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EFFECT OF ELECTROSTATIC INTERACTIONS DURING PROTEIN ULTRAFILTRATION: EFFECTS OF LIGAND CHEMISTRY AND PROTEIN SURFACE CHARGE DISTRIBUTION

A Dissertation in
Chemical Engineering

by

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ABSTRACT

The production of high value recombinant proteins requires robust, cost-effective, and high-resolution purification methods that can provide high yield and purification. Although ultrafiltration (UF) was originally viewed as a purely size-based separation process, it is now well established that the rate of protein transmission is strongly affected by electrostatic interactions. The overall objective of this thesis was to develop a more fundamental understanding of the role of electrostatic interactions in determining the transport and separation characteristics of a variety of electrically charged ultrafiltration membranes produced using a range of negative, positive, and zwitterionic ligands.

A series of novel positively-charged ultrafiltration membranes were generated by covalent attachment of ligands having similar size but containing different numbers of primary, secondary, and quaternary amines. Protein transmission through these membranes was well correlated with the apparent zeta potential of the membrane, irrespective of the detailed ligand structure. The most strongly charged membrane provided more than 40-fold selectivity for the separation of cytochrome c from a neutral dextran with similar hydrodynamic radius.

Experimental data were obtained for transmission of neutral, basic, and acid proteins using a series of zwitterionic ultrafiltration membranes generated by covalent attachment of small zwitterionic ligands to a base cellulose membrane. The sieving
coefficients well correlated with the product of the surface charge densities of the protein and membrane, consistent with a partitioning model accounting for electrostatic effects. The apparent zeta potential of these zwitterionic membranes could be described using the pK\textsubscript{a} values of an analog of the zwitterionic ligand accounting for the conversion of the primary amine to a secondary amine through the chemical linkage. The zwitterionic membranes showed minimal fouling even under conditions where the protein and membrane had opposite charge, making them attractive in bioprocessing applications.

The effects of protein surface charge distribution were studied using model proteins with similar size but different amino acid composition and surface charge distribution. The results demonstrated that the protein sieving coefficient is determined almost entirely by the net surface charge density of the protein, irrespective of the detailed distribution of charge groups over the protein surface. This behavior is very different than that seen in ion-exchange chromatography where binding is determined by the presence of localized charge patches.

The separation characteristics of the charge-modified membranes were also studied using protein charge ladders, a set of covalently modified derivatives of a single protein having different net charge. The selectivity increased with increasing membrane zeta potential, with a sharp decline in the sieving coefficient when the variant charge exceeded a critical value. These results have implications in the use of charge-modified membranes for the separation of protein variants in large-scale downstream processing.
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1.1 Background

The production of high value recombinant proteins in the biotechnology industry requires robust, cost-effective, and high-resolution purification methods that can provide sufficient yield and purification. Although there is considerable variability in the economics for different therapeutic products, Blanch and Clark (1997) reported that up to 80% of the total production cost for large-scale manufacture is associated with the downstream purification process. The development of high-dose chronic therapies using recombinant DNA derived antibodies has led to a number of new separation challenges for the biotechnology industry (van Reis and Zydney, 2007). Cell culture titers of proteins, especially monoclonal antibody (mAb) proteins, have increased dramatically over the past decade from tenths of a gram per liter to approximately 10 g/L (Thommes and Etzel, 2007). Cell culture volumes have also increased dramatically; the industry is now considering the production of 100-kg batches (Thommes and Etzel, 2007).

Electrostatic interactions are one of the most powerful tools for protein separations. For example, ion exchange chromatography (IEX) is one of the most widely used unit operations for the high resolution purification of recombinant therapeutic protein products. Protein retention in IEX occurs due to electrostatic interactions
between oppositely charged functional groups on the stationary phase and the protein, with the separation due to differences in the strength of the electrostatic interactions for the product and impurities. Both anion and cation exchange steps are used extensively in bioprocessing; at least one ion-exchange chromatography step is included in about 75% of protein purification processes (Janson and Ryden, 1998). Isoelectric focusing, gel electrophoresis, capillary electrophoresis, and dielectrophoresis all exploit electrical interactions, although these systems are typically limited to analytical (as opposed to preparative) scale separations. Several studies have demonstrated that electrostatic interactions can also be important in membrane systems; this is discussed in more detail in the next section.

1.2 Protein Purification using Membranes

Membranes are well suited for protein separations since they operate under mild conditions that will not degrade or damage most proteins. In addition, membrane separations are robust, provide high throughput, and are much easier to scale-up than chromatographic processes.

Ultrafiltration (UF), a pressure-driven process using membranes with pore size from around 1 to 15 nm, has become the standard method for protein concentration and buffer exchange in processing a variety of therapeutic proteins. It is also used as the method of choice for final formulation of nearly all recombinant protein products (van Reis and Zydney, 2007). UF was originally viewed as a purely size-based separation process with the macromolecular species retained by the membrane due to steric
exclusion from the small pores. However, it is now well established that the rate of protein transmission is determined by both steric and long-range interactions, with the latter dominated by electrostatic interactions associated with the electrical charge on the protein and/or membrane (van Reis and Zydney, 2007; Zydney, 2009).

Electrostatic interactions have been exploited to develop high performance ultrafiltration membranes with significantly greater protein retention (or selectivity) for a given value of the permeability than conventional UF membranes (Zydney, 2009; Mehta and Zydney, 2006). Electrostatic effects have also been used to achieve high resolution protein separations, with the charged membranes providing high retention of like-charged proteins while allowing relatively uncharged solutes to pass into the permeate (van Reis and Zydney, 2007; van Reis et al., 1999). Examples include the separation of bovine serum albumin (BSA) as a model impurity from hemoglobin (van Eijndhoven, 1995), the separation of BSA and immunoglobulin G (Saksena and Zydney, 1994), the purification of an antigen binding fragment from BSA (van Reis et al., 1999), the purification of a monoclonal antibody from Chinese Hamster Ovary cell proteins (van Reis and Zydney, 2007), and the purification of an antibody fragment (Lebreton et al., 2008) from E. Coli host cell proteins.

Although many commercial ultrafiltration membranes are naturally charged in aqueous solution, "intentionally" charged UF membranes have yet to be introduced for large-scale bioprocessing applications. The commercial application of such membranes requires further optimization of the membrane performance as well as a more detailed understanding of the various factors that govern the performance of these systems.
including the electrostatic interactions between the charged protein and membrane. The earliest discussion of electrostatic interactions during solute transport through porous membranes dates back to at least 1975 and a series of observations that the sieving coefficient of a synthetic polyanion (dextran sulfate) through the renal glomerular capillaries was about 20-fold smaller than that of a neutral dextran of similar size and structure (Chang et al., 1975). A few years later, Malone et al. (1978) demonstrated that the hindered diffusion coefficient of latex particles through synthetic track-etched mica membranes was significantly reduced at low salt concentrations, a phenomenon that they attributed to the stronger electrostatic interactions at low ionic strength. Subsequent studies extended these observations to pressure-driven filtration of charged proteins through a range of commercial ultrafiltration membranes with different surface charge characteristics and under different solution conditions (Mehta and Zydney, 2006; Bil’dyukevich et al., 1989; Pujar and Zydney, 1994; Millesime et al., 1995; Burns and Zydney, 1999; Nakao et al., 1988; Bakhshayeshi and Zydney, 2008).

Previous studies of electrostatic interactions during protein ultrafiltration have generally assumed that the membrane surface charge density and pore size are the only membrane properties that determine the overall performance, without any consideration of the detailed molecular structure of the charged ligands or proteins. This is in sharp contrast to the behavior seen in ion exchange chromatography where the detailed physical and chemical properties of the functional ligand and the protein can have a significant impact on the adsorption characteristics. In addition, all previous analyses of electrostatic interactions during protein ultrafiltration have implicitly assumed that the protein can be
treated as a sphere with uniform surface charge, completely neglecting the possible effects of the surface charge distribution on the magnitude of the electrostatic interactions.

1.3 Thesis Program

The overall objective of this thesis was to develop a more fundamental understanding of the role of electrostatic interactions on the transport and separation characteristics of electrically charged ultrafiltration membranes and to develop a framework for the design of high performance charge-modified membranes. This included: (1) investigating the effects of protein surface charge distribution on electrostatic interactions during protein ultrafiltration, (2) investigating the effects of ligand chemistry, and in particular the number and nature of functional groups, on the performance of charge-modified ultrafiltration membranes, (3) obtaining quantitative data on protein transport/retention for a series of semipermeable zwitterionic ultrafiltration membranes made from different ligands, and (4) examining the separation characteristics of several of the positively-charged, negatively-charged, and zwitterionic membranes using protein charge ladders.

The general theoretical background used to analyze the ultrafiltration results is presented in Chapter 2. This includes a brief review of the theoretical analysis of solute and solvent transport through narrow pore size membranes, with a particular emphasis on the effects of electrostatic interactions on solute partitioning into charged membrane
pores. A brief discussion of the theoretical analysis of the protein charge based on both the electrophoretic mobility and the amino acid sequence is also provided.

Chapter 3 describes the experimental set-up, materials, and methods used in the experimental studies described in this thesis. Specific details on some of the experimental procedures and membrane modification techniques are provided in the appropriate Chapters.

Chapter 4 examine the behavior of several novel positively-charged ultrafiltration membranes generated by covalent attachment of a series of ligands having similar size but containing different numbers of primary, secondary, and quaternary amines. Ultrafiltration experiments were performed over a range of solution ionic strength using cytochrome c as a model protein.

Quantitative data on protein transport / retention for a series of zwitterionic ultrafiltration membranes are presented in Chapter 5. Zwitterionic ultrafiltration membranes were generated by covalent attachment of small zwitterionic ligands to a base cellulose membrane. Filtration experiments were performed using basic (cytochrome c), neutral (myoglobin), and acidic (α-lactalbumin) proteins over a range of solution pH. Experimental results were analyzed using available theoretical models described in Chapter 2 for the partitioning of a charged particle into a charged pore. The extent of fouling during protein ultrafiltration through the zwitterionic membranes was also examined.

Chapter 6 examines the effects of the protein surface charge distribution on the magnitude of the electrostatic interactions that occur during protein ultrafiltration. Data
were obtained over a range of solution pH with the sieving results compared to the measured protein retention on a strong cation exchange resin. Data were obtained with cytochrome c and lysozyme, two proteins that have similar size and net charge but different amino acid composition and surface charge distribution. Limited data were also obtained with peracetylated cytochrome c formed by reaction of cytochrome c with acetic anhydride to block all free lysine groups. Experimental results were again analyzed using available theoretical models described in Chapter 2 for the partitioning of a charged particle into a charged pore.

Chapter 7 examines the separation characteristics of several of the positively-charged, negatively-charged, and zwitterionic membranes developed in this thesis using protein charge ladders. A protein charge ladder consists of a set of covalently modified derivatives of a single protein that have similar size but different net charge. Ultrafiltration data were analyzed in terms of the net charge of the individual rungs in the protein charge ladder as determined from the measured electrophoretic mobility.

Chapter 8 summarizes the major contributions of this thesis and makes several recommendations for future studies on development of high performance charged ultrafiltration membranes for protein purification.
Chapter 2

Theoretical Background

2.1 Introduction

The first part of this Chapter provides a brief review of the theoretical models that have been developed to describe the basic mass transport and separation phenomena that occur during protein ultrafiltration. Most of the discussions presented in Sections 2.2 and 2.3 are based on previous discussions presented by Zeman and Zydney (1996) and in several dissertations published under the direction of Professor Andrew Zydney (Pujar, 1996; Burns, 2000; Mehta, 2006; Molek, 2008). Part of the text in Section 2.3.2 is reproduced from: Rohani M.M., Zydney A.L., Role of electrostatic interactions during protein ultrafiltration, Advances in Colloid and Interface Science 160 (2010) 40 – 48.

The overall rate of protein transport through semipermeable ultrafiltration membranes is determined by the rate of transport from the bulk solution to the membrane surface in combination with the rate of solute transport through the membrane pores. Transport in the bulk solution is governed primarily by the system hydrodynamics while transport through the membrane pores has contributions from thermodynamics and hydrodynamics. Thermodynamic factors determine the initial partitioning of solute into the pore while the hydrodynamic factors determine the rate of solute motion inside of the pores. These phenomena are discussed briefly in this Chapter, with an emphasis on the
effect of electrostatic interactions on partitioning and transport of charged solutes into charged pores.

A brief review of the theoretical analysis of the protein net charge from both the electrophoretic mobility and the known amino acid sequence is provided towards the end of this Chapter. A more detailed discussion of the protein charge calculations is available in Menon (1999).

2.2 Bulk Mass Transport

The pressure-driven flow through a semipermeable membrane causes an accumulation of the retained protein adjacent to the upper surface of the membrane, a phenomenon called concentration polarization. Figure 2.1 shows a schematic of the concentration polarization phenomenon, including the expected concentration profile. Polarization causes the protein concentration to vary from a value of $C_b$ in the bulk solution to a much greater value at the membrane surface ($C_w$, where the subscript “w” stands for “wall”), over a distance equal to the concentration polarization boundary layer thickness, $\delta$. The increase in concentration in the solution immediately upstream of the membrane can significantly alter protein transmission due to the increase in the driving force for transport into and through the membrane. In addition, the osmotic pressure associated with this high protein concentration can reduce the effective pressure driving force, thereby reducing the solvent flux. At low transmembrane pressures, the filtrate flux is typically directly proportional to the pressure since concentration polarization effects are low, but as the pressure increases the osmotic pressure of the retained protein
will reduce the filtrate flux, with the flux eventually becoming independent of the applied transmembrane pressure. The high protein concentration at the membrane surface can also increase membrane fouling leading to irreversible changes in the membrane properties.

![Diagram of concentration polarization during protein ultrafiltration](image)

**Figure 2.1** Schematic of concentration polarization during protein ultrafiltration (Adapted from Molek, 2008).

### 2.2.1 Stagnant Film Model

The stagnant film model is the most commonly used approach to describe concentration polarization in membrane systems. This model provides an approximate analysis of the concentration profile upstream of the membrane, neglecting many of the
complexities associated with the detailed fluid flow characteristics in the particular module as well as the coupling between mass and momentum transport. In the conventional stagnant film model, protein-protein interactions are neglected and the solute diffusivity and viscosity are both assumed to be independent of the solute concentration and constant throughout the boundary layer. At steady state, the solute flux through the membrane and into the filtrate solution is set equal to the net solute flux towards the membrane:

$$-J_v C_f = -J_v C - D_\infty \frac{dC}{dz}$$

(2.1)

where $J_v$ is the filtrate flux through the membrane, $C_f$ is the concentration of solute in the filtrate solution, $C$ is the local solute concentration at a position $z$ above the membrane surface, and $D_\infty$ is the free solution diffusion coefficient of the solute. Equation (2.1) is integrated across the concentration boundary layer (from $C = C_w$ at $z = 0$ to $C = C_b$ at $z = \delta$) giving the following expression for filtrate flux upon rearrangement:

$$J_v = \frac{D_\infty}{\delta} \ln \left( \frac{C_w - C_f}{C_b - C_f} \right)$$

(2.2)

A more detailed analysis of this model, including the validity of the one-dimensional transport analysis, is provided by Zydney (1997).

In addition to altering the filtrate flux, concentration polarization also affects the rate of solute transport through the membrane. This is typically described in terms of the observed sieving coefficient, which is defined as the ratio of the solute concentration in the filtrate solution to that in the bulk solution well above the membrane ($S_o = C_f / C_b$).

