlate well with those obtained by a single radial immunodiffusion assay. Using fluorescently labeled protein A as affinity probe, which binds selectively to the Fc region of immunoglobulins, a similar assay for the determination of IgG in cultivation media was developed (124) (Fig. 19.15b). Newer examples are given in several comprehensive reviews (117, 125–128) and chip-based immunoassays are described in References 38 and 129.

### 19.3.7 The CE Capillary as Ultramicroreactor: Application to Enzyme Assays

CE has been shown to lend itself as a unique and efficient microreactor for chemical reactions followed by the on-line assay of a reaction product. In electrophoretically mediated microanalysis (EMMA) (130–133), electrophoretic mixing is utilized to merge zones containing the analyte and analytical reagents. The reaction is then allowed to proceed either in presence or absence of the applied electric field prior to the electrokinetic transport of the detectable product through the on-column detector. Two types of reaction schemes have been described, one being based on a plug–plug reaction in which sample and reactant are applied separately onto the capillary, and the second comprising a continuous reaction with application of sample into a configuration with the reactant being present in the buffer (134).

EMMA has been applied to the determination of enzymes. Enzymatic catalysis consists of a reaction

\[
E + S = ES = E + P,
\]

where E, S, ES, and P refer to the enzyme, a substrate, an enzyme–substrate complex, and the product, respectively. If complex and product have different charges, then they are likely to be separable by CE. EMMA of enzymes is typically carried out in a capillary filled with substrate, buffer, and other required reagents. The enzyme is introduced as a small sample plug by electrokinetic or hydrodynamic injection. Application of power induces the mixing process and thus the start of the reaction. Product formation stops when the
enzyme leaves the capillary. Two modes of operation differing in constant and periodic (stop flow) application of electric power have been described. With constant driving potential, the reactants are mixed and separated from product continuously. Typically, enzyme catalysis occurs much faster than electrophoretic separations. Thus, product formation is much larger than the amount of enzyme applied. This amplification provides the enormous sensitivity of the CE assay. For a system where the product is the only compound responding for detection, the height of the plateau recorded is directly proportional to the enzyme concentration and inversely proportional to the electric power applied (Fig. 19.16a, upper graph). In the zero potential (stop flow) approach, product is accumulating because there is no electrophoretic transport of the enzyme and no separation of complex and product. When power is reapplied, a peak on top of the plateau is detected, this augmenting the sensitivity of the assay (Fig. 19.16a, lower graph). Using this approach for determination of alkaline phosphatase (ALP) in a gel-filled capillary, a detection limit of $7.6 \times 10^{-12}$ M has been reported (131). The same approach was employed for the simultaneous monitoring of two enzymes, ALP and $\beta$-galactonidase (131). As a real-world application, leucine aminopeptidase levels in human serum, human urine, and Escherichia coli supernatant samples were determined using the enzyme cleaving reaction of L-leucine-4-methoxy-$\beta$-naphthylamine (nonfluorescing compound) to 4-methoxy-$\beta$-naphthylamine (4-MBNA, fluorescing compound). For the exclusion of interferences during detection, time-resolved LIF detection was used. Furthermore, 4-MBNA, which is neutral and thus separates well from the enzyme, was used as internal standard (132). Similarly, isoenzymes of lactate dehydrogenase (LDH) were...

Figure 19.15 Immunoassay data based on CE. (a) CIEF in 75 μm i.d. LPA-coated capillary (anodic chemical mobilization and LIF detection) of purified affinity probe (top graph) and its separation from the complex with met-rhGH (lower graph). Affinity probe and complex are marked with * and **, respectively. Reprinted from Reference 120, with permission. Copyright, American Chemical Society. (b) CZE determination (50 μm i.d. untreated capillary, pH 10.5 buffer, LIF detection) of increasing amounts of monoclonal mouse IgG dissolved in cultivation medium using $9.6 \times 10^{-7}$ M of FITC-labeled protein A as affinity probe. From Reference 124, with permission.
monitored in single and multiple human erythrocytes via the enzymatically catalyzed lactate to pyruvate conversion in the presence of NAD$^+$ (135). The resulting NADH activity (proportional to LDH present) was determined by LIF. In that approach, LDH isoenzymes were first separated electrophoretically prior to an incubation period of 2 min and electrokinetic transport of the product zones produced by the isoenzymes past the point of detection (Fig. 19.16b). This example effectively demonstrates the EMMA capability of rapidly measuring ultratrace enzymes within an environment as small as a red blood cell (about 90 fL). Newer examples, including those performed in chips (38, 136), are presented in various reviews (137, 138).

19.3.8 Capillary Electrochromatography

CEC of proteins and peptides is a relatively new area of active research and has recently gained increasing attention (for reviews see References 139–141). CEC combines the selectivity of HPLC with the efficiency of electrophoresis. Thus, due to its promise of high peak capacity, CEC should resolve complex mixtures, such as those generated during protein and peptide mapping. Separation in CEC is governed by multiple physicochemical parameters, including hydrophobic interactions (reversed-phase CEC), electrostatical interactions (ion-exchange CEC), electrophoresis, and electroosmosis. As a large number of operating parameters influence the magnitude of these processes, separation in CEC is a rather complex interplay of many factors. Adjustable experimental parameters for separation of proteins and peptides include the pH, ionic strength, and the content of the mobile modifier (mainly acetonitrile) in the mobile phase. CEC separations of proteins have been carried out in three formats, namely in packed-bed, continuous-bed, and open-tubular configurations. In addition to electrophoresis, different chromatographic modes have been successfully exploited, with reversed-phase and ion-exchange being the most prevalent sorbents investigated. Although mainly model proteins and peptides have been analyzed to date, the suitability of CEC has also been demonstrated with real-world samples (139). The potential of CEC to yield complementary information and even a superior separation with respect to pressure-driven HPLC and CE has been demonstrated. For example, for electrokinetically driven cation-exchange chromatography of proteins in the open-tubular format using immobilized poly(aspartic acid) on the inner wall of 75 μm i.d. fused-silica capillaries, Xu and Regnier reported that isocratic separations in the electroelution mode were equivalent to gradient elution in the HPLC mode (142). The open-tubular, frit-free format has the advantage that bubble formation, which is often observed in packed-bed CEC, can largely be avoided. For CEC of proteins, most researchers use conventional fused-silica capillaries with optical or MS detection. CEC in microfluidic devices is also being explored (42, 139). Affinity CEC performed in capillaries and microchips comprises biospecific interactions including lectin affinity, immunooaffinity, immobilized metal affinity, sugar-based affinity, protein A affinity, protein G affinity, aptamer affinity, and enzyme affinity. Affinity CEC can also be used for continuous focusing of a minor compound in a complex mixture and for sample preparation by solid-phase extraction (143, 144).

Figure 19.16 Electrokinetic capillary enzyme assays. (a) Schematic of data obtained in EMMA under continuous reaction conditions and having constant (top graph) and periodically interrupted (bottom graph) applied power. The case with $v_{ex} > v_{f}$ is considered only. The plateau, initial peak, and peak produced by zero potential are marked with *, **, and ***, respectively. Adapted from Reference 130, with permission. (b) Electropherograms of a LDH isoenzyme standard mixture (12.3, 6.7, and 10.2 nIU of isoenzymes 1, 2, and 3, respectively; top graph) and LDH isoenzymes in lysed red blood cells (equivalent of about nine cells; bottom graph). A 20 μm i.d. fused-silica capillary was employed. Reprinted from Reference 135, with permission. Copyright, American Chemical Society.
19.3.9 Capillary Electrophoresis–Mass Spectrometry

Analytical chemists are faced with the challenge of increasing sample complexity and decreasing sample quantities. MS provides key tools for the analysis of proteins in complex mixtures. Thus, not unlike other areas of separation science, significant developments of hyphenated techniques, including CE-MS and LC-CE, and their application to protein separation and analysis have been undertaken. The on-line coupling of CE with MS, first described by Smith and colleagues (145), combines the extremely high resolving power of CE with structural information (molecular masses and/or fragmentation patterns) provided by MS detection. Not surprisingly, many different CE techniques were hyphenated with quadrupole, ion trap, and time-of-flight MS, for which electrospray ionization was mostly used for the generation of positively or negatively charged parent ions or fragments (146–148). An example dealing with proteins is presented in Figure 19.17. These data demonstrate how accurately the molecular mass of lysozyme could be determined by CZE-MS using electrospray ionization. Although (i) the flow rates from the CE column are compatible with on-line coupling to MS, (ii) numerous papers have appeared, and (iii) several instrumental companies offer interfaces for MS (Table 19.4), CE-MS of proteins is not yet widely accepted for routine use. The major limitation is the relatively high concentration detection limit of CE, a handicap that can be somewhat compensated by the stacking procedures referred to above (10, 11, 15, 112) and elsewhere (149, 150), including on-line transient CITP precondensation of the solutes, which permits injection of sample volumes two to three orders of magnitude higher than is usual in CZE (148, 149). Nevertheless, using electrospray ionization detection limits for a full scan CZE-MS analysis to \( \sim 10^{-7} \) M of proteins has been demonstrated (149). CE-MS is, however, widely used for research purposes. The capillary format with CZE-MS (151) and CIEF-MS (152), the coupling of microfluidic devices to MS (Fig. 19.6) (42, 153), and multidimensional set-ups with MS as final separation and detection device (see below) provide rapid means for the analysis of the proteome and other activities. Recent years have witnessed an increased use of CE-MS for the analysis of intact proteins (154), efforts that included electrospray ionization hyphenated with ion trap and time-of-flight MS (155) and matrix assisted laser desorption/ionization (MALDI) coupled to time-of-flight MS (156). The usefulness of CE-MS techniques has been demonstrated for protein isoform

![Figure 19.17 CZE-MS data of model proteins using electrospray ionization.](image-url)
assignment, single cell analysis, metalloprotein characterization, proteomics, and biomarker screening (154).

