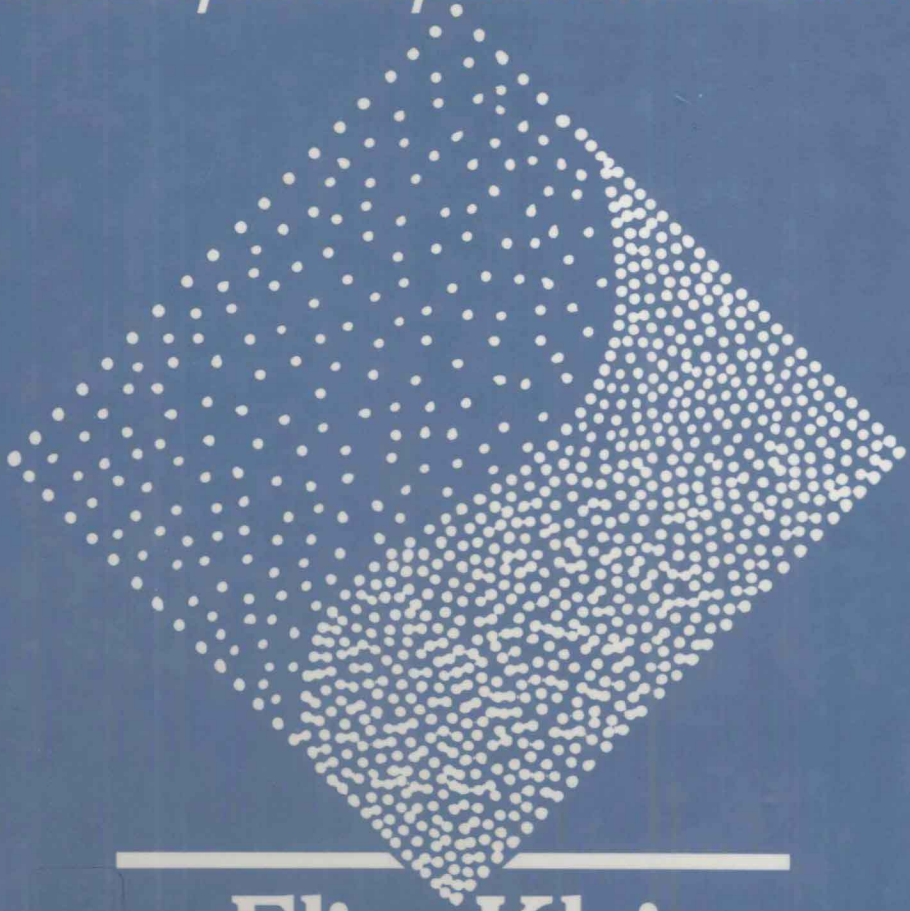
AFFINITY MEMBRANES

Their Chemistry & Performance in Adsorptive Separation Processes



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To Mania and Rushka, who sent me back to school . . .
to Beverly, who kept me there.

PREFACE

In 1957 the Artificial Kidney Program of the National Institutes of Health was looking for a "few good people," to paraphrase the Marines, with an interest in improving artificial kidney membranes. Since several schoolmates during my teenyears had died from glomerulonephritis, I became involved in the program. Fifteen years later I was a specialist in membrane synthesis and characterization. That participation led indirectly to writing this book. At the time a number of us posed the following naive question to the clinicians in the program: "What do our membranes need to remove from the patients' blood?" There was no clear answer to that question then, nor is there now, because we don't have membranes that can selectively remove toxins and thus provide the basis for clinical experiments. Thus the search for membranes with highly specific removal capabilities is not a new topic. It applies to problems in medicine, biotechnology, and industrial separations.