The observed sieving coefficient can be related to the actual sieving coefficient ($S_a$),
which is defined as the ratio of the solute concentration in the filtrate to that at the
membrane surface or "wall" \((S_a = C_f / C_w)\), by rearranging Equation (2.2) to give:

\[
S_o = \frac{S_a \exp\left(\frac{J_v}{D_{\infty} / \delta}\right)}{1 - S_a + S_a \exp\left(\frac{J_v}{D_{\infty} / \delta}\right)}
\]  

(2.3)

2.2.2 Bulk Mass Transfer Coefficient

The ratio of the solution diffusion coefficient \((D_{\infty})\) to the boundary layer thickness
\((\delta)\) in Equations (2.2) and (2.3) is typically set equal to the solute mass transfer
coefficient, \(k_m\). The mass transfer coefficient is a function of the solute diffusivity and
the hydrodynamics of the device. A semi-empirical equation for the mass transfer
coefficient in a stirred cell for laminar flow (\(Re < 32,000\)) was developed by Smith et al.
(1968) based on the rate of benzoic acid dissolution into a stirred solution as:

\[
Sh = \frac{0.567}{Re^{0.33} Sc^{0.33}}
\]  

(2.4)

where \(Sh = k_m b / D_{\infty}\) is the Sherwood number, \(Re = \omega b^2 / \nu\) is the Reynolds number,
\(Sc = \nu / D_{\infty}\) is the Schmidt number, \(b\) is the radius of the stirred cell, \(\omega\) is the stirring
speed, and \(\nu\) is the kinematic viscosity. Opong and Zydney (1991) evaluated \(\chi\) as 0.23
for a 25 mm diameter Amicon stirred cell (which is the system used in the experiments
performed in this dissertation) based on data for the filtrate flux as a function of the
transmembrane pressure at several bulk protein concentrations and stirring speeds.
The protein diffusion coefficient can be evaluated from the correlation developed by Young et al. (1980):

\[ D_\infty = \frac{k_B T}{\mu M^{1/3}} \]  

(2.5)

where \( D_\infty \) is in cm\(^2\)/s, \( \mu \) is the solution viscosity in cP, \( T \) is the absolute temperature in K, \( k_B \) is the Boltzmann’s constant \((k_B=1.38 \times 10^{-23} \text{ J/K})\), and \( M \) is the protein molecular weight in g/mol. Equation (2.5) is valid only at infinite dilution because it neglects the effects of protein-protein interactions.

2.3 Membrane Transport

The rate of solute and solvent transport through porous membranes is typically described using hydrodynamic theories presented by Anderson and Quinn (1974) and Deen (1987) in which the membranes are modeled as an array of well-defined, typically cylindrical, pores, while the solutes are considered to behave as uniform rigid spheres. The advantage of the hydrodynamic models is that the key transport parameters can be calculated directly in terms of the physical properties of the solute and the pores. Hydrodynamic theories can be easily extended to incorporate the effects of a pore size distribution (by numerical integration over the distribution (Mochizuki and Zydneey, 1993; Saksena and Zydneey, 1995)) as well the effects of electrostatic interactions.
2.3.1 Solvent Transport – Membrane Hydraulic Permeability

The rate of solvent transport through a membrane is generally described in terms of the hydraulic permeability ($L_p$):

$$L_p = \frac{\mu J_v}{\Delta P}$$  \hspace{1cm} (2.6)

where $\mu$ is solution viscosity, $J_v$ is the filtrate flux (volumetric flow rate per total membrane area), and $\Delta P$ is the transmembrane pressure. For a membrane with a uniform array of cylindrical pores, the flux can be evaluated using the Hagen-Poiseuille equation with the permeability given as:

$$L_p = \frac{N \pi r_p^4}{8 \delta_m}$$  \hspace{1cm} (2.7)

where $N$ is the number of pores per unit area of the membrane, $r_p$ is the pore radius, and $\delta_m$ is the membrane thickness. Equation (2.7) can be used to obtain a rough estimate of the membrane pore radius based on experimental measurements of the membrane permeability. Equation (2.7) assumes that end effects are negligible, which is valid for typical ultrafiltration membranes since the membrane thickness is typically more than 100 times greater than the pore radius.

The rate of solvent transport is also dependent on the membrane surface charge and solution ionic strength due to electrokinetic effects. The presence of a net surface charge on the pore wall causes an accumulation of counterions in the electrical double layer adjacent to the pore wall. The pressure-driven convective fluid flow through the charged pore will generate an unequal flux of the anions and cations, leading to the development of an induced (streaming) potential. At steady state, the streaming potential
generates a back conductive ion transport that exactly balances the convective ion flux, resulting in a situation in which there is no net current flow through the pore. The induced streaming potential reduces the magnitude of the solvent flux due to the net force on the fluid exerted by the action of the electric field on the ions (often referred to as counter-electroosmosis). A detailed review of solvent transport through electrically-charged membranes is provided by Pujar (1996) and Burns (2000).

### 2.3.2 Solute Transport: Thermodynamic Contribution

The rate of protein transport through small pore ultrafiltration membranes is typically analyzed in terms of both thermodynamic and hydrodynamic interactions, with the actual protein sieving coefficient \( S_a \) expressed as:

\[
S_a = K_C
\]

The rate of protein transport through small pore ultrafiltration membranes is typically analyzed in terms of both thermodynamic and hydrodynamic interactions, with the actual protein sieving coefficient \( S_a \) expressed as:

\[
S_a = K_C
\]

where \( \phi \) is the equilibrium partition coefficient between the bulk solution and the membrane pore, and \( K_C \) is the hindrance factor for convection which accounts for the additional hydrodynamic drag on the solute molecule due to the presence of the pore wall. Equation (2.8) assumes that protein transport through the membrane is dominated by convection, which is a reasonable approximation during protein ultrafiltration due to the relatively high Peclet numbers in these systems (Zeman and Zydney, 1996; van Reis and Zydney, 2010). In the case of pressure-driven (convective) flow, the hydrodynamic interactions have a relatively small effect on the rate of protein transport since the increase in drag associated with boundary interactions is largely balanced by the increase in local velocity as the protein is excluded from the slow-moving fluid in the region near
the pore walls (Zeman and Zydney, 1996; Dechadilok and Deen, 2009). The rate of protein transport is thus determined primarily by the equilibrium partition coefficient, which is defined as the ratio of the average protein concentration in the pore to that in the bulk (external) solution immediately adjacent to the membrane:

\[
\phi = \frac{C_{\text{pore}}}{C_{\text{bulk}}} = \frac{2}{r_p^2} \int_{0}^{r_p} \exp \left[ -\frac{\psi_{\text{total}}(r)}{k_B T} \right] r dr
\]  

(2.9)

where \( r_p \) is the pore radius, \( r \) is the radial coordinate within the cylindrical pore, \( k_B \) is the Boltzmann constant, and \( T \) is the absolute temperature. The total interaction potential, \( \psi_{\text{total}}(r) \), has contributions from steric (hard-sphere), electrostatic, and van der Waals forces. For purely hard-sphere interactions, \( \psi_{\text{total}} \rightarrow \infty \) when the solute overlaps the pore wall and is zero in the pore interior:

\[
\phi = 0 \quad r > r_p - r_s \\
\phi = \frac{2}{r_p^2} \int_{0}^{r_p-r_s} r dr \quad r < r_p - r_s
\]  

(2.10) (2.11)

where \( r_s \) is the solute (protein) radius. Thus, under these conditions the integral in Equation (2.9) reduces to:

\[
= (1 - \lambda)^2
\]  

(2.12)

where \( \lambda \) is the ratio of the solute (protein) to pore radius.

Most of the experimental data presented in this thesis are analyzed in terms of available theoretical models for the electrostatic energy of interaction. Smith and Deen (1980) developed the first rigorous analytical expressions for the electrostatic potential for a spherical solute in a cylindrical pore by solving the linearized Poisson-Boltzmann
equation using matched asymptotic expansions in cylindrical and spherical coordinates. The results for interactions at constant surface charge density are conveniently expressed as:

\[
\frac{E}{k_B T} = \left( A_s \sigma_s^2 + A_{sp} \sigma_s \sigma_p + A_p \sigma_p^2 \right)/A_{den} \tag{2.13}
\]

where \( \sigma_s \) and \( \sigma_p \) are the dimensionless surface charge densities of the solute (protein) and pore:

\[
\sigma_s = \frac{F r_p q_s}{\varepsilon_o RT} \tag{2.14}
\]

\[
\sigma_p = \frac{F r_p q_p}{\varepsilon_r \varepsilon_o RT} \tag{2.15}
\]

with \( \varepsilon_o \) the permittivity of free space, \( \varepsilon_r \) the dielectric constant of the solution, \( F \) the Faraday constant, \( R \) the ideal gas constant, \( T \) the absolute temperature, \( q_s \) and \( q_p \) the dimensional surface charge densities of the solute and pore. The coefficients \( A_s, A_{sp}, A_p, \) and \( A_{den} \) are all positive functions of the solution ionic strength, solute radius, and pore radius:

\[
A_s = \frac{4 \pi \tau \lambda^4 e^{-\tau \lambda}}{1 + \tau \lambda} \int_0^\infty K_1 \left( \tau^2 + \theta^2 \right)^{1/2} d\theta \tag{2.16}
\]

\[
A_p = \frac{2 \left[ (1 + )e - (1 )e \right]}{2 I_1^2 ( )} \tag{2.17}
\]

\[
A_{sp} = \frac{4 \left[ \left. \right| \right]}{I_1 ( )} \tag{2.18}
\]
where $I_1$ and $K_1$ are modified Bessel functions, $\tau = \kappa r_p$ is the dimensionless pore radius, and $\kappa$ is the inverse Debye length:

$$\kappa^{-1} = \left[ \frac{F^2 \sum z_i^2 C_i}{\varepsilon_0 \varepsilon_r RT} \right]^{1/2}$$

Equations (2.16) to (2.19) are valid for a solute located at the pore axis. Corresponding results are available for arbitrary radial positions (Smith and Deen, 1983) as well as for interactions at constant surface potential instead of constant surface charge density (Smith and Deen, 1983; Smith and Deen, 1980). Pujar and Zydney (1997) subsequently extended this analysis to include the effects of charge regulation using a linearized form of the charge regulation boundary condition, thereby accounting for the change in surface charge/potential of the protein and pore wall associated with the alteration in the local electrical potential field (and ion concentrations) when the protein enters the pore.

Figure 2.2 shows sample calculations for the electrostatic energy of interaction for a positively charged solute (protein) in a positively charged pore as a function of the protein and membrane surface charge densities with $r_s = 1.59$ nm (corresponding to the protein lysozyme), $r_p = 3.4$ nm, and $\kappa^{-1} = 3.06$ nm (corresponding to a 10 mM ionic strength solution). The dimensionless electrostatic energy of interaction ($\psi_E/k_B T$) is
repulsive (positive) for all values of $q_s$ and $q_p$ since all three terms in Equation (2.13) are positive. The projection of the $\psi_E/k_B T$ values along the axis with $q_p = 0$ describes the energy of interaction for a charged solute in a neutral pore. The large values of $\psi_E/k_B T$ under these conditions reflects the energetic penalty associated with the distortion of the electrical double layer surrounding the protein caused by the presence of the pore wall and is proportional to $q_s^2$ (described by the first term on the right hand-side of Equation 2.13). The projection along the axis with $q_s = 0$, corresponding to the energy of interaction for a neutral protein in a charged pore, is also positive but very small under these conditions ($\psi_E/k_B T < 1$) since the maximum surface charge density on the pore wall was assumed to be a factor of 10 smaller than that of the protein (consistent with experimental measurements obtained in typical ultrafiltration systems). The greatest contribution arises from direct charge-charge interactions, described by the second term on the right hand-side of Equation (2.13) (proportional to $q_s q_p$). Calculations performed by integrating the expression for the energy of interaction over the radial coordinate (using Equation (2.9) with appropriate expressions for $\psi_E(r)$) gives results similar to those in Figure 2.2 since the most favorable equilibrium location of the protein is near the pore centerline.
Figure 2.2  Model calculations for the dimensionless electrostatic energy of interaction for a positively charged protein in a positively charged membrane. Conditions: \( r_s = 1.59 \) nm, \( r_p = 3.4 \) nm, and \( \kappa_z = 3.06 \) nm (corresponding to 10 mM ionic strength).

2.4  Protein Net Charge Analysis

2.4.1  Protein Charge Calculations from Amino Acid Composition

The protein surface charge density is determined by the dissociation of the various ionizable amino acid residues on the surface of the protein along with the adsorption (or binding) of specific ions from the bulk electrolyte. The dissociation equilibrium of a typical amino acid residue (for example, an \( \alpha \)-carboxylic acid) is described by the intrinsic dissociation constant of that ionizable group:
\[ K_{int}^{i} = \frac{[R \ COO^-][H^+]}{[R \ COOH]} \]  

Equation (2.21) can be rewritten in terms of the pH and the number of dissociated groups \( r_i \)

\[ \text{pH} = pK_{int}^{i} + \log \frac{r_i}{(n_i - r_i)} \]  

(2.22)

where \( \text{pH} = -\log [H^+] \), \( pK_{int}^{i} = -\log [K_{int}^{i}] \), and \( n_i \) is the total number of titratable species.

The local \( H^+ \) concentration in Equation (2.21) is the value at the protein surface, which is different from the bulk \( H^+ \) concentration due to electrostatic interactions between the charged protein and the charged hydrogen ion. This effect is typically described using a classical Boltzmann distribution:

\[ H^+ = H_b^+ \exp \left( \frac{-e \psi_s}{k_BT} \right) \]  

(2.23)

where \( H_b^+ \) is the bulk hydrogen ion concentration, \( e \) is the electron charge \((1.609 \times 10^{-19} \text{ C})\), and \( \psi_s \) is the electrostatic potential at the protein surface:

\[ \psi_s = \frac{eZ}{4 \sigma r_s (1 + r_s)} \]  

(2.24)

where \( Z \) is the net charge (evaluated as the number of electronic charges) on the protein surface. Equation (2.24) is developed assuming that the protein is a hard sphere with the electrical charges distributed uniformly over the spherical surface (Overbeek and Wiersema, 1967).
The protein charge is equal to the difference between the maximum number of positive charges (N-terminal, histidine, lysine, arginine) and the sum of all the dissociated groups:

\[ Z = Z_{\text{max}}^+ - \sum_{i=1}^{n} r_i \]  

(2.25)

Equations (2.22) to (2.25) are solved iteratively to evaluate the net protein charge as a function of the bulk pH and solution ionic strength (which determines the Debye length). The development of these equations is discussed in more detail by Menon and Zydney (2000). The number and pK\(_a\) values of the various amino acids present in the proteins used in this thesis are given in Appendix A.

Typical results for the net protein charge as a function of solution pH are shown in Figure 2.3 for cytochrome c and lysozyme at both low (10 mM) and high (100 mM) ionic strengths. The dashed curves represent the calculated values of the protein charge in the absence of any charge regulation effects, i.e., assuming that the H\(^+\) concentration (and thus the solution pH) at the protein surface is equal to that in the bulk solution. The protein isoelectric point, the pH at which the net protein charge is zero, is independent of solution ionic strength (assuming that ion binding interactions are negligible) since there are no electrostatic interactions between the (neutral) protein and the H\(^+\) ions under these conditions. The predicted isoelectric point for lysozyme was pH 10.9 ± 0.1, which is in very good agreement with literature data; however, the calculated value for cytochrome c (9.9 ± 0.1) is slightly lower than that reported in the literature (10.4 ± 0.1). This small discrepancy could be due to ion binding interactions or to a shift in the pK\(_a\) of certain
amino acids due to local charge-charge interactions (Menon and Zydney, 1999; Sharma et al., 2003). The protein charge increases with decreasing pH due to the protonation of the various ionizable groups.

The difference in net protein charge at low (10 mM) and high (100 mM) ionic strength is due to charge regulation. At low ionic strength, the $H^+$ concentration at the protein surface is different than that in the bulk solution due to the electrostatic interactions between the charged protein and the charged ions. At pH below the isoelectric point ($Z>0$), the repulsive interactions reduce the local $H^+$ concentration near the protein surface leading to a reduction in the net protein charge. The opposite effect is seen at pH $> pI$ due to the increase in local $H^+$ concentration associated with the attractive electrostatic interactions. These effects are much less pronounced in the 100 mM ionic strength solution due to the electrostatic shielding provided by the bulk electrolyte.