19.3.10 Multidimensional Separations

All CE techniques discussed thus far, with the exception of CE-MS, are one-dimensional separation techniques with insufficient peak capacity for the separation of highly complex samples. Two-dimensional separations provide peak capacities corresponding to approximately the product of the peak capacities of the two methods involved (157) and many different two-dimensional separations using microcolumns have been developed (158, 159). Slab gel electrophoresis, in which separations based upon charge (IEF, first dimension) and size (SDS/PAGE, second dimension) are combined, represents the most widely employed two-dimensional method. Comprehensive LC-CE, as pioneered by Jorgenson’s group (160), is a fully instrumental format of combining an LC separation followed by CZE analysis, in which LC effluent is analyzed by CZE. For protein analysis, microcolumn size exclusion chromatography (SEC) was coupled with CZE (160). SEC-CZE gray-scale image data obtained with protein standards are presented in Figure 19.18a. Incomplete resolution of all sample components was achieved when either technique was performed independently and under the same experimental conditions, whereas complete resolution was noted for the coupled approach. SEC-CZE data of a more challenging sample, reconstituted human serum, are presented in Figure 19.18b. Again, compared to single-dimension runs under the same conditions, higher resolution was reported for the two-dimensional approach. However, due to insufficient detection sensitivity for many proteins and the loss of some proteins adsorbed to the untreated fused-silica capillary, only a relatively small number of protein spots were detected. The use of coated capillaries in which protein–wall interactions would be diminished would have greatly enhanced the performance of that two-dimensional separation system. Nevertheless, the data demonstrate the two-dimensional separation principle applied to proteins. Furthermore, Jorgenson and colleagues also described the comprehensive analysis of peptides by reversed-phase liquid chromatography (RPLC) coupled to CZE (160, 161), SEC-RPLC-CZE (162), and RPLC-CZE-MS (163). RPLC coupled to a CE-based, competitive immunoassay for glucagon was described by German and Kennedy and applied to measure glucagon secretion from single islets of Langerhans (164), and protein digests were analyzed via integration of CIEF with capillary RPLC (165) and by CIEF, capillary RPLC, and MS (166). A rapid, nongel-based, on-line combination of CIEF followed by hollow-fiber flow FFF in which proteins are first separated according to their pIs and then by molecular mass was introduced by Kang and Moon (167).

Li and colleagues (168) described the integration of IEF with parallel SDS poly(ethylene oxide) gel electrophoresis for multidimensional protein separations in a polycarbonate microfluidic network. In this comprehensive...
two-dimensional protein separation, focused proteins are electrokinetically transferred into an array of 10 orthogonal microchannels and further resolved by SDS gel electrophoresis in a parallel and high throughput format. Proteins are noncovalently labelled with Sypro Red during electrophoresis and detected on-chip via fluorescence. With planar dimensions of 2 × 3 cm, separation is complete in less than 10 min with an overall peak capacity of ≏1700. An increase of the number of separation channels in the array would enhance peak capacity via analysis of a higher number of IEF fractions in the size-based separation dimension. The coupling of IEF and CZE on an acrylic microfluidic device using a simple cross-channel network was reported by Herr and colleagues (169). Chartogne and co-workers combined CIEF, continuous-flow electrophoresis in the chip format, and MS for the analysis of model proteins in which the chip device is employed to remove the CAs that are required for IEF but are undesirable for MS (170), and Mazereeuw and colleagues hyphenated HPLC with a continuous electrophoresis chip device for the bioanalysis of biotin in human urine with on-chip LIF detection (171).

The coupling of two techniques is not limited to those that are based on completely different separations (as in LC-CZE and IEF-SDS/PAGE), but can also be accomplished on similar separation mechanisms. Free solution CZE coupled to channel gel electrophoresis with a small pore size polyacrylamide gel (40% T, 3.3% C) was shown to nicely resolve FITC-labeled peptides that could not be separated by CZE alone (172). In that approach, the CZE outlet capillary end is moved across the entrance of the gel channel, thereby preserving completely both separations. The concept of combining a single capillary, which is used for sampling, and depositing the analytes across the entrance of a rectangular channel stems from the work of Ewing’s group (173–176). It was developed with the goal of continuous monitoring of biological systems and was applied to a variety of situations, including the detection of neurotransmitters, the kinetics of small molecules and to monitor the chemical environment around a single cell (175, 177). In the same context one should also mention ITP coupled to CZE (178), an approach that has been available for quite some time, but is not much used for the analysis of proteins, and ITP-CZE-MS (179).

Immunoadfinity CE can also be classified as a multidimensional separation technique. It is based upon tandem or online coupling of highly selective analyte capture compounds, including antibodies, and high-resolution CE. Analytes of interest are adsorbed onto immobilized affinity ligands, recovered, and analyzed by CE (144). It is an emerging and promising technique for the analysis of low-abundance biomarkers in complex matrices and has the potential to be automated, for example, in chip format. For instance, a protein that exists in a biological sample in a concentration far below its limit of detection can thereby be brought to a level within its range of quantification.

CE-MS and transient isotachophoretic sample stacking prior to CZE (approaches that use ITP for sensitivity enhancement in capillaries and microfluidic devices) (10, 11, 15, 112, 149, 150) can essentially be performed on all instruments. With the exception of the EA 102 and 202 analyzers of Villa Labeco (see Section 19.2), which feature coupling of ITP with CZE for two-dimensional analysis, no commercial instruments featuring multidimensional capillary separations are currently available. All the mentioned and other ongoing research activities, however, will pave the way towards commercial instrumentation and thus routine use of instrumental multidimensional separation procedures.

19.4 OTHER APPROACHES WITH APPLICABILITY TO PROTEINS

19.4.1 Electrical Field Flow Fractionation Techniques

The overall set-up of FFF techniques is schematically presented in Figure 19.19a. The relation to an HPLC system in which the chromatographic column is replaced by the FFF trough is obvious. An electrolyte pump providing the buffer flow and a flow-through detector are mounted at the two ends of the separation capillary. A computer for the control of the pump and the power supply, as well as for data acquisition, data storage, and data handling, permits versatile programming throughout the entire process. Detection of eluted solutes occurs by measuring of specific physical properties such as optical and electrochemical responses using flow-through cells as in LC and flow injection analysis. In EFFF, developed by Giddings and colleagues (22, 180, 181), the separation channel consists of two parallel membranes, which separate the flowing stream from the electrode compartments (Fig. 19.1b). Chamber thicknesses of 0.25–0.35 mm having channel widths of 15–25 mm were used with a length of ≏50 cm. Reasonable protein separations could be achieved using flow rates of ≏5 mL/h and electric fields between 2 and 20 V/cm. However, analysis times of several hours were typical with this kind of equipment. Newer channel designs with chamber thicknesses of 130–180 μm featuring graphite plates that simultaneously act as channel walls and electrodes have been applied to particle but not protein separations (182, 183). The same is true for a micromachined system with gold electrodes featuring a channel of 56 mm length, 6 mm width, and 28 μm height (184).

Davis and colleagues, meanwhile, designed an apparatus with an annular channel utilizing concentric porous Vycor glass tubes of various diameters (185). For this device, experimental retention ratios are reported to depart considerably from predictions based on theoretical EFFF. Similar problems were encountered in EPC, in which the separation trough consists of an anisotropic ultrafiltration hollow fiber of
circular cross-section (≏50 cm long, 0.5 mm i.d., nominal molecular mass cut-off of 5000 Da). The fiber is bathed in a buffer and subjected to a transverse electric field (23, 186, 187). Carrier flow rates of ≏12 μL/min were admitted together with electric field strengths of 5–10 V/cm in order to achieve separations without complete electroretention in about 2 h. At these low voltages, retardation was found to be quite consistent with the theoretical prediction. Higher electric field strengths, however, were shown to immobilize macromolecules in the column. The totally retained components at the hollow fiber’s wall could be eluted only by decreasing the applied voltage for the case of reversible sorption. By programming temporal changes in the electric field strength, separations according to differences in electroretention values are achieved. This is an analogy to the chromatofocusing principle. For a great number of biopolymers, however, electroretention has to be avoided because of sorption nonreversibility, causing a substantial loss of material. In a more recent contribution, pH-regulated protein electroretention was described in a 22-cm-long piece of a blood dialysis fiber of regenerated cellulose with an i.d. of 190 μm, a wall thickness of 8 μm and a molecular mass cutoff of 10 kDa (188). With model proteins, field-induced protein immobilization was found to be a function of the pH adjusted in the solution surrounding the fiber and employed to separate proteins according to their isoelectric points.

In EHFFF, contact between most proteins and the channel walls is avoided (24, 189, 190) by the concomitant presence of a pH and an electrical potential gradient representing the same gradient combination as in IEF. Solute layers having different depths from the walls are thereby established (Fig. 19.19b). This method, also termed isoelectric focusing FFF (189), could not be made operational in a ribbonlike (rectangular cross-section) channel thus far. However, protein separations executed in a trapezoidal cross-section channel (Fig. 19.19c) of 0.875 mL volume and 25 cm length, and with a buffer flow rate of 40 μL/min (elution in less than 1 h) have been described (189). A fractogram obtained with three proteins, ferritin (FER), MYO, and CYTC, is presented in Figure 19.20a. For the sake of comparison, CIEF data in a set-up featuring electroosmotic zone displacement are also shown (Fig. 19.20b). The two focusing methods in flowing streams have interesting similarities as well as differences. EHFFF and CIEF are methods that utilize hydrodynamic and EOF, respectively, for the elution of focused isoelectric zones and their detection by conventional detectors developed for HPLC and CE, respectively. In the two methods, the electric field and the flow (column axis) are differently oriented to each other, the two vectors being perpendicular and parallel in EHFFF and CIEF, respectively. With today’s instruments, the experimental procedure and set-up for CIEF is simpler than that of EHFFF. For separations of proteins, CIEF provides higher resolution and efficiency. Advantages of EHFFF include the requirement for lower voltages and its simpler application to micropreparative applications. The time intervals required for separation and analysis in the two methods are comparable. Applications of FFF in proteomics were recently reviewed by Chmelík (192).
19.4.2 Analytical Continuous-Flow Electrophoresis

In CFE (Fig. 19.1c), a small stream of sample is admitted into the laminar flow of the buffer, except for IEF, when the sample is evenly mixed with the CAs prior to electrophoresis. For all techniques the liquid flow, and to a smaller extent also the applied electric field strength, defines the transit time of the sample. Resolution is given in terms of differences in mobility or pI values respectively, sample quantity and composition, current applied, flow velocity, effectiveness of cooling, length of electrophoresis chamber, and width of the flowing fluid film. If samples are recovered, resolution is dependent on the density of the outlet tubes of the separation chamber. For analytical purposes it can suffice to monitor the solutes directly through a window placed towards the end of the separation chamber with an optical scanning system. Good reproducibility and very short analysis times are achieved. Further assays can be performed on the fractions collected. Online detection with a video camera or a scanning sensor also provides the means for feedback control and automation.