In 1981 I joined the faculty of a medical school and began working with clinicians, engineers, and biochemists interested in separating biologicals. Their major tool is column chromatography of one sort or another. Chromatography is a process that produces exquisitively sensitive separations, at the expense of very low productivity. By contrast, the membranes I was familiar with before arriving at the University of Louisville provide very high productivity but rather poor separations. If large-scale biochemical separations—or even smaller-scale therapeutic devices—are to operate economically and with high specificity, it is clear that one has to find the means to combine the attractive aspects of each separation method. This text is about that search for efficient, selective separation devices.

Before writing the book, I had to learn more about affinity-based separations from the biochemical literature. Fortunately a six-month sabbatical in 1988 came at just the right time to do this. A number of friends in the United States and in Germany made it easy for me to read the right books and papers and to begin writing this book. With that information in hand my coworkers and I started lab work to produce membranes that exhibited biochemical specificity using covalently linked ligands. As the work progressed, it became clear that we had to integrate the membrane formation process with the biochemistry of the immobilized ligands in order to achieve our goals of selectivity and high productivity. We have found that the available membranes for ultrafiltration and dialysis are often incompatible with the modification reactions needed to link ligands. Moreover, many

microfiltration membranes are so chemically inert that covalent linkage is impractical. The work continues; this book is intended to interest other investigators in joining the search. More specifically, the book is written for those biochemists who want to learn how synthetic membranes are made, tested, and modified to bind a variety of ligands. It is also addressed to those synthetic membrane chemists and chemical engineers who want to learn more about the process biochemists call *affinity separation*. Progress in this field will require interest from researchers in all three disciplines.

The book is divided into three major sections. The first section (Chapters 1 to 4) deals with the chemistry of the problem: the properties of substrates, how to identify ligands of interest, how to link them to synthetic membranes, and what some kinetic limitations of frontal elution chromatography are. The second section (Chapters 5 to 8) is a detailed discussion of the various ligands used in affinity separations and the formation processes of semipermeable membranes. The last section (Chapters 9 and 10) discusses filtration processes using membranes and the kinetics of separations based on affinity membranes.

A number of colleagues were of great help in this endeavor. As is usual in a single-author book, the errors are mine but much of the credit for any success the book may enjoy belongs to them. Drs. Ulrich Baurmeister and Gunther von Sengbush of AKZO Fibers and Polymers Division, Wuppertal, Germany provided a great deal of help during my sabbatical. Dr. Dieter Rehm of Johann Wolfgang Goethe University, Frankfurt, Germany, made it possible for me to search *Chemical Abstracts* via phone lines while in Germany. That greatly facilitated the literature research. Drs. Steve Matson and Steve Kessler of Sepracor, Inc., Marlborough, Massachusetts, were most helpful with critical reviews of various sections of the book, as were Dr. Reichelt, of the AKZO central laboratories in Obernburg, Germany and Dr. Robert Petersen of FilmTec, Minneapolis, Minnesota.

My appreciation goes also to all those investigators whose published works made it possible for me to synthesize the concepts outlined here. Finally, my thanks go to Mrs. Cara Heybach, my secretary, whose organizational and computer skills made the writing chores much easier.

ELIAS KLEIN

Louisville, KY
November, 1990

AFFINITY MEMBRANES

CONTENTS

1 Introduction

This monograph is about the use of synthetic membranes for achieving biologically specific separations. The term *affinity membranes* used in the title is technically not a correct description, but then, neither is *affinity chromatography*, the term commonly applied to columnar separations. *Affinity* refers to any attractive force. It is a much more comprehensive term than the phenomena that cause the highly specific interactions among such biological molecules as enzymes, antibodies, and nucleic acids. Perhaps the most exact term for the material discussed here is *biological affinity*. However, since much of the discussion will be based on the biochemical literature in which *affinity* and *affinity chromatography* are already ingrained, these terms will be used here too. On the other hand, this text will not cover any of the bulk adsorption materials described extensively in the chemical process industry, such as activated carbon or ion-exchange resins.