Cytochrome c has a greater negative charge than lysozyme at high pH due to the greater number of acidic amino residues (a total of 12 aspartic acid and glutamic acid residues for cytochrome c compared to 9 for lysozyme). The relative plateau in the net charge between pH 6 and 9.5 reflects the relative absence of amino acid residues with $pK_a$ values in this pH range.
Figure 2.3  Calculated net charge of cytochrome c (upper panel) and lysozyme (lower panel) as a function of solution pH at low (10 mM) and high (100 mM) ionic strength.  The dashed curves represent calculated values of the protein charge assuming the H\(^+\) concentration at the protein surface equals that in the bulk.
2.4.2 Electrophoretic mobility and Protein Charge from Capillary Electrophoresis

The calculation of the net protein charge from the measured electrophoretic mobility requires a model for electrophoretic motion. The electrophoretic mobility reflects the balance between the electrical forces arising from the applied electric field and the hydrodynamic (friction) forces associated with the viscosity of the suspending medium. A large number of theoretical analyses have been presented in the literature for the electrophoretic mobility, with the differences lying primarily in the approximations made in evaluating the electrical interactions. These differences include both the detailed structure of the equilibrium electrical potential (e.g., the use of the low electrical potential, small Debye length, or flat plate approximations) and the distortion of the equilibrium structure associated with the particle and fluid motion during electrophoresis. The simplest approach is to treat the protein as a non-conducting charged sphere with a uniform surface charge density. A relationship between the electrophoretic mobility and the potential at the surface of the sphere is obtained by solving the governing equations for the electrical drag forces acting on the particle (Overbeek and Wiersema, 1967). In the simplest limiting case, when the electrical double layer thickness is much smaller than the sphere radius (i.e., \( kr_s \geq 1 \)), the electrophoretic mobility \( \mu_E \) is given by the Helmholtz-Smoluchowski equation:

\[
\mu_E = \frac{\varepsilon \zeta}{\mu}
\]

(2.26)

where \( \zeta \) is the electrostatic potential at the particle surface, \( \varepsilon \) is the electrical permittivity of the solution, and \( \mu \) is the solution viscosity (Masliyah and Bhattacharjee, 2006).
If the electrical double layer is much larger than the particle radius (i.e., $\kappa r_s \leq 1$), the electrophoretic mobility ($\mu_E$) is given by the Debye-Huckel equation assuming that the potential is low:

$$\mu_E = \frac{2 \varepsilon \zeta}{3 \mu}$$  \hspace{1cm} (2.27)

Henry (1931) obtained a more complete solution for the electrophoretic mobility that accounts for the distortion of the electric field lines by the presence of the particle, with the resulting expression valid over the entire range of Debye lengths:

$$\mu_E = \frac{2 \varepsilon \zeta}{3 \mu} f_H(\kappa r_s)$$  \hspace{1cm} (2.28)

where $f_H$ is Henry’s function which accounts for the finite double layer thickness:

$$f_H(\kappa r_s) = 1 + \frac{1}{16} (\kappa r_s)^2 - \frac{5}{48} (\kappa r_s)^3 - \frac{1}{96} (\kappa r_s)^4$$

$$+ \frac{1}{96} (\kappa r_s)^5 + \frac{1}{8} (\kappa r_s)^6 - \frac{1}{96} (\kappa r_s)^7 \left[ \exp(\kappa r_s) \int_\infty^\kappa \frac{\exp(-t)dt}{t} \right]$$  \hspace{1cm} (2.29)

where $t$ is a dummy variable over which the integration is performed. The zeta potential for a uniformly charged hard sphere can be expressed in terms of the particle net charge using Equation (2.24). The net electrical charge of the protein is thus given as:

$$Z = \frac{6\pi \mu r_s (1 + \kappa r_s) \mu_E}{ef_H}$$  \hspace{1cm} (2.30)

More details on the evaluation of the electrophoretic mobility from capillary electrophoresis experiments are provided by Menon (1999) and Molek (2008).
Chapter 3

Materials and Methods

3.1 Introduction

This chapter describes the materials, apparatus, and methods used for the experimental studies performed in this thesis. Additional details on specific materials or methods are provided in subsequent chapters as appropriate.

3.2 Membranes

3.2.1 Membrane Properties

Ultrafiltration experiments were performed using Ultracel™ composite regenerated cellulose membranes with nominal molecular weight cut-offs (MWCO) of 30 kDa or 100 kDa (Millipore Corp., Bedford, MA). Ultracel™ membranes with 10 kDa molecular weight cut-off were used for buffer exchange. Limited fouling experiments were performed with Biomax™ polyethersulfone membranes having a MWCO of 100 or 300 kDa, which were also provided by Millipore Corp. (Bedford, MA). The nominal molecular weight cut-off refers to the molecular weight of a solute which has approximately 90% rejection as determined by the manufacturer. These ultrafiltration membranes all have an asymmetric structure with a thin skin, which provides the membrane retention characteristics, and a much thicker and more porous support that
provides the membrane structural integrity. A scanning electron micrograph (SEM) of the cross section of the composite regenerated cellulose membrane is shown in Figure 3.1 (adapted from Mehta, 2006), which shows (1) a regenerated cellulose skin layer (approximately 0.5-1 μm thick – not visible in the SEM), (2) a porous cellulosic substructure (approximately 60 μm thick), and (3) a porous polyethylene substrate.

Figure 3.1  Scanning electron micrograph showing the cross section of the composite regenerated cellulose membrane (adapted from Mehta, 2006).

Figure 3.2 shows a schematic of the chemical structure of cellulose. The glucose rings within the cellulose polymer have a large number of free hydroxyl groups which increases the hydrophilicity of the membrane thus reducing protein adsorption and
fouling during ultrafiltration. These hydroxyl groups are also available for chemical modification as discussed in the next section.

Figure 3.2  Molecular structure of cellulose (adapted from Mehta, 2006).

Membrane disks with 25 mm diameter were cut from large flat sheets using a stainless-steel cutting device fabricated in our laboratory. All membranes were soaked in isopropanol for 45 min to remove any wetting/storage agents. The membranes were then thoroughly rinsed with at least 100 L/m$^2$ of deionized (DI) water.

3.2.2  Membrane Modification

Most of the methods for surface modification of cellulose membranes are based on the activation and subsequent reaction of the free hydroxyl groups on the base cellulose. Typically, these chemical modifications involve esterification or etherification of the hydroxyl groups under alkaline conditions. Surface-modified membranes used in this thesis were generated in our laboratory using different chemistries that are described briefly below and are discussed in more detail in later chapters.
Negatively charged versions of the Ultracel™ membranes were generated by covalent attachment of ligand containing sulfonic acid groups to the membrane surface using the base-activated chemistry described by van Reis (2006) and shown schematically in Figure 3.3. The membrane was initially soaked in 0.1 M NaOH for about 2 hours, followed by immersion in a 2 M solution of 3-bromopropanesulfonic acid sodium salt (Catalog #B2912, Sigma Chemical) in 0.1 N NaOH for fixed periods of time to control the extent of modification. The membrane was then thoroughly flushed with at least 100 L/m² DI water, followed by storage in 0.1 M NaOH (Rao and Zydney, 2006).

A positively charged version of the Ultracel™ membrane was created by covalent attachment of a quaternary amine functionality to the membrane surface using a proprietary chemistry provided by Millipore. The membrane was first equilibrated in 0.1 M NaOH and then chemically modified using a solution of glycidyl trimethylammonium chloride (50053, Sigma–Aldrich, St. Louis, MO) in 0.1 M NaOH. The membrane was flushed with the ligand solution for fixed periods of time to control the extent of modification. The membrane was then flushed with 0.2 M acetic acid followed by DI water, and then stored in 0.1 M NaOH. A series of positively-charged Ultracel™ membranes were generated by covalent attachment of a series of ligands having similar size but containing different numbers of primary, secondary, and quaternary amine groups. In this case, the membrane was first activated with epichlorohydrin and then reacted with a diamine solution following the chemistry initially developed by Liu et al. (2005) and subsequently modified by Mehta and Zydney (2008). A sequential reaction/activation scheme was employed to develop membranes with three secondary
amines and a terminal primary amine. A similar approach was used to generate a membrane with a terminal quaternary amine. This chemistry is described in more detail in Chapter 4.

A series of zwitterionic Ultracei\textsuperscript{TM} membranes were generated by covalent attachment of small zwitterionic ligands to a base cellulose membrane using an adaptation of the reaction chemistry presented by Riordan et al. (2009). The membrane was first activated with allyl glycidyl ether and then reacted with N-Bromosuccinimide followed by reaction with the zwitterionic ligand. This chemistry is described in more detail in Chapter 5.

![Cellulose Membrane](image)

**Figure 3.3** Schematic of reaction used to prepare negatively charged membranes.

### 3.2.3 Streaming Potential Measurement

The surface charge characteristics of the membranes were evaluated from streaming potential measurements using the apparatus shown in Figure 3.4 as originally described by Burns and Zydney (2000). The membrane was sealed between two
Plexiglas chambers which were then filled with the desired salt solution, taking care to remove all air bubbles. Ag/AgCl electrodes were then screwed tightly into the chambers (with O-rings in place to provide good sealing) to ensure reproducible placement of the electrodes relative to the membrane surface. The electrodes were placed approximately 1- 2 mm away from the membrane surface. The Ag/AgCl electrodes were prepared by placing a 1 mm diameter silver wire (Sigma Chemical Co., St. Louis, MO) and a reducing electrode in a 1 M KCl solution. The silver wires were first lightly sanded and placed in concentrated nitric acid solution for approximately 10 s. The wire was then washed with DI water and placed in a 1 M KCl solution. A DC power source was then connected to the silver electrode and a steel wire, and the current was maintained at 20 mA for 20 min to deposit a uniform Ag/AgCl layer on the wire surface. The same process was repeated for the second silver electrode. Electrodes were stored in 0.5 M KCl solution between experiments.

To measure the streaming potential of the membrane, a solution reservoir containing buffered KCl solution was attached to one chamber and a drain was connected to the other chamber taking care to insure that no air bubbles were trapped in either chamber. Air pressure was then applied to the solution reservoir, the system was allowed to stabilize for approximately 30 min, and the transmembrane voltage ($E_z$) was then measured using a Keithley 2000 Multimeter connected to Ag/AgCl electrodes facing both sides of the membrane. The system pressure was gradually increased from 14 to 34 kPa, with data obtained at four or more discrete pressures. The system was allowed to stabilize for approximately 15 min at each pressure. The apparent zeta potential ($\zeta_{app}$)
was evaluated from the slope of the voltage (streaming potential) as a function of pressure using the Helmholtz–Smoluchowski equation (Hunter, 1981):

\[
\zeta_{\text{app}} = \frac{\mu \Lambda_0}{\varepsilon_0 \varepsilon_r} \left( \frac{dE_z}{d\Delta P} \right) \tag{3.1}
\]

where \( E_z \) is the measured voltage at a given applied pressure (\( \Delta P \)), \( \mu \) is the solution viscosity, \( \Lambda_0 \) is the solution conductivity, \( \varepsilon_0 \) is the permittivity of free space, and \( \varepsilon_r \) is the dielectric constant of the solution. Note that Equation (3.1) is only valid under conditions where the double layer thickness is very small compared to the pore radius. Thus, \( \zeta_{\text{app}} \) should be treated as an apparent or effective zeta potential, which is directly related to the membrane surface charge but is different than the 'true' zeta potential associated with the pore surface. A more detailed discussion on the evaluation of the membrane charge from the streaming potential is provided by Burns (2000).
3.2.4 Dye-Binding Assay

The number of accessible charge groups on the positively-charged membranes was estimated using a dye-binding assay. Cibacron Blue 3GA (Sigma, C9534) was used as the negatively charged dye. Membranes were incubated at room temperature in a Cibacron Blue solution for a specified amount of time, with the solution gently agitated on a shaker plate. Small samples of the free solution were obtained immediately before and after exposure to the membrane, with the concentration of Cibacron Blue determined.
from the absorbance at 350 nm using a SPECTRAmax Plus 384 UV-vis
spectrophotometer (MD Corp., Sunnyvale, CA). Actual concentrations of free dye were
evaluated by comparison of the absorbance with that of known dye standards, with the
total mass of bound dye calculated from a simple mass balance.

3.3 Solution Preparation

3.3.1 Buffer Solutions

Buffer solutions were prepared by dissolving pre-weighed amounts of the
appropriate salts in deionized distilled water obtained from a NANOpure® Diamond
water purification system (Barnstead Thermolyne Corporation, Dubuque, IA) with
resistivity greater than 18 MΩ-cm. All salts were analytical reagent grade. The solution
pH was measured using a Model 402 Thermo Orion pH meter (Beverly, MA) and was
adjusted using 0.1 M sodium hydroxide or hydrochloric acid as needed. The solution
conductivity was measured using a 105A plus conductivity meter (Thermo Orion,
Beverly, MA). All buffer solutions were pre-filtered through 0.2 μm pore size Supor®
200 membranes (Pall Corp., Ann Arbor, MI) to remove particulates and undissolved
salts. The ionic strength of the buffer solution was evaluated as:

\[ I = \frac{1}{2} \sum z_i^2 C_i \]  

(3.2)

where \( z_i \) and \( C_i \) are the net charge and total concentration of each ion, respectively.
3.3.2 Protein Solutions

Protein sieving experiments were performed using cytochrome c, lysozyme, myoglobin, and \( \alpha \)-lactalbumin as model proteins. Sigma Chemical catalog numbers and key physical properties are summarized in Table 3.1. The amino acid composition and \( pK_a \) values of the various amino acids present in the proteins are provided in Appendix A.

Bovine serum albumin (BSA, Sigma A7906, Sigma-Aldrich Inc., St Louis, MO) with a molecular weight of 67 kDa and an isoelectric point of 4.7 was used as a model protein in limited fouling experiments due to the extensive prior literature on BSA fouling during ultrafiltration and microfiltration. Protein solutions were prepared by slowly dissolving the appropriate mass of powdered protein in the desire buffer, with the pH of the resulting solution adjusted by adding small amounts of 0.1 M solutions of the appropriate acid or base (e.g. HCl and KOH for a KCl salt solution) as required. Protein solutions were filtered through a 0.22 \( \mu \)m Acrodisc® syringe filter (Pall Corp.) to remove any protein aggregates immediately prior to use in the ultrafiltration experiments. Protein solutions were used within 12 h of preparation to minimize the likelihood of protein aggregation or denaturation. Protein charge ladders were synthesized by chemical modification of lysozyme, myoglobin, and \( \alpha \)-lactalbumin by reaction with either acetic anhydride or succinic anhydride, with details provided in Chapter 7. A peracetylated version of cytochrome c was formed by reaction of cytochrome c with acetic anhydride to chemically block all free lysine groups. Details of the reaction are provided in Chapter 6.
### Table 3.1 Physical properties of proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Catalog Number</th>
<th>Molecular Weight (Da)</th>
<th>Isoelectric Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c</td>
<td>C 7752</td>
<td>12384</td>
<td>10-10.5</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>L 6876</td>
<td>14307</td>
<td>11</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>M 0630</td>
<td>17600</td>
<td>6.9</td>
</tr>
<tr>
<td>α-lactalbumin</td>
<td>L 5385</td>
<td>14200</td>
<td>4.6</td>
</tr>
</tbody>
</table>

### 3.3.3 Dextran Solutions

Dextrans are polymers of glucose, joined by α-1,6 linkages, with a small number of branches attached to the main chain by α-1-3 links. Dextrans are synthesized naturally by a strain of the bacterium *Leuconostoc mesenteroides*. Dextrans have been used extensively in the past for membrane characterization (Mochizuki and Zydney, 1992) since they do not have any ionizable side groups, thus providing a purely size-based measure of the membrane sieving characteristics. Granath (1958) measured the diffusion coefficient for different molecular weight dextrans using a diffusion cell with the data correlated using a simple power law expression as:

$$D = 7.667 \times 10^{-5} \times M_w^{-0.47752}$$

(3.3)

where $D$ is the diffusion coefficient (in m$^2$/s) and $M_w$ is the molecular weight in Da. The Stokes radii can be evaluated from Equation (3.3) using the Stokes-Einstein equation as (Granath and Kvist, 1967):
\[ R_{SE} = 0.31 \times M_w^{0.47752} \]  

with \( R_{SE} \) given in Å.