CFE instrumentation (25, 26), particularly for preparative applications, is currently available only as BD Free Flow Electrophoresis System (BD Diagnostics, Franklin Lakes, NJ, USA; BD GmbH, Martinsried/Planegg, Germany). This instrument is based upon the CFE systems of Dr. Weber GmbH (Kirchheim, Germany), which comprise rectangular chambers in which laminar flow conditions are maintained by a small (0.3–0.8 mm) gap between two parallel plates. Typical chamber dimensions are 500 × 100 mm, and running conditions include electric field strengths up to 300 V/cm and sample transit times of 20–3600 sec. A smaller instrument with a 120 × 30 mm chamber and having a 0.3-mm fluid layer thickness has also been commercialized (ACE 710, Hirschmann, Unterhaching, FRG), but is no longer available. Miniaturized CFE has been designed for continuous sample pretreatment and separation. CFE quartz channel devices with an internal height and width of 48–110 μm and 2 cm, respectively, and having lengths of 2–7.8 cm have been constructed by Mesaros and colleagues (173). This approach to CFE has the potential to continuously sample and separate analytes from volume-limited microenvironments. The principle has been shown using dansylated amino acids for which sample introduction (but not separation) is carried out using conventional CZE and eluting analytes are monitored at the channel exit by LIF using two linearly arranged fiber optic arrays (173). Electrochemical detection using an electrode array has also been discussed (174). Ultrathin channels with capillary sample introduction have been used to monitor dynamic events from microenvironments, to detect analytes over long periods of time, kinetic analysis, parallel DNA separations, and electrophoretic 2D separations (193) (see also Section 19.3.10).

In another approach, Raymond and colleagues reported the successful integration of a CFE device with comparable cell dimensions (50 μm × 10 mm × 50 mm, depth × width × length) onto a silicon chip in which the separation chamber was isolated from the electrodes via two arrays of 2500 V-groove channels (194, 195). The channel systems were etched into the silicon, to which a glass cover plate was anodically bonded to form the channels. Solute detection towards the chamber end was effected by LIF using a scanning detector assembly. For that case, system performance was discussed using labelled amino acids (194) and proteins (195). Data obtained with FITC-labeled model proteins electrophoresed at various electric field strengths are presented in Figure 19.21. Detection occurred at 3.1 cm along the separation chamber length. A simple fraction collection system with sample outlets at four positions along the 10 mm
separation width was also implemented and applied to off-line analysis of the collected fractions (195). Another design comprises a plain glass substrate of 1.5 mm thickness and a thin (0.3 mm), crosslinked PDMS layer with micromachined channels composed of numerous posts, a high speed device that features a 0.2 μL chamber volume with a liquid layer thickness of 10 μm, has a sample residence time of 2 sec, and permits continuous separations to be performed in less than 100 msec (196). A similar chip was fabricated for fast IEF of peptides and proteins with which the sample could be up to 400-fold concentrated within less than 500 msec (197). Microfabricated continuous-flow electrophoresis of proteins in a 56.5 mm (width) × 35 mm (length) Pyrex chamber with a 30 μm fluid thickness and 19 outlets was described by Kobayashi and colleague (198). In that device, electroosmosis had to be suppressed with HPMC and the separation time for two model proteins was 10 sec. Collected fractions were analyzed by HPLC. Simple, miniaturized continuous-flow devices with on-column LIF detection (171) and with three outlet streams featuring UV detectors (170) have been described by the group of van der Greef and were applied to on-line separation in analytical systems in which an additional separation step of charged compounds is desired (see Section 19.3.10).

19.4.3 Electric Field Gradient Focusing

Electric field gradient focusing (EFGF) is a relatively new equilibrium gradient separation technique which is promising for protein analysis. EFGF involves a gradient in electric field strength along the length of a separation column and a pressure-driven bulk flow of fluid that is opposite to analyte migration. When the electrophoretic velocity is equal and opposite to the velocity of the fluid flow, the analyte is focused into a stationary band. EFGF was first described by Koegler and Ivory for preparative separations (199) and is expected to reach protein concentrations higher than those attainable in CIEF. Furthermore, unlike IEF, EFGF is not limited to amphoteric compounds, but is much less developed than CIEF. Devices with changing cross-sectional area, featuring gradients in buffer conductivity, using a computer-controlled array of individually addressable electrodes, with imposed temperature gradients together with buffers whose conductivities change as a function of temperature, have been built and tested in various laboratories (200). Nice protein separations were demonstrated using a voltage-controlled elution in a conductivity gradient EFGF system based on a hollow dialysis fiber (201). Recent efforts have led to protein separations in microfluidic EFGF devices with a changing cross-sectional column area over a length of 4 cm, which were manufactured from PEG-functionalized copolymers and that comprised a buffer ion-permeable but protein-excluding conductive hydrogel (202).

19.5 BRIEF DISCUSSION AND OUTLOOK

Forty years after the inception of CE, a considerable amount of progress has been made, particularly with the emergence of fused-silica capillary-based instrumentation, which is now being followed by the development of microfluidic...
chip-based instrumentation. The successful coupling of CE with MS is another important milestone for this technology. Automated instruments, reagents, and entire CE kits with complete protocols are available that provide the possibility of adopting CE as an attractive alternative to HPLC and slab gel electrophoresis. On the other hand, CFE, the FFF methods (i.e., EFFF, EPC, and EHFFF), and EFGF have only barely been explored.

CE has become a key technology in genomics, proteomics, and metabolomics, areas for which combined information will help to obtain an integrated understanding of cell biology. CE with sieving matrices is used for the separation and analysis of oligonucleotides, DNA fragments, and proteins, whereas free solution CE is applied to small molecular mass compounds, ions, peptides, and proteins. Recent milestones associated with CE comprise the completion of the sequencing of the human and other genomes (projects that would not have been completed so quickly without automated CE), the establishment of forensic DNA offender databases in many countries (for which input data are largely those produced by CE), as well as the beginning of a widespread use of CE in clinical diagnostics, forensic drug analysis, and biomedical research. Furthermore, CE provides the research community with a tool that permits breaking new ground and reaching new horizons, including the chemical characterization of nanoliter environments (e.g., analysis of the content of a single living cell), the on-line study of chemical reactions (with the capillary being a unique ultramicroreactor), and the investigation of separations and reactions on a millisecond timescale, to name but a few.

In the field of protein separation and analysis, characterization and modification of capillary walls, and the development of suitable running conditions for many different proteins were essential to understanding and preventing undesired protein–wall interactions. As a result of these efforts, CE of many proteins can now be regarded as a fast and high resolution substitute for slab gel electrophoresis, including SDS-PAGE and gel IEF, and HPLC. Although separations and solute detection can be achieved within a few minutes, sample throughput in CE is typically lower than that obtained in slab gel electrophoresis. With instrumentation comprising multiple capillaries or microfluidic channels, however, sample throughputs comparable or even higher to those obtained in multilane gel analyses are obtained. Furthermore, CE of proteins is not at all limited to the application of different electrophoretic separation techniques, such as CZE, CITP, and CIEF. The instrumental capillary and chip formats have been shown to be well suited for (i) the investigation of biospecific affinity and other reactions involving proteins, (ii) the performance of highly sensitive immunoassays and on-column reaction-based chemical analyses (e.g., enzyme assays, even within a single cell), (iii) the determination of protein mass by CE-MS (also providing structural information of the electrophoresed compound) or via analysis of their dodecyl sulfate complexes in a sieving environment, and (iv) the resolution of complex protein samples by two-dimensional techniques (LC-CE, CITP-CZE, etc.). In other words, CE is being employed in many laboratories for analysis of peptides and tryptic digests of proteins, analysis of entire proteins and assessment of their heterogeneity (including their glycosylation patterns, Fig. 19.10), examining affinities among proteins (including receptor–ligand interactions) and of proteins with other solutes, probing folding/unfolding/misfolding of proteins and determining physical properties of proteins, including mass, charge, size, mobility, pI, titration data, electrostatic interactions and affinities to various substrates. A number of reviews and book chapters can be recommended to gain further insight into these applications of protein CE (46–56, 203, 204).

Together with other technologies, such as LC-MS and protein microarrays, CE is a powerful method for the analysis of proteins. Each method has its own strengths and weaknesses and no single method is optimal in all applications. The continuing development of innovative strategies for protein separation and analysis will provide a wealth of new tools for this exciting and important scientific field. For CE, the current trends point towards miniaturization, with handheld microanalytical instruments being just around the corner (38, 39, 205), and the elucidation of specific column coatings, including those based upon an organic boronate compound for the selective separation of glycoproteins (206) and a controlled passivation of microfluidic channel walls combined with bioprobe immobilization to perform heterogeneous affinity reactions at microchannel intersections (207, 208).