This book is written for the polymer and/or membrane chemist interested in developing synthetic membranes for highly specific separations. The text is also written for the biochemist or bioengineer who wishes to learn more about the preparation and uses of synthetic membranes in biological separations. These might include membranes for (a) therapeutic devices to treat autoimmune diseases; (b) processes to recover products from gene-modified bacteria or mammalian cells; or (c) membrane-bound enzyme reactors. Each of these applications requires a degree of selectivity that is impossible to achieve with synthetic membrane separators as they are customarily produced today. The necessary attribute of specificity, required for most biological separations, is missing from current membranes. But specificity—for analyses, for isolation, or for mechanistic studies—is what biochemists have concerned themselves with for a long time. Thus this book begins with a review of what biochemists have done to provide themselves with the materials needed for highly selective purifications. Then we will examine how to provide a comparable degree of specificity to membranes and how these membranes differ from sorbents used in columnar geometries.

This book is possible now as the result of a series of coincidences in the 1960s. During the early years of that decade Loeb and Sourirajan (1) developed the first desalination membrane, made from cellulose acetate, which provided high selectivity while producing commercially useful

quantities of purified water. Reid and Breton (2) had suggested in the late 1950s that reverse osmosis was a possible means of purifying water, but apparently Loeb and Sourirajan were unaware of Reid's work (3). Fortunately, the U.S. Department of the Interior then established an external research program, the Office of Saline Water (OSW), to investigate new and technologically advanced methods of water purification. After Loeb and Sourirajan pubished their work, a number of research groups sponsored by OSW were actively investigating new techniques of membrane formation. They studied not only the cellulose acetate already reported but also a wide variety of other synthetic polymers. Not much of the early research was successful in producing new desalination products, but a great deal of understanding about the structures of synthetic membranes and the routes to their preparation was obtained. Stimulated by this search for improved reverse-osmosis membranes, a number of investigators found applications for other membrane processes suitable for less stringent separations in pharmaceutical, food processing, and heavy manufacturing industries. This was the beginning of the separation processes now generically termed *ultrafiltration*.

At the same time that desalination research was being supported by strong interest from government, industry, and academe, the National Institutes of Health (NIH) started a much smaller research program called the Artificial Kidney Program. Chronic renal failure was an incurable and fatal disease in the 1960s. Two experimental demonstrations gave hope that palliative procedures, while not effecting cures, could prolong life with a tolerable therapy. One was the development by Scribner et al. (4) of permanent plastic access to the patient's vein that remained patent for some months with continued use; the other, much earlier event, was Kolff and Berk's (5) demonstration that hemodialysis could be used to reestablish the physiological acid–base balance and remove metabolic end products, such as urea, creatinine, and inorganic phosphates and sulfates. Dialysis had already been demonstrated as a life-saving procedure in the treatment of acute renal failure, but the possibility of using repeated hemodialysis treatments to maintain life over extended periods now seemed practicable.

The first membranes used for hemodialysis were made from never-dried Visking, a cellulose film then manufactured for preserving cigarettes. Initially the hemodialysis process was extremely costly and rather primitive, with extensive labor requirements and long dialysis periods (over 10 hrs three times a week). Treatment complications included anemia and bone resorption. Yet, it was clear that the lives of many thousands of patients could be extended, with a reasonable quality of life. Much of the effort in the Artificial Kidney Program was devoted to understanding the altered physiology and endocrinology of end-stage renal patients, but improvement of the dialytic process was another major goal. The NIH program sought to define better the requirements for hemodialysis and then develop improved devices to meet those requirements. This led to new designs (6)

and materials for more efficient mass transfer, always within the constraints of shear sensitivity imposed by blood cells and plasma proteins.