A narrow molecular weight dextran standard with weight average molecular weight of 4 kDa (American Polymer Standards Corp., Mentor, Ohio) was used as a model neutral solute in Chapter 4. Dextran solutions were prepared by dissolving preweighed amounts of dextran powder in the desired buffer solution. All dextran solutions were pre-filtered through a 0.22 μm Acrodisc® syringe filter (PALL Corp., Ann Arbor, MI) to remove any undissolved dextran immediately prior to use.

### 3.4 Ultrafiltration Experiments

#### 3.4.1 Ultrafiltration Set-up

Ultrafiltration experiments were performed in an Amicon 8010 stirred cell (Millipore Corp., Bedford, MA) placed on a magnetic stir plate. A membrane disk with effective area of 4.1 cm² was placed in the bottom of the stirred cell directly on top of a porous Tyvek® support that minimized deformation of the membrane at high pressure. The stirring speed was set to 600 rpm using a Strobotac Type 1531-AB strobe light (General- Radio Co., Concord, MA). The stirred cell was connected to an air-pressurized acrylic solution reservoir, with the filtrate flux controlled by adjusting the pressure (see Figure 3.5). Constant flux ultrafiltration experiments were performed using a Masterflex 7523-20 peristaltic pump (Cole-Parmer, Vernon Hills, IL) connected to the filtrate line (downstream of the membrane) to avoid the possibility of protein denaturation or aggregation associated with the pump.
Figure 3.5  Schematic of experimental set-up for constant pressure ultrafiltration experiments. Adapted from Bakhshayeshirad (2011).

3.4.2 Membrane Hydraulic Permeability

The membrane hydraulic permeability ($L_p$) was evaluated by measuring the filtrate flux as a function of pressure using a 500 mM buffered KCl solution unless otherwise stated. The high salt concentration minimized the effect of counter-electroosmosis (Mehta and Zydney, 2006). The permeability was evaluated from the slope of the filtrate flux ($J_v$) versus transmembrane pressure ($\Delta P$) data as:

$$L_p = \frac{\mu J_v}{\Delta P}$$  

(3.5)
Data were obtained at transmembrane pressures between 14 and 35 kPa (corresponding to between 2 and 5 psi), with the filtrate flow rate evaluated by timed collection using a digital balance (Model AG104, Mettler Toledo, Columbus, OH) with an accuracy of 0.1 mg. The system was then flushed with at least 25 L/m² of water, followed by buffer at the pH and ionic strength of interest.

3.4.3 Protein Sieving

Protein sieving experiments were performed with membranes that were first soaked overnight at 4 °C in the appropriate protein solution to minimize any initial transients associated with protein adsorption on and within the membrane pores. The membrane permeability was re-evaluated after this initial adsorption. The system was then flushed with at least 25 L/m² of buffer solution at the pH and ionic strength of interest. The stirred cell and feed reservoir were then emptied and refilled with the desired protein solution. The device was then re-pressurized (for the constant pressure experiments) or the flux was set using the filtrate pump. Constant filtrate flux was maintained by a Masterflex model 7523-20 peristaltic pump (Cole-Parmer, Vernon Hills, IL).

Protein transmission was evaluated by obtaining a small sample of the filtrate solution after filtration of a minimum of 500 μL to ensure equilibrium operation and to wash out the dead volume downstream of the membrane. The filtrate port was then clamped and a small (approximately 400 μL) sample of the bulk solution was taken directly from the stirred cell to evaluate the bulk protein concentration. The stirred cell
was then carefully emptied, rinsed with the desired buffer, and the membrane hydraulic permeability was re-evaluated. All experiments were performed at room temperature (22 ± 3 °C).

Protein concentrations were determined spectrophotometrically using a SPECTRAmax Plus 384 UV-vis spectrophotometer (MD Corp., Sunnyvale, CA) with the absorbance measured at 410 nm for cytochrome c and myoglobin, at 280 nm for α-lactalbumin, and at 240 nm for BSA. Protein concentrations in the binary mixture of lysozyme and cytochrome c were determined spectrophotometrically using the basic approach described by Ghosh (2001) with details provided in Chapter 6.

Calibration curves were developed for each protein using solutions of known concentration. Actual concentrations were evaluated by comparison of the absorbance with that of known protein standards. Samples with high protein concentrations were diluted with the desired buffer to assure that readings were taken within the linear portion of the calibration curve. All samples were analyzed within 24 hr of collection, with storage at 4 °C whenever the analysis was delayed for more than 8 hr.

3.4.4 Diafiltration

Diafiltration was performed in an Amicon stirred cell (Millipore Corp., Bedford, MA) with a 10 kDa Ultracel™ membrane to remove the dioxane and any residual reactants formed during synthesis of the protein charge ladders and peracetylated cytochrome c. The stirred cell was initially filled with the protein mixture. The acrylic feed reservoir was filled with DI water and attached to the stirred cell to provide the
diafiltration solution (in this case DI water) at the same rate as the permeate solution was drawn through the membrane. The feed reservoir was air pressured to the appropriate value. The filtrate flux was measured by timed collection with small adjustments made to the applied pressure to maintain the flux at an essentially constant value (within 0.5 µm/s). More details on the protein diafiltration are given in Chapters 6 and 7.

3.5 **Dextran Diagnostics: Size Exclusion Chromatography**

Dextran concentrations in the bulk and filtrate solutions were determined by size exclusion chromatography (also known as gel permeation chromatography). An Agilent 1100 Series high performance liquid chromatography system (Agilent Technologies, Palo Alto, CA) was used with a Superdex 200, 10/300 analytical column (13 µm particle size, 1 x 10^5 MW exclusion limit) obtained from GE Healthcare (Uppsala, Sweden). The mobile phase was a 10 mM Bis-Tris buffer at pH 7 containing 0.25 M KCl at a flow rate of 0.3 mL/min. The column was initially equilibrated with a minimum of 2 column volumes of the mobile phase. The buffer was degassed before entering the system and 25 µL samples of the dextran solution were injected by the autosampler immediately upstream of the guard column. The dextran concentration in the exit stream was evaluated using a refractive index detector (Agilent 1100). Data collection was performed using ChemStation software version A.04.08 (Agilent Technologies, CA).
3.6 Ion Exchange Chromatography

Cation exchange chromatography was performed using an AKTAEexplorer 100 chromatography system from Amersham Biosciences (now GE Healthcare, Piscataway, NJ) operated by Unicorn software version 5.01. SP Sepharose Fast Flow (SP FF) resin (GE Healthcare) with 90 µm mean particle size was packed to a height of 167 mm (column volume of 5.7 mL) following the manufacturer's directions in an Omnifit glass column (6.6 mm I.D x 250 mm length, Bio-chem Valve Inc., Boonton, NJ). Protein detection was at 214 nm using the UV-visible detector provided as part of the AKTAEexplorer system.

3.7 Capillary Electrophoresis

The net protein charge was calculated from the electrophoretic mobility determined using a G1600A High-Performance Capillary Electrophoresis instrument (Agilent Technologies, Palo Alto, CA) equipped with a dual polarity variable high voltage DC power supply and variable wavelength UV–vis diode array detector. Detection was by UV absorbance at 214 nm. Negatively-charged fused silica capillaries (Agilent Technologies, Catalog Numbers G1600-62211, G1600-61211, Palo Alto, CA) were used for analysis of the negatively-charged proteins. Both capillaries had 50 µm inner diameter with lengths of 80.5 and 65 cm, respectively. Positively-charged eCAP™ Amine capillaries (Beckman Coulter, Inc., Catalog Number 477431, Fullerton, CA) with an inner diameter of 50 µm and a total length of 65 cm were used for positively-charged proteins. Mesityl oxide was used as a neutral marker. Injection of 15-30 nL samples was
done by application of a 3.5 kPa pressure for 25 s. Data were obtained at a constant applied electric field of 25 kV, with the field direction chosen so that the direction of the bulk flow was toward the detector. The current was less than 45 µA, so Joule heating was negligible. Electropherograms were recorded and analyzed using 3D-CE ChemStation Software (version A.09.03, Agilent Technologies, Palo Alto, CA).

3.8  **X-ray Photoelectron Spectroscopy (XPS)**

XPS analysis of the surface modified membranes was performed using a Kratos Analytical Axis Ultra instrument (Kratos Analytical Inc., Chestnut Ridge, NY) available in the Materials Research Institute at The Pennsylvania State University. Data were obtained using monochromatic Al Kα as the X-Ray source (1486.6 eV photons).

3.9  **Attenuated Total Reflectance Spectroscopy (ATR)**

Fourier transform infrared spectroscopy (FTIR) in attenuated total reflectance (ATR) mode was performed on surface modified membrane samples using a Bruker IFS 66/S FTIR spectrophotometer (Bruker Optics, Billerica, MA) coupled with a Horizon™ ATR accessory (Harrick Scientific Products Inc., Pleasantville, NY) available in the Materials Research Institute at The Pennsylvania State University. The ATR accessory was equipped with a multi-reflection ZnSe ATR element with a 45 degree entrance face (50 x 10 x 2 mm).
Chapter 4

Development of High Performance Charged Ligands to Control Protein Transport through Charge-Modified Ultrafiltration Membranes


4.1 Introduction

Several recent studies have demonstrated that protein transport through semipermeable ultrafiltration (UF) membranes is strongly affected by electrostatic interactions between the charged membrane and the charged protein. These electrostatic interactions can be exploited to develop high performance ultrafiltration membranes with significantly better selectivity (for a given value of the permeability) than conventional UF membranes (Zydney, 2009; Mehta and Zydney, 2006; Christy et al., 2002; van Reis et al., 1999). For example, Mehta and Zydney (2006) showed that the transmission of positively-charged cytochrome c through a series of positively-charged membranes was a strong function of the membrane charge, in this case controlled by the number of quaternary amine groups attached to the base cellulose, with the protein transmission reduced by a factor of 100 as the membrane surface charge density increased from 0.2 to 7 mC/m². However, these studies have implicitly assumed that the membrane surface
charge and pore size are the only membrane properties determining the overall performance, without any consideration of the detailed molecular structure of the charged ligand.

Mehta and Zydney (2008) demonstrated that the spacer arm length (determined by the number of alkyl groups between the membrane and the functional ligand) can also have a strong effect on the performance characteristics of electrically charged membranes. Membranes with greater spacer arm length had greater retention of a like-charged protein even though the hydraulic permeabilities of these membranes were essentially identical. The increase in protein retention was consistent with the observed increase in membrane zeta potential associated with the reduction in electrical interactions between the two amine groups on the larger ligand. Subsequent studies showed that protein retention increased with increasing number of amine groups for membranes modified with a series of polyethyleneamines, although it was not possible to determine if this effect was due primarily to the number of charge groups or the overall length of the ligand (Mehta, 2006).

In contrast to the relatively limited data on the role of ligand properties in determining electrostatic interactions during membrane filtration, it is well-established that the detailed physicochemical properties of the functional ligand can have a significant effect on binding interactions during ion exchange chromatography (Riordan et al., 2009; Yao and Lenhoff, 2005; DePhillips and Lenhoff, 2001). For example, Riordan et al. (2009) examined the effects of ligand properties on virus retention at relatively high salt concentrations using a series of anion exchange membrane adsorbers.
Ligand charge, density, and molecular structure all contributed to virus binding, with the presence of primary amines and guanidinium groups as particularly significant factors. DePhillips and Lenhoff (2001) showed that protein retention on strong cation exchangers, generated by covalent attachment of sulfonic acid groups, was significantly greater than that on the corresponding weak cation exchanger (developed using carboxylic acids) even though the resins had identical surface charge densities. Several recent studies have reported that novel tentacle-type ligands can provide higher binding capacities than conventional ion exchange resins (Bruch et al., 2009; Yao and Lenhoff, 2005; DePhillips and Lenhoff, 2001). This increase in binding capacity has generally been attributed to the flexibility of the polyelectrolyte chains (“tentacles”), allowing multipoint binding interactions between the protein and multiple charge groups on the functional ligand.

The overall objective of the work presented in this Chapter was to examine the behavior of a series of charged composite regenerated cellulose ultrafiltration membranes generated by covalent attachment of ligands having similar physical size but with different numbers of primary, secondary, and quaternary amine groups. The membrane surface charge density was evaluated using both streaming potential and dye-binding measurements, with the number of nitrogen amines determined by X-ray photoelectron spectroscopy (XPS). Ultrafiltration experiments were performed over a range of solution ionic strength using cytochrome c as a model protein. The results provide important insights into the relationship between ligand properties and membrane performance, providing a framework for the development of novel charged ultrafiltration membranes for high performance bioprocessing applications.
4.2 Materials and Methods

The general procedures for the protein filtration experiments were described in Chapter 3 and are summarized below for convenience.

4.2.1 Solution Preparation

KCl solutions were prepared by dissolving preweighed amounts of KCl (BDH Chemicals, BDH0258) in deionized water obtained from a NANOpure® Diamond water purification system (Barnstead Thermolyne Corporation, Dubuque, IA) with a resistivity greater than 18 MΩ-cm. Salt solutions were buffered with 10 mM Bis-Tris (MPBiomedicals, 101038) as described in Chapter 3.

Cytochrome c (Sigma, C7752) with a molecular weight of 12.4 kDa and an isoelectric point of 10.4 was used as a model protein because of the extensive prior literature on cytochrome c transport through ultrafiltration membranes (Mehta and Zydney, 2008; Mehta and Zydney, 2006; Balakrishnan et al., 1993). Protein solutions were prepared by slowly dissolving the protein powder in the desired buffer at pH 7, with the resulting solution filtered through a 0.22 μm Acrodisc® syringe filter (Pall Corp.) to remove any protein aggregates immediately prior to use. Protein solutions were used within 12 h of preparation to minimize the likelihood of protein aggregation or denaturation. The protein concentration was determined spectrophotometrically using a SPECTRAmax Plus 384 UV-vis spectrophotometer (MD Corp., Sunnyvale, CA) with the absorbance measured at 410 nm. Actual concentrations were evaluated by comparison of the absorbance with that of known protein standards.
Dextran with a weight average molecular weight of 4 kDa (American Polymer Standards Corp., Mentor, Ohio) was used as a model neutral solute. The Stokes radius of the dextran was evaluated as $R_{SE} = 15.3$ Å using the correlation presented by Granath (1958) as described in Chapter 3, which is essentially the same size as cytochrome c. The dextran powder was dissolved in Bis-Tris buffer with added KCl at pH 7. Dextran concentrations were determined by size exclusion chromatography (Agilent 1100 HPLC system) with refractive index detection using a Superdex 200, 10/300 gel permeation column obtained from GE Healthcare (Uppsala, Sweden) as described in Chapter 3. The mobile phase was a 10 mM Bis-Tris buffer at pH 7 containing 0.25 M KCl at a flow rate of 0.3 mL/min.

4.2.2 Membrane Preparation

Ultrafiltration experiments were performed using positively-charged versions of Ultracel™ composite regenerated cellulose membranes with 100 kDa nominal molecular weight cut-off (Millipore Corp., Bedford, MA) made by covalent attachment of different amine-containing compounds to the free hydroxyls on the base cellulose. The specific ligands were chosen so that they had similar total length as determined by theoretical calculations using both Gaussian 2003 and Spartan 04 Software (Table 4.1). This made it possible to study the effect of the ligand chemistry independent of the ligand length.
Table 4.1  Number of amine groups, calculated size, and apparent zeta potential (at pH 7) for the charge-modified 100 kDa UltraCel™ membranes produced using the ligands shown in Figure 4.2.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Number of Amine Groups</th>
<th>Ligand Size* (nm)</th>
<th>Apparent Zeta Potential, ζ_{app} (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>2</td>
<td>1.4</td>
<td>5.3</td>
</tr>
<tr>
<td>b</td>
<td>4</td>
<td>1.8</td>
<td>6.2</td>
</tr>
<tr>
<td>c</td>
<td>6</td>
<td>1.9**</td>
<td>4.4</td>
</tr>
<tr>
<td>d</td>
<td>4</td>
<td>1.7</td>
<td>3.4</td>
</tr>
<tr>
<td>e</td>
<td>2</td>
<td>0.4**</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Calculated using Gaussian 2003 Software  
** Calculated using Spartan 04 Software

Membrane disks with 25 mm diameter were cut from large flat sheets using a stainless-steel cutting device fabricated in our laboratory. All membranes were soaked in isopropanol for 45 min to remove any wetting/storage agents. The membranes were then thoroughly rinsed with at least 100 L/m² of deionized water and stored for 24 h in a 0.1 M NaOH solution prior to surface modification.
Figure 4.1  Schematic of reactions used for charge modification: (1) activation with epichlorohydrin, (2) reaction with diamine, (3) re-activation with epichlorohydrin for sequential reactions. Adapted from Mehta and Zydney (2008).