19.6 CONCLUSIONS

Electrophoretic separations in capillaries of small inner diameters, microchannels, and thin fluid films have received considerable attention within the last four decades. In this period a number of instrumental approaches for capillary zone electrophoresis, capillary isoelectrophoresis, capillary isoelectric focusing, capillary gel electrophoresis, capillary electrochromatography, continuous flow electrophoresis, electrical field gradient focusing, and electrical field flow fractionation or electroporalization chromatography have emerged. High separation efficiencies have been demonstrated in conjunction with high resolution on-column and/or end-column sample detection. For the separation and analysis of proteins, CE is complementing other existing techniques, particularly high performance liquid chromatography and slab gel electrophoresis. For protein analysis, applications range from the basic electrophoretic modes of operation to specific techniques, including affinity electrophoresis, immunoassays, reaction-based chemical analysis, hyphenation with mass
19.8 REFERENCES

PART V

SEPARATION METHOD OPTIMIZATION
20

HIGH THROUGHPUT SCREENING TECHNIQUES IN PROTEIN PURIFICATION

KAROL M. LACKI AND EGGERT BREKKAN
GE Healthcare Bio-Sciences AB, R&D Department, SE-751 84 Uppsala, Sweden

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20.1 INTRODUCTION

In the past, the development of chromatographic separation has been performed by tedious experimental methods involving series of experiments on either a manual or fully automated chromatographic system. The introduction of chromatography systems, such as FPLC™, improved this workflow by allowing predefined runs to be performed in a sequential manner. Further introduction of automated systems such as the ÅKTA™ family improved the workflow even further by allowing a fully automated experiment to be performed without operator assistance. However, these systems were designed for fairly large columns and high flow rates, which in turn required large sample volumes to be used. At the same time, a natural development of high-throughput techniques has occurred in different fields, including the design of new materials, improved cell culture conditions, and (last but not least) advances in laboratory automation. Liquid handling systems such as Freedom EVO™ (Tecan Group Ltd, Männedorf, Switzerland), Biomek™ (Beckman Coulter, Inc., Fullerton, CA) and STAR (Hamilton Robotics, Inc., Reno, NV) have become standard equipment in most large research facilities. In addition, miniaturization of analytical methods has also contributed to a need to re-evaluate the old paradigms around the development of protein purification processes. In this chapter we will review recent advances in what we consider high-throughput methods for performing simple purification tasks and for the development of optimized chromatography protein purification processes. The following definition of high throughput has been used for this discussion: a high throughput method is any method that will maximize the amount of information per unit time and unit mass of sample used. Consequently, because of this definition we have excluded from our discussions all the methods that require more than 500 μL of chromatography resin per
single experiment. For an interested reader, a few recent applications of such methods can be found elsewhere (1–3).

In this chapter we will look at the use of high throughput methods in the development of industrial scale protein purification processes. We will also consider a link between the high throughput methods and steadily increasing expectations from the regulatory authorities that new purification processes are developed based on better understanding and/or firm knowledge of effects of critical process parameters on product quality attributes. In addition, the increasing cost for developing new drugs is also putting pressure on process development groups to search for more efficient processes in the development of workflows that would not only reduce development time and material requirement but also provide more information about the developed process. It has been argued (4) that a rigorous implementation of the Quality by Design (QbD) initiative into a process for designing/optimization of a chromatography step is prohibitive from a time and sample consumption perspective, even with small chromatographic columns. Thus, in order to reach QbD goals and at the same time keep development cost to a necessary minimum, new paradigms in process development should be sought. A simple approach to reach these goals is to introduce high throughput experimental approaches that rely on either significantly scaled down variants of currently used experimental methods, or on other experimental methods that can provide more information about the separation step in question using less sample. In either case, the methods employed should preferably be operated in a parallel manner in order to increase the productivity of a process development laboratory.

High throughput (HT) screening techniques have been used in the small drug discovery field for decades, but they were not utilized in the field of biologics until the beginning of this century. Since then, examples of successful HT applications have been reported in many areas related to the development of manufacturing processes for a biopharmaceutical drug, such as miniaturization of upstream (5) and development of downstream purification processes (6), including solubility (7), partitioning (8), refolding (9), and chromatography studies (10, 11).

Use of this type of technique is also gaining popularity in the field of sample purification. Examples include purification of membrane proteins (12) and recovery of histidine-tagged recombinant proteins from unclarified samples (13, 14).

Any type of HT experimentation can be looked at through a prism of its primary goal. It has been argued that the use of HT techniques can be divided into primary screening, also called discovery, and optimization (15). Applying the same reasoning for the field of protein purification would indicate that the primary screening studies will look at finding leads, or giving a simple yes/no answer, for instance in identifying the avidity of different ligands under identical conditions or under a very sparse parameter space. Optimization, on the other hand, would entail exploration of a wide/diverse parameter space where the parameters are varied in a continuous manner. The experimental workflow is more or less the same for the two types of studies (Fig. 20.1). It starts with a formulated hypothesis, followed by choice of parameters, design of the study, experimental work, sample analysis, results, and finally evaluation of the results and formulation of the final hypothesis. Of course, if the new hypothesis is not good enough, iterations may be necessary until the desired level of information is attained. The step dedicated to design of experiments (DOE) may be given some consideration here. From the screening perspective, it could be argued that the current background knowledge would allow at least the formulation of a list of parameters to investigate and the tentative direction of their effects, which could lead to a more efficient

Figure 20.1 Outline of HT experimentation approaches used in protein purification studies. Work from GE Healthcare Life Sciences, reproduced with permission.
design of the screening experiments. By the same token, any study focusing on optimizing conditions need not rely on a detailed mapping of the parameter space, but may in fact be based on a predefined design, seeking a mathematical relationship between the parameters and the chosen objective function. With a mathematical model verified, optimum conditions within the investigated experimental space can be found easily. Taking this argument into account and applying it to the field of preparative protein purifications, it can be postulated that the final optimization should be performed using the system that resembles the final purification process as closely as possible, of course at a reasonable scale. In Figure 20.2, the information flow, starting with the initial hypothesis through to the final preparative scale chromatography process, is depicted. As the experimental parameter space shrinks towards the potential design space, the information density and process reliability should increase, leading to a more robust and fully controllable operation.

In this chapter, a brief technical review is provided of available HT techniques used for the development of chromatographic purification steps. This part is followed by a more detailed description of a method based on batch uptake. A few examples of studies utilizing HT methods in characterizing different purification steps are provided. Throughout the chapter we will discuss a complete workflow related to HT methods for designing a purification process, starting with the design of experiments, samples characterization/analysis, and finally data evaluation. At the end, we will discuss what we believe will be future trends, and the development needs in the field of HT protein purifications. Since the field of HT process development is rapidly evolving, the material presented in this chapter refers to the state of this field as per end of 2009.

20.2 TECHNICAL OVERVIEW

In the past, the development of a protein purification step relied on laboratory scale columns and automated chromatography systems to perform a series of experiments in which different separation conditions were tested. Because the columns were relatively large, the amount of sample required to perform these tests was also fairly significant. In addition, considering the intrinsic hardware limitations of a typical automated chromatography system that does not allow the performance of many experiments in parallel, the time factor associated with the development of the chromatography step was also significant. For instance, in order to develop a relatively simple capture step using a 1 mL column packed with a chromatography resin having a modest binding capacity, as much as 0.5 g of a valuable sample could be required, and the time for completion of such a study could be up to 5 days (16, 17). The low throughput could be considered unacceptable, especially in industrial settings where process development groups are working with multiple processes simultaneously. Obviously, the throughput and sample consumption issues can be addressed by the introduction of parallel and scaled-down experimental techniques, respectively. In recent decades, such techniques have been developed in many laboratories, and are described in detail in the scientific literature and also in many commercialized cases. In general, these techniques can be divided into flow-based systems, such as microcolumns or prefilled pipette tips, or techniques utilizing a batch incubation principle, such as microtiter plates prefilled with chromatography resins.

The microcolumn method is based on a linear downscale of a standard chromatographic method, as the geometry of the bed is maintained. Microcolumns ranging from 50 to 200 µL are available from Atoll GmbH (Wingarten, Germany) (18, 19). According to Atoll, the columns can be packed with any bioprocess resin. Undoubtedly, the biggest operational advantage associated with this method is a reduction in the sample consumption required for development of a chromatographic step. To achieve a higher experimental throughput with these columns it is necessary to operate several, typically eight, in parallel, because the operating time for these columns, that is, the time for the equilibration step, sample load, elution, and so on, should be scale-invariant.
This can be achieved with the help of a column holder and an automated pipette or by converting a laboratory multichannel liquid handler, such as a Freedom EVO™ system, into a multipump delivery system, where each channel (a tip and a syringe) of the liquid handler acts as a pump delivering liquid to one microcolumn. In either case, effluents from each column are collected as drops in a series of wells in a collection microtiter plate, where each well in the collection plate will represent a separate fraction. One cumbersome aspect of this approach is that care must be taken to assure that the size of each fraction can be determined, as the size can vary because the droplets collected may differ in volume. The extent of this variation will depend on many factors including effluent composition (affecting the surface tension) and the flow rate used. Furthermore, to attain residence times that are relevant for chromatographic applications, the dispensing speed of the syringes very often has to be reduced below the specification for the given robotic system, which although mechanically feasible puts a question mark on the robustness/reproducibility of the fraction collection and liquid delivery methods. Nevertheless, several examples of interesting applications using microcolumns have been reported (19, 20). As most of them were based on 200 μL columns, the sample consumption in these applications was rather large.

To date, no application work performed with columns smaller than 200 μL has been reported, which is most likely related to the difficulty in collecting representative fractions from smaller columns.

The other method in which convective flow is attained utilizes small pipette tips filled with chromatography resin. Currently, prefilled pipette tips are available from PhyNexus, Inc. (San Jose, CA) and from Millipore Corp. (Billerica, MA), with the latter tips being recommended for sample preparation prior to mass spectrometric analysis. When working with these tips, a sample is brought in contact with the chromatography resin by continuously aspirating and dispensing the sample from and into a sample reservoir for a predefined contact time. By repeating this procedure several times, the dynamics of an adsorption process can be characterized by monitoring changes in sample concentration with time. In some aspect this method bears some resemblance to the so-called shallow-bed method that has been used in studies of adsorption systems (21, 22). Because sample is pushed through the bed of resin particles by a pressure-driven process, convective flow around the particles may resemble what happens in a packed bed, even though a packing pattern in a tip and in a chromatography column will be different. Nevertheless, the conditions of convective flow may favor this technique over a simple batch incubation method (see below) when working with adsorption of large molecules. In such a case, intraparticle mass transport is practically absent, as the adsorption process occurs exclusively on the external surface of resin particles, thus being enhanced by convective flow. A very successful application of this technique was reported by Wenger and colleagues (23), who used PhyNexus tips for purification of virus-like particles.