As a result, a number of research programs were started to develop membranes with specific mass-transfer characteristics but, more importantly, with a concern for their biocompatibility. Protein adsorption to membranes, protein binding, and activation of the enzymatic clotting cascade became of interest to membrane chemists and engineers. Not surprisingly, some of the research groups were involved in both desalination and hemodialysis research. From these investigators have come a series of membrane developments that today encompass different membrane configurations (tubes, hollow fibers, and spiral-wound membranes) and differing membrane types (e.g., interfacial composites, homogeneous asymmetric membranes, thin-film dialytic, and phase-separated membranes). In addition to the improvements in therapeutic equipment, the last 20 years has brought better understanding of the mechanisms involved in forming membranes from synthetic polymers and about the surface interactions between the membrane and the solution being processed. As a consequence, there are now membranes available for separating plasma from cells (7) and lipoproteins from the immunoglobulins and other plasma components (8); some membranes can even achieve partial separations between albumin and IgM in serum. The more selective membranes needed for separating albumin from IgG purely mechanically appear to be an elusive goal, perhaps not even theoretically attainable.

Quite independent of this research, a scientific explosion was occurring in the field of biochemical separations. Reports describing new techniques of separation and identification, developed specifically for the complexities of natural mixtures, appeared monthly. Nishikawa (9), who traces the historical development of enzyme isolation studies, points out that it was not until 1944 that an enzyme, amylase, was purified significantly by an adsorptive technique. The introduction of ion exchance resins in the 1950s allowed biochemists to separate enzymes and other proteins by class-specific chromatographic procedures. In 1953 Lerman (10) first demonstrated that a covalent linkage to cellulose could be utilized to immobilize enzymes without inactivating their catalytic functions. His work was not widely recognized. It was not until the early 1960s that a rapid proliferation of biospecific separations developed using the general principle of adsorbing a ligate to a ligand that had somehow been immobilized. Near the end of that decade (1968) Cuatrecasas et al., (11) published the paper describing the simple application of the cyanogen bromide coupling reaction, previously developed by Axen and Porath (12), for immobilizing several enzymes. In the same paper they introduced the term *affinity chromatography*. Both affinity chromatography and membrane preparation have been active fields of research. Although conducted independently of each other, they share a common objective—solute separations.

High productivity and relatively low specificity are the characteristics of

membrane separations. The solute sizes being isolated by membrane separators classify the separation process. A good *reverse osmosis (RO) membrane* will retain all ionized electrolytes, as well as organic species heavier than 100 daltons (D). In return for this "tight" retentive structure, these membranes require high pressure differentials to produce practical flows of purified solvent. But even these membranes are not very specific in the retention of small organic molecules. For example, reverse osmosis membranes that are capable of recovering 99%+ pure water from saline solutions are ineffective in separating methanol or ethanol from water.

Ultrafiltration membranes, which readily allow permeation of small molecules, provide much higher solvent fluxes and are used with less than 100-psi pressure difference. They are generally not considered suitable for separating solutes that differ in molecular weight by less than a factor of 10. When applied to protein separations, some ultrafilters can separate albumin (62,200 D) and IgG (150,000 D) from IgM (650,000 D), but not albumin from IgG. Scott et al. (13) found that monoclonal antibodies could not be separated efficiently, by ultrafiltration alone, from low-molecular-weight culture components, especially those of 20,000 D or more. Thus, ultrafiltration membranes alone are not adequate even for simple serum protein fractionation or for antibody recovery.

Microfilters, which only retain particles of colloidal and larger sizes, are widely used to sterilize pharmaceutical fluids and to remove cellular debris and other colloidal matter from aqueous streams, as well as particulates from some solvents. However, microfilters are generally incapable of separating any truly dissolved species from its solvent.

Each of these membrane classes—reverse osmosis, ultrafiltration, and microfiltration—is capable of processing large quantities of fluids rapidly and has found applications in separations that do not require high selectivity. When compared with the highly specific separation resulting from the interaction of an antigen with its corresponding antibody, or an enzyme with its substrate, the goal of the membrane chemist becomes quite clear: how to impart solute specificity to membrane separations on a process scale without losing the membrane's high productivity. This monograph is an attempt to outline how this goal might be achieved using biologically selective specificity. Some membrane research reports published recently (14,15) address this same goal. For the most part, however, work on affinity membranes has been directed at small laboratory applications using dead-end filtration to recover minute amounts of ligate.