The membranes were activated by reaction with epichlorohydrin obtained from Sigma Chemical (St. Louis, MO). Each membrane disk was placed in a 25 mL capped plastic jar containing 10 mL of 0.1 M NaOH and 5 mL of epichlorohydrin and incubated at 45 °C (Step 1 in Figure 4.1). The membranes were removed after 2 h and rinsed with DI water. The epoxide groups on the activated membranes were then reacted with one of the following amines: pentaethylenehexamine, 1,4-diaminobutane, 1,6-diaminohexane, 1,10-diaminodecane, or 2-aminoethyl trimethyl ammonium chloride (all from Sigma Chemical, St Louis, MO). The molecular structures of the different amine compounds are shown schematically in Figure 4.2.

A charge-modified membrane with two amine groups, an internal secondary amine and a terminal primary amine, was produced by placing the membrane in 20 mL of a 0.5 M solution of 1,10-diaminodecane at pH 11 ± 0.2, with the pH adjusted using 4 M
HCl as needed. The reaction occurred at 45°C for 24 h, with the membrane then removed and thoroughly rinsed with DI water for at least 1 h. An analogous membrane structure but with five secondary amines along the length of the functional ligand (6 total amines) was produced by reaction with a 1 M solution of pentaethylenehexamine. A membrane with three secondary amines and a terminal primary amine was generated using a sequential reaction / activation scheme. In this case, the membrane was first activated with epichlorohydrin and then reacted with a 0.5 M solution of 1,4-diaminobutane. The terminal amine on this structure was then activated by reaction with epichlorohydrin, with the final membrane produced by a second reaction using 1,4-diaminobutane. A similar approach was used to generate a membrane with a terminal quaternary amine. In this case, the activated membrane was initially reacted with a 0.3 M solution of 1,6-diaminohexane followed by epichlorohydrin and then a 0.3 M solution of 2-aminoethyl trimethyl ammonium chloride. This sequential reaction procedure was used since we were unable to identify a commercially available ligand of appropriate length containing a terminal quaternary amine (with a reactive primary amine for conjugation to the epichlorohydrin). Experiments were also performed with a membrane made by reacting a 0.5 M solution of the 2-aminoethyl trimethyl ammonium chloride directly with the epichlorohydrin-activated membrane to give a "short" quaternary amine functionality.
Figure 4.2  Molecular structures of the amine ligands attached to the glucose monomers (R) in the membranes generated by: (a) reaction with 1,10-diaminodecane, (b) sequential reaction with 1,4 diaminobutane, (c) reaction with pentaethylenehexamine, (d) sequential reaction with 1,6-diaminohexane followed by 2-aminoethyl trimethyl ammonium chloride, and (e) reaction with 2-aminoethyl trimethyl ammonium chloride.

4.2.3 Attenuated Total Reflectance Spectroscopy (ATR)

The effectiveness of the epichlorohydrin activation and the subsequent reaction with a free amine were examined using Fourier transform infrared spectroscopy (FTIR) in attenuated total reflectance (ATR) mode. All spectra were collected using a Bruker IFS 66/S FTIR spectrophotometer (Bruker Optics, Billerica, MA) coupled with a
Horizon™ ATR accessory (Harrick Scientific Products Inc., Pleasantville, NY) equipped with a multi-reflection ZnSe ATR element with a 45 degree entrance face (50 x 10 x 2 mm). The internal reflection element was carefully cleaned before each measurement, with all spectra referenced to the clean ATR crystal. Membrane samples were initially cut into small (approximately 2 cm x 1 cm) pieces using a razor blade, gently dried using a Kimwipe, and placed onto the ATR crystal. The mounted samples were dried using a heat gun and scanned 1600 times with a resolution of 6 cm⁻¹.

4.2.4 X-ray Photoelectron Spectroscopy (XPS)

The density of amine groups in the charge-modified membranes was estimated by evaluating the elemental composition of the membranes using X-ray photoelectron spectroscopy. The membranes were flushed with deionized water, dried gently using a Kimwipe, cut into small (approximately 1.2 cm x 0.5 cm) pieces using a razor blade, and mounted on a sample platen. XPS analysis was performed using a Kratos Analytical Axis Ultra instrument (Kratos Analytical Inc., Chestnut Ridge, NY) available in the Materials Research Institute at The Pennsylvania State University. Data were obtained using monochromatic Al Kα as the X-Ray source (1486.6 eV photons) at a pass energy of 40 eV. The pressure in the analysis chamber was 10⁻⁸ Torr. The XPS data were analyzed using CasaXPS Software (version 2.3.12Dev9) by integrating the peak areas and applying the appropriate relative sensitivity factors to account for the x-ray cross section and the transmission function of the spectrometer.
4.2.5 Dye-Binding Assay

The number of accessible charge groups on the membranes was estimated using a dye-binding assay as described in Chapter 3. Cibacron Blue 3GA (Sigma, C9534) was used as the negatively charged dye. Membranes were incubated at room temperature in 10 mL of a 0.1 g/L Cibacron Blue solution at pH 5 for 4 h, with the solution gently agitated on a shaker plate. Small samples of the free solution were obtained immediately before and after exposure to the membrane, with the concentration of Cibacron Blue determined from the absorbance at 350 nm using a SPECTRAmax Plus 384 UV-vis spectrophotometer (MD Corp., Sunnyvale, CA). Actual concentrations were evaluated by comparison of the absorbance with that of known dye standards, with the total mass of bound dye calculated from a simple mass balance.

4.2.6 Streaming Potential Measurement

The surface charge characteristics of the membranes in 10 mM buffered KCl solutions were evaluated from streaming potential measurements following the procedure described by Burns and Zydney (2000) as described in Chapter 3. The apparent zeta potential ($\zeta_{app}$) was evaluated from the slope of the voltage (streaming potential) as a function of pressure using the Helmholtz–Smoluchowski equation (Equation 3.1). It is important to note that Equation (3.1) is only valid under conditions where the double layer thickness is very small compared to the pore radius. In actuality, the double layer thickness in a 10 mM ionic strength solution is 3.1 nm, which is slightly less than half the value of the pore radius (evaluated as 6.9 nm using Equation (4.2) assuming Poiseuille
flow through a uniform array of cylindrical pores). Thus, \( \zeta_{\text{app}} \) should be treated as an apparent or effective zeta potential, which is directly related to the membrane surface charge but is different than the 'true' zeta potential associated with the pore surface.

### 4.2.7 Ultrafiltration

Ultrafiltration experiments were performed in an Amicon 8010 stirred cell with effective membrane area of 4.1 cm\(^2\) (Millipore Corp., Bedford, MA) as described in Chapter 3. The stirring speed was set to 600 rpm using a Strobotac Type 1531-AB strobe light (General- Radio Co., Concord, MA). The membrane hydraulic permeability \( (L_p) \) was evaluated by measuring the filtrate flux as a function of pressure using a 500 mM KCl solution buffered with 20 mM Bis-Tris at pH 7 as described in Chapter 3. The high salt concentration minimized the effect of counter-electroosmosis. The permeability was evaluated from the slope of the data using Equation (2.6). The system was then flushed with at least 25 L/m\(^2\) of buffer at the ionic strength of interest.

Protein ultrafiltration experiments were performed using a 2 g/L cytochrome c solution at pH 7 over a range of solution ionic strength. Each membrane disk was soaked overnight at 4 °C in the appropriate protein solution to minimize any initial transients associated with protein adsorption on or within the membrane pores. The filtrate flux was maintained at 7 \( \mu \)m/s using a Masterflex 7523-20 peristaltic pump (Cole-Parmer, Vernon Hills, IL) connected to the filtrate line (downstream of the membrane) to avoid the possibility of protein denaturation or aggregation associated with the pump. The feed reservoir that is connected to the stirred cell was air-pressurized so that a positive gauge
pressure was maintained throughout the system. Relatively low protein concentrations and low filtration velocities were employed during the ultrafiltration to minimize effects of concentration polarization and fouling. This was also confirmed experimentally by measuring the membrane hydraulic permeability before and after the ultrafiltration, with the permeability values differing by less than 10% for all runs.

Protein samples were collected from the filtrate and bulk solutions as described in Chapter 3. The observed sieving coefficient was calculated as

\[ S_v = \frac{C_f}{C_b} \]  

(4.1)

where \( C_f \) and \( C_b \) are the protein concentration in the filtrate and bulk solutions, respectively. The stirred cell was then carefully emptied, flushed with at least 25 L/m² of buffered KCl solution, and refilled with a fresh protein solution at a new ionic strength. The same procedure was used to evaluate the sieving coefficients for the 4 kDa dextran. All experiments were performed at room temperature (22 ± 3°C).

4.3 Results and Discussions

4.3.1 Membrane Modification

In order to study the specific effects of the ligand chemistry, and in particular the number and nature of the amine groups, on the performance of the charge-modified membranes, it was important to try to keep the length of the different ligands and the density of the surface modification nearly uniform across all membranes. All of the charge functionalities examined in this Chapter had nearly the same number of internal
carbon atoms (calculated based on the total ligand structure accounting for the epichlorohydrin activation and the use of sequential modification steps). The expected length of each ligand was calculated using either Gaussian 2003 or Spartan 04 Software with results summarized in Table 4.1. The molecular structures were manually constructed in Gaussview and initially relaxed using the Dreiding force field. The structures were minimized in Gaussian 2003 with density functional theory (DFT) using the hybrid UB3LYP exchange-correlation functional. The Lanl2DZ basis set was also invoked. Each molecule’s complete atomistic structure was considered in the quantum mechanical calculations. All calculations were performed on a Linux PC cluster using four 3.06 Xeon CPUs with 4GB RAM. The ligand generated by reacting the epichlorohydrin-activated membrane with 1,10-diaminodecane was approximately 1.4 nm in length, while the other ligands have lengths between 1.7 and 1.9 nm. The length of the short quaternary amine ligand, formed by reacting the epichlorohydrin-activated membrane directly with 2-aminooethyl trimethyl ammonium chloride, was only 0.4 nm.

The step-wise reaction chemistry described in Figure 4.1 was tracked by FTIR-ATR spectroscopy. FTIR spectra were obtained in ATR mode for the upper surface of membranes before modification, after activation with epichlorohydrin, and after charge-modification by reaction with the given ligand. Figure 4.3 shows typical results for the membrane made with the pentaethylenehexamine; the spectra for the epichlorohydrin-activated membrane and the charge-modified membrane are shown after subtracting off the absorbance of the un-modified cellulose membrane as a baseline. The reference spectrum for EPI in the liquid phase is also shown for comparison. The epoxide peaks
are visible in the 750-850 cm\(^{-1}\) region for the epichlorohydrin-activated membrane, confirming the attachment of the epichlorohydrin to the base cellulose. The peaks at 840 and 850 cm\(^{-1}\) correspond to the symmetric and anti-symmetric ring deformation in EPI, respectively (Kalasinsky and Wurrey, 1980). These peaks were essentially invisible for the membrane after exposure to the pentaethylenehexamine for 24 hr, suggesting that nearly all of the epichlorohydrin sites on the activated membrane are converted to the corresponding ether. Similar results were obtained with the other ligand chemistries. It was not possible to quantitatively evaluate the extent of modification from the ATR spectra due to the large noise associated with the relatively low density of epoxide groups in the activated membranes.
The number of ligands attached to the membrane surface was controlled by adjusting the reactant concentration to obtain similar degrees of modification with the different chemistries. The extent of modification was estimated from data for the membrane hydraulic permeability, assuming that membranes with similar permeabilities (after modification with ligands having the same size) should have the same extent of
modification. Permeability data were obtained using 500 mM KCl buffered with 20 mM Bis-Tris; the high salt concentration should eliminate any effects of counter-electroosmosis arising from the membrane surface charge. Membrane disks were cut from adjacent regions on large flat sheets. These disks were only modified if the initial permeability was $3.3 \pm 0.1 \times 10^{-12}$ m; membranes with unusually high or low permeability were simply discarded. The membranes were then activated and reacted with the appropriate amine compound, with the permeability of the charge-modified membrane evaluated after thoroughly flushing the membrane to remove any residual chemicals or adsorbed ligands. The ligand concentration was then increased or decreased based on the measured permeability, with the goal of achieving a final permeability of $2.7 \pm 0.1 \times 10^{-12}$ m. This change in permeability corresponds to an average reduction in mean pore size of 0.4 nm as determined from the Hagen-Poiseuille equation (Zeman and Zydney, 1996):

$$r_p = \left( \frac{8 \delta_p L_p}{\varepsilon} \right)^{\frac{1}{3}} = \left( \frac{8 \delta_p L_p A}{N_p \pi} \right)^{\frac{1}{3}}$$  \hspace{1cm} (4.2)

assuming that the membrane is composed of a parallel array of uniform cylindrical pores.

The second expression in Equation (4.2) was developed using the definition of the membrane porosity ($\varepsilon$) where $A$ is the membrane cross-sectional area, $N_p$ is the total number of pores, $r_p$ is the average pore radius, and $\delta_p$ is the pore length. The change in pore radius was estimated from Equation (4.2) assuming that $N_p$ remains constant using an initial porosity of $\varepsilon = 0.5$ and a pore length of $\delta_p = 1 \mu$m based on the thickness of the membrane skin layer (Rao and Zydney, 2005). The membrane with the small quaternary
amine ligand was generated using the same reactant concentration and time as that employed for ligand (a).

The extent of membrane modification was verified by X-ray photoelectron spectroscopy (XPS). Figure 4.4 shows typical XPS spectra for the upper surface of an unmodified composite regenerated cellulose membrane and a charge-modified version produced by reaction with pentaethylenhexamine. The nitrogen peak occurs at a binding energy of approximately 398 eV. This peak was essentially absent in the XPS spectrum for the base cellulose membrane, but is clearly visible for the modified membrane confirming the attachment of the pentaethylenhexamine. Quantitative analysis of the full XPS spectra gave nitrogen atomic concentrations of 0.06 % for the unmodified membrane and 1.6 % for the membrane after attachment of the pentaethylenhexamine. The small amount of nitrogen present in the unmodified membrane is probably associated with trace impurities; this value was subtracted from the measured atomic percent of the charge-modified membranes in all subsequent calculations.
Figure 4.4  XPS spectra showing nitrogen peak for an unmodified UltracelTM 100 kDa membrane and a charge-modified membrane generated by reaction with pentaethylenehexamine.

The degree of modification was estimated from the atomic fraction of nitrogen ($F_N$) as

$$F_N = \frac{af}{b + df}$$  \hspace{1cm} (4.3)

where $f$ is the fraction of glucose rings in the base cellulose that were modified with an attached ligand (assuming that all epichlorohydrin groups were reacted with the amine compound), and $a$, $b$, and $d$ are constants evaluated from the atomic structure of the
modified membrane. For example, the membrane modified with pentaethylenehexamine has \( a = 6 \) (accounting for the 6 amine groups along the length of the ligand) with \( b = 11 \) based on the number of carbon and oxygen atoms in the glucose ring (6 C and 5 O), and \( d = 20 \) accounting for the ether and alcohol functionality associated with the epichlorohydrin activation (3 C and 1 O) and the structure of the ligand (10 C and 6 N). This gives \( f = 0.029 \) for the pentaethylenehexamine-modified membrane, consistent with a very low degree of charge modification.