The third technique used in HT studies of chromatographic separations is based on a combination of the well-known principle of batch adsorption and the HT advantages that the microtiter plate format provides. Examples of successful applications of this technique include, among others, investigations of a second step in a MAb purification process either by cation exchange (24) or by HIC (7) chromatography, screening of purification conditions (6), characterization of a multicomponent adsorption system (10), and estimation of dynamic binding capacities (25). The plates can be either prepared in house or purchased prefilled. In the former case a rather work-intensive development of a reliable method for filling the plates should be anticipated, while in the latter case, the selection of resins available in the plate format may be limited. The broadest selection of plates filled with modern chromatography resins is available from GE Healthcare BioSciences AB (Uppsala, Sweden). The plates are sold under the brand name of PreDictor™ plates, and are available in a variety of configurations specifically designed for different types of studies. These plates were developed based on a proprietary design of the bottom filter that minimizes hold-up volume and filter surface area, yet provides the expected functionality of a microtiter filter plate, and a very precise method for filling wells with a predefined volume of resins ranging from as little as 2 μL per well to 50 μL per well (26).

Recently a review of microscale methods has been written in which authors have looked at the pros and cons of the three formats available for HT investigations of chromatographic separations (4). In addition to a comprehensive review of technical guidelines for each of the methods, attributes such as type of study, ease of automation, flexibility, and cost were considered. A qualitative comparison of the three methods based on these attributes is given in Table 20.1.

As shown in Table 20.1, methods employing microtiter plates appear most appealing from the flexibility and low cost perspective. Although difficulty in automation has been highlighted for the microtiter plate method as a potential drawback, it is most likely related to the longer lead times necessary for the programming of the robot to handle the additional experimental steps present in the workflow.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Micropipette</th>
<th>Microriter Plates</th>
<th>Minicolumns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding capacities</td>
<td>Dynamic</td>
<td>Static</td>
<td>Dynamic</td>
</tr>
<tr>
<td>Automation</td>
<td>Easy</td>
<td>Difficult</td>
<td>Easy</td>
</tr>
<tr>
<td>Flexibility</td>
<td>High</td>
<td>Very high</td>
<td>High</td>
</tr>
<tr>
<td>Cost</td>
<td>High</td>
<td>Very low</td>
<td>Very high</td>
</tr>
</tbody>
</table>

TABLE 20.1 Qualitative Comparison of HT Methods for the Development of Chromatographic Separations (Based on Reference 41)
for the plate method when compared to programming of the robot to accommodate the other two formats. In fact, the only difference in experimental workflows between the three methods is the need to separate the resin particles and the liquid phase at different stages of the microtiter filter plate based experiment, which is a fairly simple procedure. Furthermore, even from the hardware perspective this additional step should not be considered problematic, as most robotic systems currently available on the market can be easily equipped with standard modules for the separation of phases either by filtration or centrifugation. Additional accessories developed specially to facilitate working with PreDictor™ plates are available from Tecan (27). As microtiter plates can also be used to indirectly study dynamic binding capacities (25), the plate format seems most suited for HT development of a typical chromatography step. Last, but not least, the plate format is also well suited for a manual workflow, and can thus be used in any laboratory with a low investment into basic equipment such as vacuum manifolds, shakers, and automated pipettes. An indirect confirmation of the above conclusion can be found by reviewing the HT work reported to date in the area of protein separation, as shown in Table 20.2. As can be seen in the table, a majority of the reported studies, 80%, used microtiter filter plates filled with chromatography particles. Obviously, the availability of microcolumns and prefilled tips can be a factor that would need to be considered when drawing conclusions from Table 20.2, but nevertheless the plate-based method seems most popular. An interesting observation from the table is that there is a fair variation in the volume of resin used in different studies, from 2 to 100 μL. Furthermore, sample volume can also vary significantly in the range from 150 to 800 μL. The reader should bear in mind that the effect of incubation times are practically feasible when performing HT studies using plates, as is depicted in Figure 20.5 by the vertical lines representing the chosen incubation times. A finite number of different incubation times can be studied in an experiment which the liquid phase is removed from each well by centrifugation or vacuum filtration (Fig. 20.3). The same procedure is applied for the wash and elution cycle. Each time the phases are separated the removed liquid is collected and analyzed to determine its composition. A straightforward mass balance is then used to calculate binding capacities, efficiencies of wash and elution steps (Table 20.3). In principle, the microtiter plate method can be used to investigate all steps in a typical column chromatography cycle such as equilibration, sample loading, wash, and elution (Fig. 20.4).

20.3.1 Kinetics

The microtiter plate method can also be used to investigate mass transfer phenomena occurring in a given adsorption system. In order to do that, the effect of incubation time on the extent of protein adsorption must be investigated. Different incubation times can be studied in an experiment employing only one microtiter plate. This is done by adding the sample to different groups of wells at different times, with samples to be incubated for the longest and the shortest amount of time added first and last, respectively. The principle of this method is described elsewhere (25). In any kinetic study it is of paramount importance to ensure that a correct phase ratio is used, in other words, that the rate of intraparticle mass transport can be measured. The simulated effect of phase ratio on the shape of protein uptake curves is shown in Figure 20.5a. Simulations were performed using typical mass transfer coefficients and equilibrium adsorption isotherms encountered in protein adsorptions studies (28). Only a finite number of different incubation times are practically feasible when performing HT studies using plates, as is depicted in Figure 20.5 by the vertical lines representing the chosen incubation times. A few important cases that could be used to further guide the reader when designing uptake experiments will be presented. If the phase ratio, defined as the ratio of liquid to resin volume present in a single well, is too low (curve A) the experimental point obtained for the shortest incubation time will not lie on the initial slope of the uptake curve and, therefore, this point cannot be used to reliably asses the real rate of intraparticle mass transfer. A similar situation can also occur when the rate of mass transfer is very high or adsorption occurs only on the external surface of adsorbent particles. In order to correctly capture the initial slopes of uptake curves yet keep the same incubation times, either the initial concentration or the phase ratio (or both) can be increased, as shown by curves B, C, and D, respectively. The other way to obtain the correct initial uptake rates would be to consider shorter incubation times, but, as mentioned previously, not all incubation times are practically feasible considering the finite length of different operations in the experimental workflow described above. In the case when adsorption occurs only on the external surface of adsorbent particles, studies of the rate of uptake in the batch system seem meaningless, as for all practical
### TABLE 20.2 Examples of Successful Applications of HT Screening Techniques in the Development of Purification Processes

<table>
<thead>
<tr>
<th>Study</th>
<th>Volume of Resin per Single Experiment, (μL)</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purification of virus-like particles (VLP)</td>
<td>Micropipette 40, 80</td>
<td>A laboratory scale chromatographic purification of VLP was miniaturized, leading to a 10-fold increase in throughput and reduction in sample consumption and labor</td>
<td>23</td>
</tr>
<tr>
<td>Screening for capacities and elution for different resins</td>
<td>Microcolumn 200</td>
<td>Characterization of binding (frontal analysis) and elution conditions for different resins</td>
<td>20</td>
</tr>
<tr>
<td>Resin screening. Wash and elution studies</td>
<td>Microtiter filter plate 100</td>
<td>Determination of an optimum purification sequence by employing a high throughput process development (HTPD) approach to recombinant protein development; screening of 12 resins</td>
<td>6</td>
</tr>
<tr>
<td>Screening of conditions for separation of four proteins</td>
<td>Microtiter filter plate 300</td>
<td>Description of protein purification parameter screening approach for optimization of yields and recoveries, and so on, by varying pH and ionic strength of solution</td>
<td>42</td>
</tr>
<tr>
<td>Resin screening</td>
<td>Microtiter filter plate 250</td>
<td>Example of application of high throughput screening (HTS) technique in screening of libraries of different resins. In two days a resin can be characterized and optimum operating conditions can be found</td>
<td>43</td>
</tr>
<tr>
<td>Purification of antibodies</td>
<td>Microcolumn 200</td>
<td>Combination of HTS screening with mass spectrometric detection using SELDI–TOF MS. Gradient elution studies in small columns; resin screening; discussion of HT analytical techniques</td>
<td>44</td>
</tr>
<tr>
<td>General paper</td>
<td>Microtiter filter plates 50, 100</td>
<td>General description of experimental methodology and modeling approaches; the basic reference for all studies using plates; comprehensive discussions of hold-up volume effects, mixing efficiency and phase ratio; discussion of analytical techniques included</td>
<td>31</td>
</tr>
<tr>
<td>CIEEX step in monoclonal antibody purification</td>
<td>Microtiter filter plate 50</td>
<td>Example of accelerating development of an ion exchange step in a MAb process; gradient elution studies, screening of optimum conditions and resins; comprehensive study generating 48 different combinations of pH, resin, and counter ion concentrations</td>
<td>24</td>
</tr>
<tr>
<td>Purification of monoclonal antibodies</td>
<td>Microtiter filter plate 150</td>
<td>Optimization of conditions for an anion exchange step</td>
<td>45</td>
</tr>
<tr>
<td>Purification of monoclonal antibodies</td>
<td>Microtiter filter plate 8,100</td>
<td>General description of microtiter plate methodology with examples of uptake curves, adsorption isotherms, solubility, DOE, and analytical methods</td>
<td>30</td>
</tr>
<tr>
<td>Development/characterization of ion exchange step</td>
<td>Microcolumn 200</td>
<td>General description of a fully automated method using microcolumns and Tecan LHS including genetic algorithm for planning and evaluation experiments, gradient elution runs; examples include optimization of buffer consumption and optimization of a multilinear gradient elution step</td>
<td>39</td>
</tr>
<tr>
<td>Ligand and resin screening</td>
<td>Microtiter plate</td>
<td>Screening of binding and elution conditions for purification of a glycoprotein on several resins; part of a fully developed approach to efficient process development based on ligand screening, optimization, and scale-up</td>
<td>46</td>
</tr>
<tr>
<td>Screening of conditions for purification of monoclonal antibodies</td>
<td>Microtiter plate 100</td>
<td>Screening of optimum conditions for purification of monoclonal antibodies using hydroxyapatite; investigation of salt type and concentration, and pH during early manufacturability studies</td>
<td>47</td>
</tr>
</tbody>
</table>

(Continued)
applications, this rate can be assumed instantaneous and the 
diffusion through the stagnant hydrodynamic layer around 
particles is the rate-limiting mass transfer step. This rate can 
be calculated by estimating values of the external mass trans-
fer coefficient using engineering correlations (28, 29). In 
Figure 20.5b, uptake curves, expressed as the change in the 

solid-phase concentration (capacity), are shown. When the 
rates of change in the solid- and liquid-phase concentrations 
approach zero, the system is believed to reach a state of equi-
librium. This state is described by the concept of the adsorp-
tion isotherm, which always needs to be considered when 
performing HT studies using plates.