Chapter 2 will trace the development of affinity chromatography. Subsequent chapters will provide a background of support materials for some biologically useful separations and the chemistry used for ligand immobilization. Then we will turn our attention to the preparation and characteristics of membranes that could be used for affinity membranes. Finally, some projections will be made on the relative productivity of affinity membranes vis-à-vis affinity columns. Intuitively one can foresee that, as the cross section of an adsorptive column increases and its depth decreases (at con-

stant bed volume), the permitted rate of perfusion (because of reduced pressure drop) will increase. What is not so obvious is that the diminished diffusion path in a membrane structure compared with the diffusion path in a bead column will have an even greater effect on performance than does the difference in geometry.

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2 Affinity Adsorption

The 1968 paper by Cuatrecasas et al. (1) is generally credited with creating the stimulus that led to the rapid proliferation of *affinity chromatography*. Certainly the description of a simple activation and coupling procedure (which has now been largely displaced by improved methodologies) provided a great impetus to introducing the technique as a general analytical method for biochemicals. However, the phenomena that permit the separations Cuatrecasas and co-workers described had already been discovered many years before during the development of modern enzymology and immunology. Early in the century, Willstatter (2), observed that lipases adsorbed to lipids and to inorganics, such as aluminum hydroxide gel or kaolin. He also recognized that the enzymes desorbed more readily from the inorganics than from the enzyme's substrate, telling something about the relative association constants. Similarly, Starkenstein (3) in 1910 had encountered biospecific adsorption of amylase bound to starch particles, although he did not recognize it. Ambard (4) also noted this adsorption and later used it in an assay for amylase without fully appreciating its utility in the purification of the enzyme. The specificity of this adsorption allowed Holmbergh (5) to report that α-amylase adsorbed to insoluble starch particles suspended in aqueous ethanol, while β-amylase did not. This may have been the first recognition of the solvent effect on ligand–ligate binding. Hockenhull and Herbert (6) used this finding in 1944 to purify an amylase some 300-fold from its crude isolate, demonstrating perhaps the first affinity purification of a biological product from a complex mixture of other proteins.

The concept of forming specific complexes between an enzyme and one of its insoluble substrates soon became a useful tool for isolating active enzymes. The initial attempts to exploit this concept relied on insolubilizing the substrate itself, as had proven so useful in the case of starch. For example, Bar-Eli and Katchalski (7) linked a polytyrosyl side chain to trypsin and cross-linked this derivative by reacting it with a diazotized copolymer of *p*-amino-phenylalanine and leucine. Although the first trypsin derivative was still water-soluble, the second was insoluble and could be isolated by centrifugation or by filtration. The insoluble complex exhibited

the same catalytic activity as soluble trypsin, such as proteolytic activity and inhibition of this activity by soybean trypsin inhibitor (8).

However, not all substrates (or inhibitors) that bind specifically to an enzyme are amenable to insolubilization, whether by cross-linking or binding to some solid phase. It was therefore the introduction of ion exchange resins as the binding matrix, used by Paleus and Neilands (9) to purify cytochrome C, that broadened the range of possible solid-phase ligands. The use of charged resins provided a generic class of ligand that, although less specific in targeting ligates, permitted many separations to be performed with a single matrix. Rapid development of ligand-based purification thereafter led to a number of modified polymers, generally with ion exchange properties. That proved very useful for protein–enzyme purifications. These applications relied more on varying the dissociation constants by selective alteration of elution conditions than on any specific interactions between resin and substrate.

In a review in 1953 Zittle (10) suggested that adsorbents of absolute specificity might be found by use of solids that resemble the substrate sufficiently to permit enzyme–substrate bonding, yet not be subject to the catalytic action of the enzyme. Lerman (11) demonstrated this concept the same year by covalently attaching azophenols to cellulose. Since aromatic amines coupled to phenols inhibit tyrosinase, the immobilized inhibitors bound tyrosinase specifically, permitting a single-step purificatin of the enzyme from an ammonium sulfate precipitate of a mushroom extract.