The calculated values for the nitrogen content and the fraction of modified glucose rings for the different membranes are shown in Table 4.2. The membranes modified with ligands (b), (c), and (e) had essentially equal fractions of modified glucose rings \( (f = 0.03) \). In contrast, the XPS data for the membranes modified with ligands (a), and (d) had slightly higher degrees of modification with \( f \) ranging from 0.06 to 0.07, even though membranes (a) to (d) all had essentially the same hydraulic permeability after modification. The implications of these differences are discussed in more detail subsequently.
Table 4.2 Degree of modification and nitrogen content (from XPS) and the volumetric charge density (from dye-binding) along with the calculated surface charge density for charge-modified 100 kDa Ultracel™ membranes produced using the ligands in Figure 4.2.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>f</th>
<th>N Content</th>
<th>$Q_m$ (C/m³)</th>
<th>Surface Charge Density (C/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>0.067</td>
<td>0.13</td>
<td>2.7 x 10⁶</td>
<td>0.52</td>
</tr>
<tr>
<td>b</td>
<td>0.030</td>
<td>0.12</td>
<td>4.6 x 10⁶</td>
<td>0.47</td>
</tr>
<tr>
<td>c</td>
<td>0.029</td>
<td>0.17</td>
<td>3.4 x 10⁶</td>
<td>0.66</td>
</tr>
<tr>
<td>d</td>
<td>0.062</td>
<td>0.25</td>
<td>3.3 x 10⁶</td>
<td>0.96</td>
</tr>
<tr>
<td>e</td>
<td>0.035</td>
<td>0.07</td>
<td>1.7 x 10⁶</td>
<td>0.27</td>
</tr>
</tbody>
</table>

4.3.2 Effective Surface Charge

The effective surface charge characteristics of the charge-modified membranes were evaluated from streaming potential measurements obtained with the fluid flow directed through the membrane pores. Figure 4.5 shows typical experimental data for the measured streaming potential ($E_z$) as a function of the transmembrane pressure for the charge-modified 100 kDa Ultracel™ membranes along with results for an unmodified membrane. The data were highly linear for each membrane, with $r^2$ values greater than 0.98, and repeat measurements with the same membrane were quite reproducible (slopes within ±15%). The non-zero intercepts, which caused the displacement between the data for different membranes, arise from asymmetries in the Ag/AgCl electrodes and
have no effect on the data analysis. The unmodified membrane has a negative slope, i.e., a negative surface charge, which is probably related to the preferential adsorption of negative ions from the electrolyte solution or possibly to the presence of trace carboxylic acid groups in the cellulose. The modified membranes are all positively-charged, with the membrane modified with ligand (b), formed by sequential reaction with 1,4-diaminobutane, having the greatest apparent zeta potential.

**Figure 4.5** Streaming potential data for an unmodified 100 kDa UltraceI™ membrane and a series of charge-modified membranes with ligands having similar length but different number of amine groups (labels correspond to chemical structures shown in Figure 4.2).
The slope of the streaming potential versus pressure data was used to calculate the apparent zeta potential via Equation (3.1). The effective surface charge density within the membrane pore \( q_p \) was then calculated from the apparent zeta potential \( \zeta_{app} \) using the Helmholtz – Smoluchowski equation:

\[
q_p = 4C_0F\kappa^{-1}\sinh\left(\frac{F\zeta_{app}}{2RT}\right)
\]  

(4.4)

where \( C_0 \) is the bulk electrolyte concentration, \( F \) is Faraday’s constant, \( R \) is the ideal gas constant, \( T \) is the absolute temperature, and \( \kappa^{-1} \) is the thickness of the electrical double layer.

\[
\kappa^{-1} = \left[\frac{F^2\Sigma z_i^2 C_i}{\varepsilon_0\varepsilon_r RT}\right]^{-1/2}
\]  

(4.5)

where \( z_i \) and \( C_i \) are the valence and concentration of each ion, \( \varepsilon_0 \) is the permittivity of free space, and \( \varepsilon_r \) is the dielectric constant of the solution. Equation (4.4) neglects the effects of surface conductance, a finite double layer thickness, and the detailed pore geometry and pore size distribution of the membrane; these assumptions are consistent with the use of Equation (3.1) to evaluate the apparent membrane zeta potential. Thus, the calculated values of \( q_p \) represent an effective (approximate) surface charge density of the membrane pores.

The calculated values of the apparent zeta potential and surface charge density of the membranes used in this study are shown in Table 4.1 and Table 4.2, respectively. The apparent zeta potential for the membrane with only two amine groups \((n=2)\) was 5.3 mV, and this increased to 6.2 mV for the membrane that was formed by the sequential
reaction with 1,4-diaminobutane (n=4). However, the apparent zeta potential for the membrane modified with pentaethylenehexamine (n=6) was only 4.4 mV, in contrast to the very similar degree of modification calculated from the XPS data in Table 4.2. The low surface charge density for ligand (c) was likely due in part to the incomplete protonation of the closely spaced amine groups along the backbone of this ligand due to intramolecular electrostatic interactions. An estimate of the degree of protonation of the different amines based on their respective pKₐ values (determined from an online computer package at http://www.acdlabs.com/products/pc_admet/physchem from ACD/Labs, Toronto, Canada) indicated that only slightly more than 50% of the amines in ligand (c) were actually charged at pH 7 compared to more than 75% of the amines for ligand (d) and essentially all of the amines for ligands (a), (b), and (e).

In addition to the streaming potential measurements, the effective surface charge of the different membranes was also evaluated using a quantitative dye-binding assay. The membranes were exposed to a 0.1 g/L Cibacron Blue solution at pH 5 for 4 h, with the amount of bound dye determined from the change in Cibacron Blue concentration in the free solution. Control experiments performed with the unmodified cellulose membrane showed very low levels of non-specific binding. The volumetric charge density of the membrane was evaluated directly from the mass of bound Cibacron Blue (M) assuming that each dye molecule binds to a single amine group as:

\[ Q'_m = \frac{MF}{MW_{CB}V'_m} \]  
(4.6)

68
where $M$ was evaluated by subtracting off the amount of Cibraron Blue bound to the un-modified membrane, $MW_{CB} = 774$ Da is the molecular weight of Cibacron Blue, and $V_m = 1.64 \times 10^{-8}$ m$^3$ is the total volume of the cellulosic part of the membrane disk, assuming that the dye binds throughout the membrane skin and the cellulosic substructure (total thickness of approximately 40 µm; the porous polyethylene support is assumed to have no binding of the dye). The volumetric charge density of the modified membranes ranged from $1.7 \times 10^6$ to $4.6 \times 10^6$ C/m$^3$, with the lowest charge density obtained with the membrane modified with ligand (e). This is consistent with the lower number of amine groups along the length of this ligand. It is also possible that the Cibacron Blue could interact with more than one amine group on a single ligand, which could lead to an underestimation of the number of charge groups for the ligands with multiple amines. Note that the dye-binding assay provides a measure of the charge density throughout the entire thickness of the cellulosic layer of the membrane while the XPS spectra only probes to a depth of 10 nm from the upper surface of the membrane.

The effective surface charge densities of the charge-modified membranes estimated from the streaming potential, dye-binding, and XPS data are summarized in the final 3 columns of Table 4.2. Details of the calculations are provided in the Appendix B. The surface charge densities calculated from the dye-binding and XPS data are similar, although the membrane modified with ligand (b) had the largest charge density as determined from the dye-binding while the membrane modified with ligand (d) had the largest charge density based on the XPS results. The membrane modified with ligand (b) had the greatest surface charge density determined from the streaming potential.
measurements while the membrane modified with ligand (e) had the lowest surface charge density, both of which are consistent with results from the dye-binding assay.

It is important to note that the surface charge densities determined from the streaming potential measurements were nearly 3 orders of magnitude smaller than those determined by either the dye-binding assay or XPS. As discussed previously (and in Appendix B), the evaluation of the surface charge densities using the different methods involved a number of assumptions and approximations regarding the membrane pore size, the detailed fluid flow, and the location of the charge groups within the pores. However, it is very unlikely that any of these would be able to explain the much smaller values of the charge determined from the streaming potential measurements. One possible explanation for this unexpected behavior is that many of the positively-charged amine groups might be so strongly associated with counterions that they act as neutral sites during the streaming potential experiments – the streaming potential provides a measure of the concentration of mobile counterions contained within the pores while the XPS and dye-binding assays likely include all nitrogens (the sulfonic acid groups on the Cibacron Blue should easily displace any bound counterions in the buffer solution). In addition, the analysis of the streaming potential data using Equations (3.1) and (4.4) implicitly assumes that the plane of shear (i.e., the no-slip boundary condition) is coincident with the location of the surface charge groups. This assumption is almost certainly not valid for the ligands containing multiple charge groups, all but one of which were located along the length of the ligand (and thus at different radial positions in the pore). The relatively large ligands used in this study (ligand length around 1.5 nm
compared to a pore radius of 6.9 nm) might also allow the electrolyte solution to flow through the annular region between the location of the terminal amine group and the outer radius of the unmodified pore, although it seems very unlikely that this could explain the three orders of magnitude difference in the calculated surface charge densities.

4.3.3 Protein Ultrafiltration

Figure 4.6 shows typical experimental data for the observed sieving coefficients of cytochrome c through the different charge-modified membranes as a function of solution ionic strength. Also shown for comparison are the sieving coefficients for an unmodified 100 kDa Ultracel™ membrane. In each case, the data were obtained using 2 g/L solutions of cytochrome c at pH 7 at a constant flux of 7 µm/s (25 L/m²/h) using a stirring speed of 600 rpm. The observed sieving coefficient ($S_o$) was evaluated from the cytochrome c concentrations in the filtrate and bulk solutions using Equation (4.1). The $S_o$ values provide a measure of the intrinsic membrane properties since the low filtrate flux and high stirring speed minimize concentration polarization effects in the stirred cell. At high ionic strength (250 mM), the sieving coefficients were nearly identical for all of the charge-modified membranes, with values only slightly below that obtained with the un-modified Ultracel™ membrane. The observed sieving coefficients for the charge-modified membranes decrease with decreasing ionic strength, with the sieving coefficients in the 2 mM ionic strength solution being as much as 33-fold smaller than those at high salt concentrations. This effect was completely absent with the unmodified
Ultracel\textsuperscript{TM} membrane, with the sieving coefficient remaining greater than 0.9 at all solution ionic strengths. Thus, the strong reduction in sieving coefficient at low salt concentrations seen in Figure 4.6 is a direct result of the strong electrostatic repulsion between the positively-charged membranes and the positively-charged protein. Note that cytochrome c has a net positive charge of approximately 6 electronic charges at pH 7, corresponding to a surface charge density of 33 mC/m\textsuperscript{2} as determined from electrophoretic mobility data.

![Graph](image.png)

**Figure 4.6** Observed sieving coefficients for cytochrome c as a function of solution ionic strength through an unmodified Ultracel\textsuperscript{TM} 100 kDa membrane and a series of charge-modified membranes at pH 7 (labels correspond to chemical structures shown in Figure 4.2).
The greatest protein retention was obtained with the membrane formed by sequential reaction with 1,4 diaminobutane, which has 3 secondary amines along the length of the ligand and one terminal primary amine. This was also the membrane that had the greatest surface charge density as determined from both the streaming potential and dye-binding assays. Similarly, the lowest protein retention (of the membranes modified with ligands around 1.5 nm in length) were obtained with the membrane modified with the pentaethylenehexamine (ligand c) and the long quaternary amine (ligand d), which were the membranes with the lowest surface charge density among this group based on the measured zeta potential and dye-binding assays (but not the XPS data). Even less protein retention was obtained for the membrane modified with the short quaternary amine (ligand e). For example, the sieving coefficient in the 2 mM ionic strength solution for the membrane made with ligand (e) was about 2-fold greater than that for the modified membrane having the same terminal quaternary amine but with a longer total length (ligand d). This higher protein transmission is consistent with the very low surface charge density, and probably slightly larger effective pore size, for the membrane modified with the shorter quaternary amine ligand.

The effect of membrane charge on the protein sieving coefficient can be seen more clearly in Figure 4.7, which shows the observed sieving coefficient as an explicit function of the apparent zeta potential (determined from the streaming potential measurements) for the four ligands with approximate lengths of 1.5 nm at three different ionic strengths. The sieving coefficient was essentially independent of the membrane zeta potential at high ionic strength due to the absence of any significant electrostatic
interactions because of the shielding provided by the bulk electrolyte under these conditions. In contrast, the observed sieving coefficient in the 2 mM ionic strength solution decreased from $S_o = 0.1$ for the membrane modified with pentaethylenehexamine ($\zeta_{app} = 4.4 \text{ mV}$) to a value of only $S_o = 0.02$ for the membrane generated by sequential reaction with the 1,4 diaminobutane ($\zeta_{app} = 6.2 \text{ mV}$). The sieving coefficients appear to be very well correlated with the apparent zeta potential. Plots of $S_o$ as a function of the extent of modification determined by the dye-binding assay showed similar behavior but with somewhat greater scatter; the results using the XPS data showed a very low degree of correlation. These results suggest that the apparent zeta potential provides the best measure of the effective membrane surface charge characteristics in the context of protein ultrafiltration.

In order to obtain additional insights into the separation performance of the charge-modified membranes, experiments were performed to evaluate the "electrostatic selectivity", $\psi$, which is defined as the ratio of the sieving coefficient for a neutral solute of approximately the same size to that for the charged cytochrome c under a given set of experimental conditions. A 4 kDa dextran was used as the neutral solute with the dextran sieving coefficients evaluated using the same membranes used for the cytochrome c experiments (but in the absence of any cytochrome c).
Observed sieving coefficients for cytochrome c at 2, 5, and 250 mM ionic strength as a function of the apparent zeta potential for the different charge-modified membranes.

Figure 4.8 shows the electrostatic selectivity between cytochrome c and the 4 kDa dextran for the charge-modified membranes as a function of the apparent zeta potential in 2 mM and 250 mM ionic strength solutions. At high ionic strength, there was essentially no selectivity between cytochrome c and the 4 kDa dextran, with $\psi \approx 1$ at all values of the apparent zeta potential. The similar sieving coefficients for the neutral dextran and the charged cytochrome c at high ionic strength are consistent with the very similar size
of the two solutes. The selectivities were significantly greater in the 2 mM ionic strength solution, with the greatest selectivity obtained for the charge-modified membranes having the greatest apparent zeta potential. The maximum selectivity under these conditions was $\psi = 36$ for ligand (b), which has 4 amine groups (formed by sequential reaction with the 1,4-diaminobutane).

**Figure 4.8** Electrostatic selectivity of the charge-modified Ultracel™ 100 kDa membranes at 2 and 250 mM ionic strength as a function of the apparent zeta potential. The electrostatic selectivity was evaluated as the ratio of the sieving coefficients for a 4 kDa neutral dextran to that for the positively-charged cytochrome c.
4.4 Conclusions

The work presented in this Chapter provides one of the first experimental studies of the possible effects of the detailed molecular structure of a series of charged ligands containing different numbers of primary, secondary, and quaternary amines on the performance characteristics of charge-modified ultrafiltration membranes. These positively-charged membranes provide significantly greater retention of the positively charged cytochrome c at low ionic strength due to the strong electrostatic exclusion under these conditions. Protein transmission was well correlated with the apparent zeta potential of the membrane, determined from streaming potential measurements obtained with flow through the membrane pores, without any significant contribution from the number of amine groups along the length of the membrane or the presence of a quaternary versus primary amine. For example, the observed sieving coefficient in a 2 mM ionic strength solution decreased from $S_o = 0.1$ for the membrane modified with pentaethylenehexamine ($\zeta_{app} = 4.4$ mV with 6 amine groups) to a value of only $S_o = 0.02$ for the membrane modified by sequential reaction with 1,4 diaminobutane ($\zeta_{app} = 6.2$ mV with 4 amine groups). There was a corresponding increase in the electrostatic selectivity with increasing apparent zeta potential, with the more heavily charged membrane providing nearly 40-fold selectivity for the separation of cytochrome c from a neutral dextran with similar hydrodynamic radius.