### TABLE 20.2 (Continued)

<table>
<thead>
<tr>
<th>Study</th>
<th>Format</th>
<th>Volume of Resin per Single Experiment, (μL)</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| Adsorption isotherms under different condi-
tions | Microtiter plate   | 35                                          | Determination of single and multicomponent adsorption isotherms for monoclonal antibody on a cation exchange resin under variety of conditions including pH and salt concentrations | 10   |
| Dynamic binding capacity determination     | Microtiter plate   | 2, 5, 10, 50                                  | Methodology for estimating dynamic binding capacity using data obtained in a microtiter plate is described; qualitative and quantitative examples are provided for ion exchange and Protein A resins, respectively | 25   |
| Dynamic binding capacities and isotherm de-
termination | Microtiter plate   | 235                                         | Description of experimental methodology to predict dynamic binding capacities from microscale experiments; combination of experimental results and modeling approaches | 48   |
| Screening for ligands/capacities           | Microtiter-based   | 1.6 cm²                                      | Screening for binding capacities of cellulose-based adsorptive membranes; flow-through membrane achieved by means of centrifugal force | 49   |
| (His)- or (GST)- tag based purification     | membrane adsorber  | 25 μg                                       | HT method for purifying protein directly from crude cellular lysates; up to 96 proteins can be purified in 3 h | 50   |
| Designing purification sequence            | Microtiter plate   | 150                                         | Selection of chromatography media; methodology for use of single buffer system is described; analytics based on SELDI-TOF MS and Protein Chip arrays are described | 51   |
| In-process monitoring                      | Microcolumn        | 200                                         | Use of minicolumns for in-process monitoring (cell culture); quantification of process product quality by cation exchange chromatography; description of an automated setup for analytical chromatography | 19   |
| Protein stability                          | Microtiter plate   | None                                        | Protein unfolding curves generated by autotitration of denaturant; in principle, similar methodology could be used to develop a hydrophobic interaction chromatography step by analyzing the solubility of a protein | 52   |
| Refolding of inclusion bodies              | Microtiter plate   | None                                        | Completely automated screening procedure for refolding of inclusion bodies using a 96-well format; 96 refolding buffers were tested using a fractional factorial approach; the screening procedure was validated with 24 proteins in the framework of two Structural Genomics projects; the tests used for this purpose included the use of quality control methods such as circular dichroism, dynamic light scattering, and crystallogenesis | 53   |

Note: VLP: virus-like particle; HTPD: high throughput process development; HTS: high throughput screening; SELDI-TOF MS: surface-enhanced laser desorption/ionization-time of flight mass spectrometry; HT: high throughput; CIEX: cation exchange chromatography; LHS: liquid handling system; GST: glutathione S-transferase.
20.3.2 Isotherm

The adsorption isotherm is a fundamental property of any adsorptive system composed of adsorbent, adsorbing components, and bulk liquid. The rate of mass transfer provides information on how the phase composition changes with time, but the adsorption isotherm describes the final equilibrium state that can be reached in a given adsorptive system. If the concentrations of an adsorbing solute in both phases are known at equilibrium, that is, when the rates of mass transfer are zero, a plot relating these concentrations will describe an adsorption isotherm (Fig. 20.6). The adsorption isotherm can also be constructed using the mass balance equation (Table 20.3) by expressing masses through concentrations in the respective phases and their volumes, and accounting for any solute carried over between different experimental steps as shown by Equation 20.1:

\[
q_i = q_{i-1} + \frac{V_{\text{liquid}}}{V_{\text{resin}}} (c_{0,i} - c_i) + \frac{V_{\text{ret}}}{V_{\text{resin}}} (c_{i-1} - c_i),
\]

(20.1)

where \(q_i\) is the capacity after the \(i\)th incubation, \(q_{i-1}\) is the capacity before the \(i\)th incubation started, \(V_{\text{liquid}}\) is the sample volume added to a well at the beginning of the \(i\)th incubation, \(V_{\text{resin}}\) is the resin volume in a well, \(c_{0,i}\) is the concentration in the sample added in the \(i\)th incubation, \(c_{i-1}\) and \(c_i\) are the initial and final concentrations of the protein in the liquid during the \(i\)th incubation, respectively, and \(V_{\text{ret}}\) is the retained volume of buffer entrapped in resin particles and in pores of the filter at the bottom of each well.

Equation 20.1 represents the general mass balance equation that can be used to construct an adsorption isotherm experimental steps as shown by Equation 20.1:

Table: Table 20.3 Step Attributes to be used in HT Studies using Microtiter Plates

<table>
<thead>
<tr>
<th>Name</th>
<th>Expression</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass balance</td>
<td>( M_{\text{initial}} = M_{\text{nonadsorbed}} + M_{\text{washed}} + M_{\text{eluted}} )</td>
<td>Equation assumes 100% recovery</td>
</tr>
<tr>
<td>Yield(Y_{\text{FT}})</td>
<td>( Y = \frac{M_{\text{nonadsorbed}}}{M_{\text{initial}}} \times 100% )</td>
<td>Yield for flow-through step represents amount of nonbound as compared to amount added</td>
</tr>
<tr>
<td>Yield(Y_{\text{BE}})</td>
<td>( Y = \frac{M_{\text{eluted}}}{M_{\text{initial}}} \times 100% = \frac{M_{\text{eluted}}}{M_{\text{initial}} - M_{\text{nonadsorbed}} - M_{\text{washed}}} \times 100% )</td>
<td>Yield for a bind elute step represents the mass recovered in the elution step as compared to the amount adsorbed</td>
</tr>
<tr>
<td>Recovery</td>
<td>( R = \frac{M_{\text{nonadsorbed}} + M_{\text{eluted}} + M_{\text{washed}}}{M_{\text{initial}}} \times 100% )</td>
<td>Recovery describes overall mass balance over the whole HT experiment. Can only be calculated if all steps quantified</td>
</tr>
<tr>
<td>Step efficiency</td>
<td>( E = \frac{M_{\text{final}}}{M_{\text{before}}} \times 100% )</td>
<td>Efficiency can be defined for any step and can pertain to removal of impurities, adsorption, elution, and so on</td>
</tr>
</tbody>
</table>
from experimental data collected using microtiter filter plates if the concentration of a protein is measured in either the solid or the liquid phase at equilibrium. For a single incubation experiment \((i = 1)\), Equation 20.1 simplifies to Equation 20.2 as the initial concentrations of the protein in the solid (resin) and liquid (retained volume) phases before addition of the sample are equal zero:

\[
q_i = \frac{V_{\text{Liquid}}}{V_{\text{resin}}} (c_{0,i} - c_i) - \frac{V_{\text{ret}}}{V_{\text{resin}}} c_i.
\]  

(20.2)

If the equilibrium adsorption isotherm is known it is possible to use Equation 20.1 to calculate the experimental

---

**Figure 20.4** Schematic of the workflow of a batch uptake experiment occurring in the wells of a microtiter plate, showing the same steps as in a column experiment: equilibration, sample addition, wash, and elution. Work from GE Healthcare Life Sciences, reproduced with permission. (See color insert.)

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**Figure 20.5** Simulated uptake curves describing change on liquid (a) and solid (b) concentrations. Curve A: phase ratio of 4, \(c_0 = 1\) g/L. Curve B: phase ratio of 4, \(c_0 = 10\) g/L. Curve C: phase ratio of 10, \(c_0 = 1\) g/L. Curve D: phase ratio of 33, \(c_0 = 2\) g/L. The respective curves in both panels are linked via Equation 20.1. Work from GE Healthcare Life Sciences, reproduced with permission.
conditions (phase ratios) necessary to reach a desired level of capacity for a given sample concentration and incubation step. The principle of this approach is based on the concept of an operating line as depicted in Figure 20.6, where the operating lines for both low and high phase ratios are shown. The two cases are based on the same initial sample concentration. In the case of a low phase ratio, the protein is depleted from the liquid phase, resulting in a low concentration, which gives a low equilibrium capacity. In order to reach a higher capacity, more protein needs to be added to the system, and the process must be repeated until the desired capacity is reached. Using a higher phase ratio one sample addition may suffice to reach the desired capacity. For the sake of graphical representation, Figure 20.6 was prepared assuming that the retained volume, $V_{\text{ret}}$, in Equation 20.1 is negligible. $V_{\text{ret}}$ is provided for all types of PreDictor plates, but can be quantified for custom-made plates following a procedure described elsewhere (11).

20.3.3 Mixing

An important aspect of the experimental workflow for any HT experiments using plates is to secure adequate mixing conditions under which the measured capacities are independent of mixing intensity. This is especially important when the kinetics of protein uptake is studied. Without sufficient agitation, the rate limiting mass transfer is different in the wells than in a chromatography column. There are many factors that affect the mixing efficiency, such as well aspect ratio (diameter/height), liquid volume, liquid viscosity/ density, particle density, and orbital amplitude of the shaking table. For instance, for a specific aspect ratio there will be an optimum liquid volume in the well that will result in a vortex that will “sweep” the bottom filter, thus lifting gel particles deposited on the filter during vacuum filtration or a centrifugation process. Too high liquid volume will result in either too weak sweeping motion or a cross-contaminations between neighboring wells due to vortices escaping the wells. The recommendation, with respect to agitation speed and liquid volume for PreDictor plates, is an agitation speed of 1100 rpm with a 3-mm circular centripetal movement and a liquid volume between 100 and 300 $\mu$L in the wells. Similar agitation speeds and liquid volumes are recommended for chromatographic screening studies (25, 30, 31).