At nearly the same time Isliker (12) demonstrated the use of cross-linked ion-exchange beads as an immobilization technique. He began by adsorbing blood-group antigen isolated from the stroma of type A red cells onto anion exchange resins. The antigen, a polysaccharide, was partially cross-linked by the use of formaldehyde to make it insoluble, but it still remained active enough to abstract its corresponding hemagglutinin from plasma. Hyperimmune plasma could be freed of more than 90% of its hemagglutinin by perfusion over this ion-exchange column. To utilize soluble antigens in a column format, Isliker reacted the anion exchange beads with sulfonyl chloride to form the corresponding acid chloride. The highly reactive beads were then coupled covalently to several different antigens with varying retention of their biological specificity. An interesting finding in this early paper was that high concentrations of the activating site (i.e., the acid chloride) did not necessarily lead to high recovery of antibody, although they did cause greater levels of antigen to be immobilized. Isliker attributed this to multipoint attachment of the antigen creating steric hindrance that interfered with subsequent complex formation.

By 1967 the interactions between substrate and enzyme were well enough understood for Baker (13), who synthesized permanent inhibitors for enzyme active sites, to formulate requirements for enzyme-specific adsorbents. He made the distinction, so important today, between *affinity sorbents* and nonspecific binding. He correctly pointed out that the polymeric carriers

should be nonionic and inert; that spacing of the ligand was important; and that the chemistry used to immobilize the inhibitor should not interfere with the enzyme–inhibitor interaction.

Affinity purifications of antibodies started somewhat earlier but were not more widely applied than the purification of enzymes. This was surprising since the ability of antibodies to recognize haptens had already been shown in the 1920s. Initial attempts at purifying antibodies relied on salt precipitation methods. Perhaps because the understanding of the structure and function of antibodies was clouded by their very complexity, the concept of purification was premature. When Willstatter's methods were used to purify antibodies, the results were discouraging. Precipitin isolations of antibody–antigen aggregates worked better than adsorption of antibodies to unrelated substrata. As early as 1936 Landsteiner and Van der Scheer (14) reported the immobilization of haptens by coupling to a solid. They isolated subpopulations of antibodies from a mixture of cross-reactive antibodies. To prepare the solid phase, they diazo-coupled the hapten to chick erythrocyte walls. This "affinity isolation" occurred before there was even agreement that the immunoglobulins were proteins. By 1936 Meyer and Pic (15) reported a systematic study of antibody recovery by the use of antigen-coated kaolin. The antigens were not covalently bound but were held by strong adsorptive forces. In 1951 the use of immobilized ligands had progressed to the use of *p*-aminobenzyl-cellulose to insolubilize bovine albumin. The bound albumin was able to bind antialbumin from sera of rabbits previously immunized with bovine albumin. Cambell et al. (16) were able to insolubilize 1.5% antigen on the substrate, which could bind about 1½ times its weight of antibody.

Thus, the use of biospecific adsorption, or complex formation, began much earlier with the immunologists than with the enzymologists, perhaps out of need alone. It became the basis of what today one might call immunospecific purification. It did not expand as rapidly from the laboratory into industrial use because of the difficulty of isolating large quantities of the needed reagents. Consequently, industrial scale-up of biochemical purifications grew more rapidly in the field of enzyme chemistry. Certainly the use of ion-exchange materials available in the 1950s offered the enzymologist much more potential in separating various enzymes than it did to the immunologists for separating an immunoglobulin that recognized a specific antigen. While enzymes could be further purified on the basis of some physical characteristic, this was not the case for antigen-specific immunoglobulins.

The scientific basis of these ligand immobilizations has grown at a very rapid rate since then. Nevertheless, it is important to note that the entire field of enzyme and other protein purification, based on insoluble complex formation, is less than 50 years old, with the majority of the understanding coming in the last 25 years. In his excellent review, Nishikawa (17) presents a table of reviews. In the 1960s there were nine reviews dealing with affinity