The underlying factors causing the differences in apparent zeta potential for the membranes made with the different charged ligands is unclear. The zeta potential for the membrane with 4 amine groups (formed by sequential reaction with 1,4-diaminobutane)
was greater than that for the membrane with only two amine groups (formed by reaction with 1,10-diaminodecane) as expected. However, the apparent zeta potential for the membrane with three secondary amines and a terminal quaternary amine was only 3.4 mV, even though this membrane actually had a higher degree of modification than the membrane with 4 amine groups (formed by sequential reaction with 1,4-diaminobutane) based on the XPS spectra. One possibility is that the quaternary amine group is so strongly associated with a negative counterion that the membrane behaves as if it is more weakly charged, both in terms of its apparent zeta potential and protein retention. Note that the membrane with a short quaternary amine ligand (formed by direct reaction of the activated membrane with 2-aminoethyl trimethyl ammonium chloride) had by far the lowest apparent zeta potential \((\zeta_{app} = 1 \text{ mV})\) and the lowest degree of protein retention, consistent with this basic hypothesis. In addition, the apparent zeta potential only provides a very crude measure of the membrane surface charge. More quantitative analysis accounting for the detailed effects of the ligand molecular structure on fluid flow, ion distributions, and electrostatic interactions would be needed to fully understand the effects on membrane performance.

The very similar results obtained with membranes having a terminal quaternary amine (ligand d) and a terminal primary amine (ligand c) suggest that the detailed chemistry of the charged ligand may have minimal effect on the membrane performance, beyond that associated with the difference in apparent zeta potential. However, this behavior might be very different at higher pH where the primary amine would become de-protonated (and thus uncharged) while the quaternary amine would be expected to
retain its charge due to the very high pK_a value. Further studies would be needed to determine whether the apparent membrane zeta potential remains the critical parameter describing the surface charge, and the strength of the electrostatic interactions during ultrafiltration, under these conditions.
Chapter 5

Development of Surface Modified Membranes with Zwitterionic Ligands to Control Protein Transport and Fouling during Ultrafiltration

Note: Part of the material presented in this Chapter was previously published in: Rohani M.M., Zydney A.L., Protein Transport through Zwitterionic Ultrafiltration Membranes, Journal of Membrane Science, 2011, Submitted.

5.1 Introduction

A number of recent studies have demonstrated that zwitterionic surfaces are highly resistant to protein adsorption (Shi et al., 2008; Liu et al., 2010; Sun et al., 2006; Chen et al., 2005; Chen and Jiang, 2008). The exterior membrane of biological cells has a high concentration of zwitterionic phospholipids (Chen et al., 2005; Vermette and Meagher, 2003); the resistance of these biological surfaces to protein adsorption and thrombogenesis was the initial motivation for using zwitterionic ligands to reduce protein adsorption to synthetic surfaces (Chen et al., 2005; Vermette and Meagher, 2003; Lewis, 2000; Ishihara and Takai, 2009). The pioneering work in this area was done by Holmlin et al. (2001) using self-assembled monolayers of alkanethiols on gold. Mixed monolayers containing equal amounts of trimethylammonium and sulfonate groups adsorbed less than 1% of a protein monolayer when exposed to solutions of fibrinogen or
lysozyme. Similar results were obtained using ligands that combined a positive and negative moiety as part of a single molecular structure. The high resistance to protein adsorption has typically been attributed to the strong hydration of the zwitterionic surfaces via a combination of electrostatic and hydrogen bonding interactions (Liu et al., 2010; Sun et al., 2006; Chen et al., 2005; Ishihara et al., 1998; Harder et al., 1998).

The idea of using zwitterionic surfaces to reduce membrane fouling was first examined by Jiang and co-workers using copolymer membranes formed from sulfobetaine and either polyethersulfone (Wang et al., 2006) or acrylonitrile (Sun et al., 2006). In both cases, the membranes were highly resistant to fouling during ultrafiltration of bovine serum albumin (BSA) solutions. Subsequent studies have examined the behavior of membranes formed by grafting zwitterionic polymers (Liu et al., 2010) or small ligands (Shi et al., 2011) to the surface of previously cast membranes. Liu et al. (2010) used static water contact angle and thermogravimetric analysis to show that these zwitterionic membranes were more hydrophilic than unmodified cellulose membranes due to increased binding of water molecules. Shi et al. (2011) examined the behavior of polyacrylonitrile membranes modified with a series of short-chain zwitterionic amino acids (lysine, glycine and serine). The membrane modified with lysine showed the greatest resistance to protein fouling, consistent with the compact hydration layer seen using molecular dynamic simulations.

In contrast to the extensive analysis of the fouling characteristics of these zwitterionic membranes, there have been no quantitative studies of the effects of the zwitterionic surface on protein retention. The retention characteristics are of critical
importance in both conventional ultrafiltration for protein concentration / buffer exchange (van Reis and Zydney, 2010; Mehta and Zydney, 2005) and in applications of ultrafiltration for high performance protein separations (Zydney, 2009). Mehta and Zydney (2005) showed that negatively-charged ultrafiltration membranes, produced by covalent attachment of small sulfonic acid groups to a base cellulose membrane, provided a much better combination of permeability and selectivity (product retention) than commercially-available (un-modified) membranes. A number of studies have demonstrated that charged ultrafiltration membranes can also be used for high resolution protein separations, exploiting differences in electrostatic interactions between the membrane and product / impurity (van Reis and Zydney, 2007; Lebreton et al., 2008; van Reis et al., 1999).

The primary objective of the studies presented in this Chapter was to obtain quantitative data on protein transport / retention for a series of zwitterionic ultrafiltration membranes generated by covalent attachment of small zwitterionic ligands to a base cellulose membrane. The extent of membrane modification was examined using X-ray photoelectron spectroscopy (XPS), with the effective membrane surface charge determined using streaming potential measurements. Filtration experiments were performed using basic (cytochrome c), neutral (myoglobin), and acidic (α-lactalbumin) proteins over a range of solution pH, with the data compared to results obtained with an unmodified membrane and with a negatively-charged membrane produced by chemical modification with a sulfonic acid ligand. The extent of protein retention was highly correlated with the product of the effective surface charge densities of the membrane and
protein. Fouling experiments were also performed to investigate the fouling characteristics of these membranes using BSA as a model protein. The zwitterionic membranes showed minimal fouling over a wide range of filtration conditions, including conditions where the protein and membrane had opposite net charge. The very low fouling characteristics of the zwitterionic membranes, coupled with the high degree of protein retention at pH > pI arising from the strong electrostatic repulsion between the like-charged protein and membrane, could be very attractive for the separation of high value proteins using these surface-modified ultrafiltration membranes.

5.2 Materials and Methods

5.2.1 Membrane Preparation

Zwitterionic membranes were produced by chemical modification of Ultracel\textsuperscript{TM} composite regenerated cellulose membranes with 30 kDa nominal molecular weight cut-off (Millipore Corp., Bedford, MA). Membrane disks with 25 mm diameter were cut from large flat sheets, soaked in isopropanol for 45 min to remove any wetting/storage agents, and thoroughly rinsed with at least 100 L/m\textsuperscript{2} of deionized water.

Membranes were modified using an adaptation of the reaction chemistry presented by Riordan et al. (2009) and shown schematically in Figure 5.1. The hydroxyl groups on the base cellulose were activated by soaking the membrane disk in a 5\% (v/v) allyl glycidyl ether solution (Sigma Chemical, St. Louis, MO) in 0.3 M NaOH overnight at room temperature. The membranes were then rinsed with DI water and reacted with a 10 g/L aqueous solution of N-Bromosuccinimide. The membranes were removed after 2
h, rinsed with DI water, and then reacted by incubation in 0.5 M solutions of the zwitterionic ligands taurine, homotaurine or glycine (all from Sigma Chemical). A negatively charged version of the Ultracel™ membrane was generated by chemical attachment of a small sulfonic acid group to the free hydroxyl as described in Chapter 3, with the extent of modification controlled by reaction time. The molecular structures of the different membranes are shown schematically in Figure 5.2 where R is a glucose ring of the cellulose membrane. Membranes were soaked overnight at 4 °C in the appropriate protein solution prior to performing the ultrafiltration experiments to minimize any initial transients associated with protein adsorption on and within the membrane pores.
Figure 5.1  Schematic of chemical reactions used for membrane modification: (1) activation with allyl glycidyl ether, (2) reaction with N-bromosuccinimide, and (3) reaction with zwitterionic ligand. Adapted from Heeboll-Nielsen et al. (2004).
Figure 5.2 Molecular structures of the zwitterionic membranes where R is the glucose monomer in the base cellulose: (a) taurine, (b) homotaurine, (c) glycine, and (d) 2-bromopropane sulfonic acid sodium salt.

5.2.2 X-ray Photoelectron Spectroscopy (XPS)

The extent of surface modification was examined from the elemental composition as determined by X-ray photoelectron spectroscopy (XPS). The surface-modified membranes were flushed with deionized water, dried gently using a Kimwipe, cut into small (approximately 1.2 cm x 0.5 cm) pieces using a razor blade, and mounted on a
sample platen. XPS analysis was performed using a Kratos Analytical Axis Ultra instrument (Kratos Analytical Inc., Chestnut Ridge, NY) available in the Materials Research Institute at The Pennsylvania State University. Data were obtained using monochromatic Al Kα as the X-Ray source (1486.6 eV photons) at a pass energy of 20 eV and a pressure of 10⁻⁸ Torr. XPS data were analyzed using CasaXPS Software (version 2.3.12Dev9) by integrating the peak areas and applying the appropriate relative sensitivity factors to account for the x-ray cross section and the transmission function of the spectrometer. All binding energies were referenced to the C₁s peak at 285 eV.

5.2.3 Streaming Potential Measurement

The effective surface charge characteristics of the membranes were evaluated from streaming potential measurements using 10 mM buffered KCl solutions, following the procedure described by Burns and Zydney (2000) as described in Chapter 3. Flow was directed through the membrane pores to obtain a measure of the effective charge on the surface of the pores. The apparent zeta potential ($\zeta_{app}$) was evaluated from the slope of the voltage (streaming potential) as a function of pressure using the Helmholtz–Smoluchowski equation (Equation (3.1)).
5.2.4 Ultrafiltration

Ultrafiltration experiments were performed in an Amicon 8010 stirred cell with effective membrane area of 4.1 cm$^2$ (Millipore Corp., Bedford, MA) as described in Chapter 3. The stirring speed was set to 600 rpm using a Strobotac Type 1531-AB strobe light (General- Radio Co., Concord, MA). The membrane hydraulic permeability ($L_p$) was evaluated by measuring the filtrate flux as a function of pressure using a 500 mM KCl solution buffered with 20 mM Bis-Tris at pH 7. The high salt concentration minimized the effects of counter-electroosmosis. The permeability was evaluated from the slope of the data using Equation (2.6).

Sieving data were obtained with cytochrome c, myoglobin, and $\alpha$-lactalbumin, which have similar size but very different charge characteristics. Sigma Chemical catalog numbers and key physical properties are summarized in Table 5.1. Bovine serum albumin (BSA, Sigma A7906, Sigma-Aldrich Inc., St Louis, MO) with a molecular weight of 67 kDa and an isoelectric point of 4.7 was used as a model protein in fouling experiments due to the extensive prior literature on BSA fouling during ultrafiltration and microfiltration (Bakhshayeshi and Zydney, 2008; Kwon et al., 2008).

Protein solutions were prepared by slowly dissolving the protein powder in the desired buffer, with the resulting solution filtered through a 0.22 $\mu$m Acrodisc® syringe filter (Pall Corp.) to remove any protein aggregates immediately prior to use. 1 mM phosphate (J.T. Baker, 026001), acetate (Sigma, S7670), Bis-Tris (MPBiomedicals, 101038), and borate (Sigma, S9640) were used as buffers for pH < 4, between 4 and 5, 6 to 8, and pH > 9, respectively. Buffered KCl solutions were prepared as described in
Chapter 3. Protein solutions were used within 12 h of preparation to minimize the likelihood of protein aggregation or denaturation. The protein concentration was determined spectrophotometrically using a SPECTRAmax Plus 384 UV-vis spectrophotometer (MD Corp., Sunnyvale, CA) with the absorbance measured at 410 nm for cytochrome c and myoglobin, at 280 nm for α-lactalbumin, and at 240 nm for BSA. Actual concentrations were evaluated by comparison of the absorbance with that of known protein standards.

Protein sieving experiments were performed using 1 g/L protein solutions as follows. A membrane disk was first soaked overnight at 4 °C in the desired protein solution. The membrane was then placed in the stirred cell and the device pressurized to approximately 10 kPa (corresponding to 1.5 psi) to maintain a positive gauge pressure throughout the system. The filtrate flux was set at 4-5 µm/s using a Masterflex 7523-20 peristaltic pump (Cole-Parmer, Vernon Hills, IL) connected to the filtrate line (downstream of the membrane). Protein samples were collected from the filtrate and bulk solutions as described in Chapter 3. The observed sieving coefficient was calculated as

\[ S_o = \frac{C_f}{C_b} \]  

(5.1)

where \( C_f \) and \( C_b \) are the protein concentrations in the filtrate and bulk solutions, respectively. The stirred cell was then carefully emptied, flushed with at least 25 L/m² of buffered KCl solution, and refilled with a fresh protein solution at a new pH. All experiments were performed at room temperature (22 ± 3 °C).
**Table 5.1**  Physical properties of cytochrome c, myoglobin, and α-lactalbumin

<table>
<thead>
<tr>
<th>Protein</th>
<th>Catalog Number</th>
<th>Molecular Weight (Da)</th>
<th>Equivalent Radius (nm)(^a)</th>
<th>Isoelectric Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c</td>
<td>C 7752</td>
<td>12384</td>
<td>1.5</td>
<td>10.4</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>M 0630</td>
<td>17600</td>
<td>1.7</td>
<td>6.9(^b)</td>
</tr>
<tr>
<td>α-lactalbumin</td>
<td>L 5385</td>
<td>14200</td>
<td>1.6</td>
<td>4.6(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Radius of sphere of equivalent volume  
\(^b\) Isoelectric point data adapted from references (Ebersold and Zydney, 2004; Molek and Zydney, 2007)

Protein fouling experiments were performed using the zwitterionic ultrafiltration membranes along with unmodified Ultracel™ (composite regenerated cellulose), Biomax™ (polyethersulfone) membranes (Millipore Corp., Bedford, MA), and a negatively-charged version of the Ultracel™ membrane with sulfonic acid ligand. Data were obtained with 1 g/L protein solutions at 5 mM solution ionic strength at the pH of interest. After measuring the membrane permeability, the system was flushed with at least 25 L/m\(^2\) of buffer at the pH of interest. The stirred cell and solution reservoir were then emptied and rapidly refilled with protein solution. The system was re-pressurized and protein filtration was performed for about an hour, with the filtrate flux measured as a function of time using timed collection. The fouling experiments were performed at constant pressure, with the stirred cell connected to a feed reservoir that was pressurized with air at 14 psi (corresponding to 96 kPa). Filtrate samples (approximately 200 μL) were collected periodically for subsequent analysis by UV spectrophotometry.
samples were taken directly from the stirred cell right at the beginning and after finishing the experiments. After completion of the ultrafiltration, the stirred cell was carefully emptied, rinsed with DI water, followed by buffered KCl solution. The system was then filled with fresh Bis-Tris buffer and re-pressurized, and the membrane permeability was re-evaluated to obtain a measure of the extent of irreversible fouling. All experiments were performed at room temperature (22 ± 3 °C).