20.4 IMPLEMENTATION OF DESIGN OF EXPERIMENTS IN THE HIGH THROUGHPUT PROCESS DEVELOPMENT (HTPD) AREA

The HT formats, whether plates or microcolumns, are designed for parallelization and low sample consumption. Therefore, a larger experimental space can be explored, giving a better understanding of the process. In the HT formats the same experimental designs can be used as in other formats, but it is affordable to do more experiments. More factors (input variables), more factor levels, or more advanced designs (more experimental points) can be used to maximize the information gathered or the reliability of the result. The experimental design can vary from simple multilevel designs to more statistical designs (32, 33), as illustrated in Figure 20.7. Dedicated Design of Experiments (DOE) software, such as Design Expert (Stat-Ease, Minneapolis, MN) and MODDE (Umetrics, Umeå, Sweden) will be useful for planning of experiments and evaluation of results. Recently, a generalized software solution for HTPD applications has become available from GE Healthcare. The company introduced a software package under the name Assist to plan and evaluate HT experiments performed using PreDictor plates.

Irrespective of the experimental design that is used, replication is recommended to secure the reliability of the data. For statistical DOE, replication is also necessary to discriminate between lack of fit and experimental error. The analytics and the number of outputs (responses) being used also affects the number of replicates that should be used. Because of the small scale of the HT techniques, replication of condition may be necessary in order to obtain enough sample for the analytics.

The quality of the data obtained can also be affected by the way data are analyzed. For plate experiments, the amount of bound target molecule can either be calculated from the decrease in concentration/mass of nonbound target molecule via the mass balance equation (Eq. 20.1) or directly from the
eluted amount of bound target molecule (Eq. 20.3):

\[
q = \frac{\sum_{i=1}^{N} (V_{\text{elution},i} \cdot c_{\text{elution},i})}{V_{\text{resin}}}, \quad (20.3)
\]

where \(V_{\text{elution},i}\) is the volume of the \(i\)th eluate, \(c_{\text{elution},i}\) is the concentration of target molecule in the \(i\)th eluate, and \(N\) is the number of elution steps.

If the capacity is quantified by use of the mass balance equation, the error in \(c\) will propagate to the capacity via the phase ratio. This means that for a given error in \(c\), the error in \(q\) will be larger for a high phase ratio than for a low phase ratio. If \(c\) is calculated via the mass balance equation via \(q\), then the reverse is true for the propagation of the error in \(c\). For a given error in \(q\), a low phase ratio will give higher error in \(c\) than a high phase ratio (Fig. 20.8).

Carrying out of the experimental work is traditionally the bottleneck, and when HT techniques are applied, the bottleneck is not removed, but rather is moved towards the analytics. The number of samples to analyze is large and depending on the analytical methods and degree of automation, obtaining the results may take time. This needs also to be considered when designing the experiments. For instance, if different tests are being considered for each sample and one of the tests can be independently performed much faster than the others tests, a sequential approach to sample analysis should be considered. In this approach only the sample that passes criteria set for one of the tests should be analyzed further.

20.5 ANALYTICS

As mentioned in the previous section, HT experiments will generate a large number of samples to be analyzed. This requires analytical techniques that not only provide sufficient
accuracy and precision but also a reasonable throughput, so the analytical part of HT studies does not become a bottleneck. In Table 20.4 are listed examples of analytical methods used in HT studies related to protein separations. Obviously, the more complex the composition of a sample the more sophisticated method should be used, and the more time will be required per sample to obtain quantitative data. For instance, for a one-component system, the sample concentration can be easily measured using spectrophotometric methods. These methods can also be used if impurity levels are low (ppm levels). However, if for instance the sample to be analysed contains monomer and aggregates, a typical analysis based on size exclusion chromatography (SEC) would require 15–20 min per sample, which gives a total analysis time of more than 24 h for one microtiter plate. Methods based on light scattering have been tested (34), but the sample preparation step focusing on removal of interfering particulates by filtration seems to question the HT character of this method. Recently, HT methods based on electrochemiluminescence (Meso Scale Discovery, Gaithersburg, MA) for determination of host cell proteins and protein A have been successfully implemented into process development work at Genentech (35).

Despite advances in HT techniques for sample analysis, analytics are and probably will be considered a bottleneck in HT studies. It could be argued that the more difficult the analysis, the lower the throughput of a given method and in such a case the analytical effort should be based on a sequential sample selection approach. The sequential approach should be based on a hierarchical analysis workflow, in which the samples are pushed through the analysis starting with the simplest, and the quickest method, and finished with a method that is slowest and/or most expensive. In each step of the sequential method samples are discarded based on some preset criteria; for example, if the recovery calculated for a sample is below a certain threshold, then any further analysis of this sample may not be justifiable, and vice versa. Applying the sequential approach will lead to a reduction in the overall number of analyses, which in some cases may remove the bottleneck associated with this part of the HT studies without sacrificing the information content extracted from an experiment.

### 20.6 APPLICATIONS

#### 20.6.1 Development and Optimization of a Capture Step

In downstream processing the goal for the initial purification step is to remove as many impurities as possible and to concentrate the product, thus reducing the volume of the process stream. From the process perspective it is important to know under which conditions the capture step can be operated either at maximum productivity or at minimum cost. In order to find these conditions the typical process development work focuses on optimizing the loading, wash, and elution steps with respect to dynamic binding capacity, yield, and purity. Resin lifetime studies are also performed.

#### 20.6.1.1 Estimating Effect of Residence Time on Dynamic Binding Capacity Using Microtiter Plates

Rates of protein uptake measured during experiments focusing on the effect of incubation time on binding capacity can be used to describe the dynamics of protein uptake in a chromatography column (25, 36), which in turn can be used to estimate the effect of residence time on the dynamic binding capacity at different process conditions. Using microtiter plates this effect can be estimated in either a qualitative or quantitative manner (25). For the qualitative estimation, it is only necessary to measure binding capacities at two incubation times. This method is useful when investigating effects of process conditions, such as pH and/or ionic strength, on the dynamic

<table>
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<tr>
<th>Assay</th>
<th>Methods</th>
<th>Samples/day</th>
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</thead>
<tbody>
<tr>
<td>Host cell proteins (HCP)</td>
<td>ELISA</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Electrochemiluminescence</td>
<td>&gt;96</td>
</tr>
<tr>
<td></td>
<td>Gyros</td>
<td>112</td>
</tr>
<tr>
<td>Aggregate content</td>
<td>Dynamic light scattering</td>
<td>&gt;96</td>
</tr>
<tr>
<td></td>
<td>Size exclusion chromatography</td>
<td>4</td>
</tr>
<tr>
<td>Concentration</td>
<td>UV microtiter plate reader</td>
<td>&gt;96</td>
</tr>
<tr>
<td></td>
<td>Biolayer interferometry</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Chip electrophoresis</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Surface plasmon resonance</td>
<td>4</td>
</tr>
<tr>
<td>Total protein content</td>
<td>Bradford assay, Bicinchoninic acid assay (BCA)</td>
<td>&gt;96</td>
</tr>
</tbody>
</table>

*The values listed should be seen as a rough guideline. Throughput depends on degree of automation and parallelization. Sensitivity of the different methods is not assessed and will of course also affect the selection of method.
binding capacity. In these studies, relative differences in dynamic binding capacities at short residence times can be estimated from differences in binding capacities obtained in microtiter plates after short incubations times. Similarly, the relative differences in dynamic binding capacities at longer residence times would be described using data obtained at the long incubation time in the plate experiment. The principle of the qualitative method is based on similar contact times, where the contact time is defined as the time during which a resin is exposed to the sample, which in the case of column experiments would be the duration of the loading step (25).

Examples of results obtained using the qualitative method are shown in Figures 20.9 and 20.10. In Figure 20.9, capacity maps representing the effect of pH and salt concentration on the binding capacity of amylglucosidase on Capto™ DEAE obtained at two incubation times are shown. In Figure 20.10, some data points extracted from Figure 20.9 are compared with dynamic binding capacities obtained at the same conditions in 2 mL chromatography columns packed with Capto DEAE.

The agreement between the results obtained with the two formats is striking not only with respect to trends, but also from the capacity level perspective, although the latter may not always be the case. It is also worth mentioning that the capacity maps are a very powerful method for identifying optimum conditions to maximize binding capacities and to determine if these conditions are independent of the residence time under which the capture step will be operated. In this example, the optimum salt concentration decreases with the increase in the incubation time as shown in Figure 20.9.

In contrast to the qualitative method, the quantitative method relies on the determination of mass transfer rates from an entire uptake curve described by several data points. Detailed methods on how to obtain a whole uptake curve using a single microtiter plate have been described elsewhere (25, 30, 31, 36). In Figure 20.11, an example is shown of uptake curves describing the rates of adsorption of a monoclonal antibody (MAb) on MabSelect SuRe™ obtained at different initial MAb concentrations. These data were used to determine a characteristic time constant for the adsorption step by fitting a mass transfer model to the data. The model was then adjusted to describe the same adsorption process in a chromatography column, and the effect of residence time on dynamic binding capacity was estimated using the values of model parameters determined from the batch uptake experiment. The model equations used to describe the microtiter plate generated uptake curves and the effect of residence time on the dynamic binding capacity are given by Equations 20.4 and 20.5, respectively:

\[
\frac{D_mC_0}{R^2q_m} t = I_1 - I_2
\]  

(20.4)

where

\[
I_1 = \frac{1}{6\Lambda} \ln \left[ \frac{\lambda^3 + \eta^3}{\lambda^3 + 1} \right] + \frac{1}{\Lambda \sqrt{3}} \times \left[ \tan^{-1} \left( \frac{2\eta - \lambda}{\lambda \sqrt{3}} \right) - \tan^{-1} \left( \frac{2 - \lambda}{\lambda \sqrt{3}} \right) \right] 
\]  

(20.4a)

\[
I_2 = \frac{1}{3\Lambda} \ln \left( \frac{\lambda^3 + \eta^3}{\lambda^3 + 1} \right) 
\]  

(20.4b)

\[
\Lambda = \frac{V_{\text{resin} q_m}}{V_{L_s} C_0} 
\]  

(20.4c)

![Figure 20.9](image-url)  

Figure 20.9  Binding capacity maps showing effect of pH and ionic strength on adsorption of amylglucosidase on Capto™ DEAE measured in PreDictor™ plates filled with 2 μL resin per well after (a) 2 min; (b) 60 min; and (c) 20 h of incubation. Work from GE Healthcare Life Sciences, reproduced with permission. (See color insert.)
\[ h = \frac{1}{C_0} \left( \frac{1}{C_0} \right)^{\frac{3}{2}} (20.4d) \]

and \( C(t) \) is the protein concentration at time \( t \) after sample addition, \( C_0 \) is the initial protein concentration, \( V_{\text{resin}} \) is the resin volume, \( V_{\text{liq}} \) is the liquid volume, \( q_m \) is the equilibrium concentration in the solid phase, \( D_e \) is the effective intraparticle diffusion coefficient, and \( R_p \) is the radius of the adsorbent particle.

Solving the above equations will yield a relationship between the change in the liquid concentration, \( C(t) \), as a function of time. Model parameters that need to be estimated using Equation 20.4 are \( D_e \) and \( q_m \).

Assuming a negligible effect of the packing quality on the size of the mass transfer zone in a column, the following equations for the dynamic binding capacity as a function of process conditions (column length and flow rate) can be obtained (28):

\[
q_{10\%} \approx \frac{q_m'}{0.364N - 0.0612N^2 + 0.0042N^3} \left[ 1 - 1.03N^{-1} \right]_{N \geq 2.75} \quad (20.5)
\]

where

\[
N = \frac{15(1 - \varepsilon)D_e}{R_p^2 \tau_{\text{app}}} \quad (20.5a)
\]

and \( q_m' \) and \( q_{10\%} \) are the equilibrium capacity for a given feed concentration calculated using the adsorption isotherm of choice and dynamic binding capacity at 10\% breakthrough, respectively. \( \tau_{\text{app}} \) is the apparent residence time defined as the ratio between column volume and flow rate.

It should be mentioned at this point that Equations 20.4 and 20.5 describe a mass transfer model based on the so-called shrinking core adsorption process. The shrinking

---

**Figure 20.10** Effect of pH at 80 mM salt (a) and ionic strength at pH 7.4 (b), expressed through total chloride concentration, on binding capacities of amylglucosidase on a prototype of Capto™ DEAE measured in PreDector™ plates filled with 2 μL of resin and in 2 mL chromatography columns and different contact times. Plates: \( \triangle \), 15 min; 60 min; 12 h. Columns: \( \square \), 1 min residence time (~60 min loading); \( \blacksquare \), 5 min residence time (~200 min loading). Work from GE Healthcare Life Sciences, reproduced with permission. (See color insert.)

**Figure 20.11** Uptake curves for different initial concentrations of MAb (total) on MabSelect SuRe™. Work from GE Healthcare Life Sciences, reproduced with permission.
core process is not a realistic mass transfer mechanism, but it is a fairly reasonable representation of an adsorption process that is governed by the pore diffusion mechanism and a favorable adsorption isotherm. If the rate-limiting mass transfer is not based on the pore diffusion, different mass transfer models should be used to describe batch uptake data and to predict column behavior.

20.6.1.2 Effectiveness of Wash and Elution Steps

The uptake curves shown in Figure 20.11 can also be used to determine experimental conditions that can be used for wash and elution studies. In these types of studies it is important to saturate the resin particles to a predetermined capacity level that would represent the capacity obtained in a chromatography column under the chosen process conditions (residence time). This is done by following the procedure described when discussing the concepts of the operating line and adsorption isotherm. An apparent adsorption isotherm (Fig. 20.12) can be generated using the data obtained at the longest incubation time (60 min) shown in Figure 20.11. According to these data, for 20 μL resin per well and a sample concentration of 1.0 g/L, it was necessary to add 300 μL of sample to reach a capacity of ~30 g/L. After loading MabSelect SuRe with the feed containing antibody monomer and aggregates, the effect of pH and salt concentration on selective elution of monomer from MabSelect SuRe was investigated. The results obtained, represented in terms of overall yield and monomer content (Fig. 20.13), showed that salt concentration had no effect on either yield or purity, purity decreased with decreasing pH, whereas the yield showed the opposite trend. Using the same data it can be shown that the monomer yield has an optimum with respect to pH, giving the highest yield between pH 3.5 and 4.0 (data not shown).

It should be mentioned at this point that the level of details in the data shown above was only achievable because of the HT approach used in this study. If similar studies were to be performed using small scale columns, the sample consumption as well as experimental time necessary to complete the studies would be prohibitive for such an approach.

20.6.1.3 Effect of CIP Treatment on Resin Performance

Lifetime studies for chromatography resins are one of the most important studies to be made during process development. At the same time, looking at possible fouling of a column as well as investigating efficiencies of different cleaning solution requires long and tedious studies that could consume a significant amount of sample. However, it has recently been shown that the cleaning efficiency of various CIP conditions on the extent of carry-over as well as their effect on binding capacity can be evaluated using the 96-well filter microtiter plate format (37).

In this study, MabSelect SuRe and MabSelect™ (50 μL resin/well, 1 plate per resin) were artificially fouled by incubating them with an E. coli lysate spiked with polyclonal IgG followed by overnight incubation with 2.9 M ammonium sulfate, 0.6 M phosphoric acid, pH 2.5, as fouling agent. Numerous potential CIP agents were evaluated for cleaning efficiency of the artificially fouled media by incubating the media with the CIP agents for 15 min. After incubation, resin samples were then removed and boiled with SDS sample buffer for analysis using SDS-PAGE. Details of the...
procedure are provided in Reference 38. The results obtained are shown in Figure 20.14.

The effect of cleaning solutions on resin stability, defined as the change in binding capacity measured before and after exposure to the cleaning chemicals, was investigated using PreDictor MabSelect SuRe and PreDictor MabSelect 6 µL resin/well plates. By storing the resins in a CIP solution for 18 h, conditions equivalent to the total CIP exposure time during 180 cycles (with 30 min CIP/sanitization-in-place (SIP) every fifth cycle) were generated. After storage, IgG binding capacities were measured by incubating the two resins with 0.3 mg IgG per well for 60 min and measuring the amount of eluted IgG. The study was designed as a DOE study using MODDE software for the experimental set-up and evaluation of data. The results obtained (Fig. 20.15) show that MabSelect SuRe (alkali stabilized Protein A) maintained the IgG binding capacity at higher NaOH concentrations compared to MabSelect (recombinant Protein A). Obviously, a more extensive protocol for a resin lifetime study can be easily implemented into the HT workflow when using plates filled with chromatography resin.

20.6.2 Screening of Ligand Libraries

If the desired purity is not achievable using standard chromatography resins, unique selectivities necessary for separation of impurities from the product can be found by screening of multimodal ligand libraries. Libraries of multimodal cation and anion exchangers and HIC resins can be quickly screened using PreDictor plates. Providing the libraries are diversified, the probability of finding a combination of ligand and process conditions (pH, salt, etc.) that will enable the difficult separation is usually fairly high. Figure 20.16 shows results obtained from a screening experiment in which a library of...
multimodal anion exchange ligands was screened for binding capacities at different pH levels. The results clearly show that different capacity levels were obtained for different ligands with the same binding conditions. Furthermore, the results obtained for ligands 2, 7, and 19 indicated a possible selectivity reversal at different pHs, indicating the diversity of this ligand library.

Combination of ligand libraries and HT methods could lead to finding conditions under which difficult separations would become feasible even at a process scale, where high yields and throughputs are necessary.

### 20.7 SUMMARY AND FUTURE TRENDS

HT methods for exploring large experimental spaces to develop better manufacturing processes are here to stay. They improve the productivity of process development groups, and reduce the development cost. Depending on the method chosen, sample requirements and development time can be reduced by up to 50 and 10 times, respectively (36). In principle, the HT tools commercially available at the moment allow for the development of all types of chromatographic separations, regardless of whether these separations are based on bind-elute or flow-through steps. However, the success of these HT methods will depend on correctly chosen experimental protocols and, therefore, it is of paramount importance to validate all in-house experimental protocols employing HT methods. For instance, for the microtiter plate based methods it is especially important to make sure that the reproducibility of resin volume in a single well is very high. From this perspective one could argue that the use of commercially available plates may be a faster way towards a fully validated HT approach to process development, as the vendors of these plates have developed reliable production methods (26). In addition, understanding results obtained using HT formats and their applicability for predicting process scale separations will also be important.

Undoubtedly, although any HT method will increase the number of conditions tested per unit time and per unit mass of sample, they will generate new bottlenecks either related to analytics or to the data evaluation part of the experimental workflow. Therefore, an efficient de-bottlenecking of the HT process development workflow will determine the future of this approach from the process development perspective. The de-bottlenecking will most likely focus on the three following areas: improved analytics delivering quick analysis of large number of samples generated; availability of dedicated software packages to facilitate planning and evaluation of HT studies; and simple but fully automated laboratory systems designed with HT process development in mind. The analysis part should be based on HT, analytical methods and algorithms that would allow a smart selection of a smaller set of samples from the whole sample population. The sample set that would still correctly capture all trends within the large data set. The dedicated software should include the ability to predict small or large scale column performance from either batch or other formats. The automated laboratory system should include buffer preparation capabilities, the step that is currently perceived as one of the major bottlenecks in HT workflows, and maybe even an artificial intelligence module for planning and execution of experiments (39).

With these three areas addressed, the next big leap in the area of HT screening methods for the development of chromatographic separations will be related to further downscaling of these techniques. After all, it can be argued that a chromatographic separation can be characterized using a single chromatography particle as shown by studies using confocal microscopy (40).

### 20.8 REFERENCES

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