5.3 Results and Discussions

5.3.1 Membrane Modification

The extent of modification was examined by X-ray photoelectron spectroscopy (XPS). Figures 5.3 and 5.4 show XPS spectra for the upper surface of an unmodified Ultracel™ membrane and a series of membranes produced by reaction with the different zwitterionic ligands. The spectra in Figure 5.3 were obtained at energies near the nitrogen peak (binding energy of approximately 400 eV) while those in Figure 5.4 are focused on the sulfur peak (binding energy of approximately 168 eV). The nitrogen and sulfur peaks are both absent in the XPS spectrum for the base cellulose membrane, but are clearly visible for the membranes formed using taurine and homotaurine. The zwitterionic membrane produced using glycine showed no visible sulfur peak (data not shown), but had 2 peaks near the N binding energy, while the negatively-charged membrane produced with the sulfonic acid ligand only showed a S peak. The N 1s spectra for the homotaurine and glycine show two split peaks at 400 eV and 402 eV, while the membrane modified with taurine showed only a single N peak at binding
energy of about 402 eV. The two nitrogen peaks are associated with the different protonated states of the amine group, consistent with previous results showing peaks at 399.4 and 401.4 eV for the un-protonated and protonated amines (Lawrie et al., 2007), respectively. The different degree of protonation for the different ligands is discussed in more detail subsequently.

**Figure 5.3** XPS spectra showing the nitrogen peak for an unmodified Ultracel™ 30 kDa membrane and the zwitterionic membranes generated by reaction with taurine, homotaurine, and glycine.
Figure 5.4  XPS spectra showing the sulfur peak for an unmodified Ultracei\textsuperscript{TM} 30 kDa membrane, a sulfonic acid modified membrane, and the zwitterionic membranes generated by reaction with taurine and homotaurine.

The atomic composition of the different membranes determined directly from the area under the C, O, N, and S peaks are summarized in Table 5.2. The unmodified cellulose membrane has 44% oxygen compared to the predicted composition of 45% oxygen (C\textsubscript{6}O\textsubscript{5}); this small discrepancy is within the expected accuracy of the XPS measurements. The membrane modified with the sulfonic acid ligand had the lowest carbon content, consistent with the short alkyl chain on this ligand (3 C, 1 S, and 3 O as shown in Figure 5.2). The membrane modified with the long homotaurine ligand (9 C, 6 O, 1 N, and 1 S) had the highest carbon content and the lowest oxygen as expected. The nitrogen and sulfur content for the membranes modified with homotaurine and taurine
should be identical; the small differences in the data likely reflect the inherent difficulty in measuring the very low concentration of these atoms in the surface-modified membranes.

In order to obtain membranes with similar degrees of surface modification, the reactant concentrations were adjusted so that the permeabilities of the modified membranes were all within ±5%. A set of membrane disks were initially cut from adjacent regions on large flat sheets with permeabilities of \( L_p = 7.2 \pm 0.1 \times 10^{-13} \text{ m} \); disks with higher or lower permeability were simply discarded. The membranes were then chemically-modified, with the goal of achieving a final permeability of \( 4.6 \pm 0.1 \times 10^{-13} \text{ m} \). This approach could not be used for the much smaller sulfonic acid ligand since an equivalent permeability would require a much higher degree of surface modification. In this case, the extent of modification was adjusted so that the apparent zeta potential at pH 10 was similar to that of the zwitterionic membrane formed with taurine.

The actual degree of modification was estimated from the measured atomic composition based on the known structure of the modified membranes (Figure 5.2) as discussed in Chapter 4. The best fit value of \( f \), equal to the fraction of glucose rings modified with the ligand, was determined by minimizing the sum of the normalized squared residuals:

\[
E = \sum_{i=1}^{N} \left( 1 - \frac{C_{i,\text{pred}}}{C_{i,\text{exp}}} \right)^2
\]

with the summation performed over the four elements (C, O, N, and S). Results are shown in the last column of Table 5.2. The membranes all had essentially the same
degree of modification \( f \approx 0.06 \pm 0.02 \), although the membrane modified with glycine did have a slightly larger value of \( f \) than the other 3 membranes.

**Table 5.2** Atomic composition (percent) and calculated degree of modification for the unmodified, zwitterionic, and sulphonic acid modified 30 kDa Ultracel\(^{TM}\) membranes determined from XPS data.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>O 1s</th>
<th>N 1s</th>
<th>C 1s</th>
<th>S 2p</th>
<th>( f )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified</td>
<td>44</td>
<td>--</td>
<td>56</td>
<td>--</td>
<td>-</td>
</tr>
<tr>
<td>Taurine</td>
<td>44</td>
<td>0.5</td>
<td>54</td>
<td>1.4</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>Homotaurine</td>
<td>39</td>
<td>0.9</td>
<td>59</td>
<td>0.5</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>Glycine</td>
<td>42</td>
<td>0.7</td>
<td>57</td>
<td>--</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>Sulfonic</td>
<td>46</td>
<td>--</td>
<td>53</td>
<td>0.5</td>
<td>0.05 ± 0.01</td>
</tr>
</tbody>
</table>

**5.3.2 Effective Surface Charge**

The effective surface charge characteristics of the zwitterionic membranes were evaluated from streaming potential measurements obtained with the fluid flow directed through the membrane pores as described in Chapter 3. Figure 5.5 shows typical experimental data for the measured streaming potential \( E_z \) as a function of the transmembrane pressure for the charge-modified 30 kDa Ultracel\(^{TM}\) membranes along with results for an unmodified membrane at pH 7 using 10 mM KCl solutions buffered with 1 mM Bis-Tris. The data were highly linear for each membrane, with \( r^2 \) values greater than 0.98; repeat measurements with the same membrane were also quite reproducible (slopes within 15\%). The non-zero intercepts arise from asymmetries in the Ag/AgCl electrodes and have no effect on the data analysis (Burns and Zydney, 2000).
The unmodified membrane has a small negative slope, corresponding to a small negative surface charge, which is likely due to the preferential adsorption of anions from the electrolyte solution or to the presence of trace carboxylic acid groups in the cellulose. The membrane modified with the sulfonic acid ligand has the greatest slope, corresponding to the most negative surface charge; the intermediate value seen with the taurine-modified membrane is due to the partial positive charge contributed by the amine functionality of the zwitterion. This is discussed in more detail subsequently.

![Streaming potential data for an unmodified 30 kDa UltracelTM membrane, the taurine-modified membrane, and the sulfonic acid modified membrane at pH 7 in a 10 mM KCl solution buffered with 1 mM Bis-Tris.](image)

**Figure 5.5** Streaming potential data for an unmodified 30 kDa UltracelTM membrane, the taurine-modified membrane, and the sulfonic acid modified membrane at pH 7 in a 10 mM KCl solution buffered with 1 mM Bis-Tris.
The slope of the streaming potential versus pressure data was used to calculate the apparent zeta potential via Equation (3.1), with the results summarized in the final 2 columns of Table 5.3. The apparent zeta potential for the modified membranes were very similar at pH 10, with values ranging from -7.4 to -8.9 mV. In contrast, there were significant differences in the apparent zeta potential at pH 7, with the largest negative value obtained with the sulfonic acid ligand, followed by the 3 zwitterionic ligands. The taurine-modified membrane has a significant negative zeta potential at pH 7 ($\zeta_{app} = -3.9$ mV) even though taurine is electrically neutral under these conditions; the pK$_a$ of the ammonium group is 9.08 while that of the sulfonic acid is 1.5 (Madura et al., 1997), yielding the zwitterionic (neutral) form at pH 7.

In order to obtain additional insights into the charge characteristics of the zwitterionic membranes, Table 5.3 summarizes the pK$_a$ values for a series of Trizma compounds (TES, TAPS, Tricine) that have structures that are analogous to the zwitterionic ligands used for the membrane modification but with the primary amine replaced by a secondary amine attached to a tri-hydroxymethylated carbon as shown in Figure 5.6. TES and TAPS have sulfonic acid moieties and are thus analogous to taurine and homotaurine while tricine has a carboxylic acid is thus an analog of glycine. The conversion of the primary amine in taurine to a secondary amine in TES causes a significant reduction in the pK$_a$ of the amine group from 9.06 to 7.34. The net fractional charge on TES at pH 7 is predicted to be -0.31 based on the pK$_a$ values of the amine and sulfonic acid groups, reflecting the partial protonation of the secondary amine at this pH. The pK$_a$ of the amine in TAPS is significantly larger than that in TES due to the greater
spacing (number of alkyl groups) between the sulfonic acid and amine functionalities (similar to the behavior seen with homotaurine and taurine). This increase in the $pK_a$ leads to an increase in degree of protonation of the secondary amine and a corresponding reduction in the negative charge on the homotaurine modified membrane, consistent with the measured values of the apparent zeta potential (Table 5.3).

**Table 5.3**  $pK_a$ values for the different ligands and the corresponding Trizma analogs. Predicted charge of the membranes at pH 7 was evaluated using the $pK_a$ values of the Trizma analogs. Last two columns show the experimental values for the apparent zeta potential at pH 7 and 10.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$pK_{a1}$</th>
<th>$pK_{a2}$</th>
<th>Trizma Analog</th>
<th>$pK_{a2}$</th>
<th>Predicted Charge (pH 7)</th>
<th>Zeta Potential (pH 7)</th>
<th>Zeta Potential (pH 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurine</td>
<td>1.5</td>
<td>9.06</td>
<td>TES</td>
<td>7.34</td>
<td>-0.31</td>
<td>-3.9</td>
<td>-8.4</td>
</tr>
<tr>
<td>Homotaurine</td>
<td>-0.99</td>
<td>10.2</td>
<td>TAPS</td>
<td>8.6</td>
<td>-0.02</td>
<td>-2.5</td>
<td>-7.7</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.35</td>
<td>9.78</td>
<td>Tricine</td>
<td>8.326</td>
<td>-0.05</td>
<td>-2.3</td>
<td>-8.9</td>
</tr>
<tr>
<td>Sulfonic acid</td>
<td>-2.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-1.0</td>
<td>-6.2</td>
<td>-7.4</td>
</tr>
<tr>
<td>Unmodified</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-1.6</td>
<td>-2.7</td>
</tr>
</tbody>
</table>

$pK_{a2}$ values adapted from references (Goldberg et al., 2002; Good and Izawa, 1972; McGlothlin and Jordan, 1976; Roy et al., 1973)

TES = N-Tris(hydroxymethyl)methyl-2-aminooethanesulfonic acid
TAPS = N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid
Tricine = N-Tris(hydroxymethyl)methyl-glycine

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Figure 5.6 Molecular structures of taurine (left) and TES (right).

The charge characteristics of the zwitterionic membranes are examined in more detail in Figure 5.7 which shows the measured values of the apparent zeta potential at pH 7 as a function of the partial charge predicted using the pK\textsubscript{a} values for the Trizma analogs at the same pH. The partial charge of the Trizma analogs were calculated using the Henderson-Hasselbach equation:

$$pH = pK_a^i + \log \frac{r_i}{(n_i - r_i)}$$

(5.3)

where $r_i$ is the number of dissociated groups and $n_i$ is total number of the ionizable groups. The net charge ($Z$) is then calculated by summing over all the ionizable groups:

$$Z = Z_{\text{max}}^+ - \sum_i r_i$$

(5.4)

where $Z_{\text{max}}^+$ is the maximum positive charge on the ligand (at very low pH), which is equal to 1 for the Trizma analogs with one amine group.

The results are highly linear when plotted in this fashion with $r^2 = 0.98$, suggesting that the charge characteristics of the surface-grafted membranes can be effectively described in terms of the protonation / deprotonation of the acidic and basic
components of the zwitterionic ligand. Note that a similar plot using the predicted charge based on the pKₐ values of the actual ligand (e.g., glycine instead of the Trizma analog tricine) showed very poor correlation with the apparent zeta potential data due to the change in the protonation of the amine group caused by the chemical attachment to the base membrane.

**Figure 5.7** Correlation between the apparent zeta potential and the calculated fractional charge based on the pKₐ values for the corresponding Trizma analogs.
5.3.3 Protein Ultrafiltration

Figure 5.8 shows experimental data for the observed sieving coefficients of α-lactalbumin at pH 10 with 1 mM borate buffer at both low (10 mM) and high (500 mM) ionic strength. The α-lactalbumin sieving coefficients at high salt concentration are greatly reduced in the zwitterionic membranes compared to that for the unmodified membrane due to the significant constriction of the membrane pores associated with the relatively large size of the zwitterionic ligands. This effect is much less pronounced for the sulfonic acid modified membrane due to the smaller size of this ligand (Figure 5.2). The sieving coefficients at low ionic strength are strongly affected by the electrostatic repulsion between the negatively charged protein and the negatively charged membranes under these conditions. The electrostatic interactions are relatively weak for the unmodified membrane due to the low apparent zeta potential. In contrast, α-lactalbumin transmission through the zwitterionic and sulfonic acid modified membranes is dramatically reduced at low ionic strength due to the strong electrostatic repulsion. The sieving coefficients for the 3 zwitterionic membranes are not statistically different given the errors associated with the determination of the protein concentration in the very dilute filtrate samples obtained in these experiments.
Figure 5.8  Observed sieving coefficients for $\alpha$-lactalbumin at pH 10 in both low (10 mM) and high (500 mM) ionic strength solutions through an unmodified UltracelTM 30 kDa membrane, a sulfonic acid modified membrane, and a series of zwitterionic membranes.

Typical experimental data for the observed sieving coefficients of cytochrome c (pI = 10.4), myoglobin (pI = 6.9), and $\alpha$-lactalbumin (pI = 4.6) through the different zwitterionic membranes are shown in the top, middle, and bottom panels of Figure 5.9, respectively. These proteins have similar size (effective radii between 1.5 and 1.7 nm), but very different surface charge; the calculated values of the protein charge at pH 7 range from $Z = +6.5$ for cytochrome c to $Z = -0.1$ for myoglobin and $Z = -4$ for $\alpha$-lactalbumin as determined directly from their respective amino acid composition (Menon...
and Zydney, 2000). In each case, the sieving coefficient data were obtained using a 1 g/L solution of the specific protein in a buffered KCl solution with 10 mM total ionic strength at a filtrate flux of 4-5 µm/s and a stirring speed of 600 rpm. Results are plotted in terms of the observed sieving coefficient ($S_o$), which should provide a direct measure of the intrinsic membrane transport properties since concentration polarization effects are negligible under these conditions. There was no evidence of any flux decline or membrane fouling during the experiments; the measured values of the membrane permeability were essentially identical before and after the ultrafiltration experiments. This behavior is discussed in more detail in the final section of this Chapter.

Transmission of cytochrome c through the zwitterionic modified membranes was close to 100% at pH values below the protein isoelectric point (pH <10), but decreased sharply at pH 11 due to the strong electrostatic repulsion between the negatively charged protein and the negatively charged membrane at pH > pI. This effect was relatively small with the unmodified Ultracel™ membrane due to its small negative charge. In contrast, the sieving coefficients for the taurine- and glycine-modified membranes at pH 11 were less than 0.02 which is more than an order of magnitude smaller than that for the unmodified membrane. The data for the membrane modified with the sulfonic acid ligand lie between those for the unmodified and zwitterionic ligands, which is likely due to the smaller size of this ligand (and thus the slightly larger pore size for the modified membrane), consistent with the results in Figure 5.8. Note that the sieving coefficients for the zwitterionic membranes at low pH actually lie above those for the unmodified Ultracel™ membrane, which is probably due to a small attractive electrostatic interaction.
between the oppositely charged protein and membrane under these conditions. This attractive interaction did not lead to any observable membrane fouling.

The sieving coefficient results for myoglobin (middle panel) were similar to those for cytochrome c, but with the values shifted down and to the left. In this case, the myoglobin transmission at low pH is somewhat less than 100%, consistent with the slightly larger size of myoglobin \( r = 1.7 \text{ nm} \) compared to cytochrome c \( r = 1.5 \text{ nm} \). The greatest transmission at low pH is seen with the membrane modified with the small sulfonic acid ligand. A sharp decline in myoglobin transmission is observed above pH 7, consistent with the isoelectric point of this protein. The myoglobin sieving coefficient through the taurine-modified membrane at pH 10 is only \( S_o = 0.001 \), corresponding to 99.9% protein rejection. Slightly lower retention was seen with the membranes modified with glycine and homotaurine, while the sulfonic acid-modified membrane has \( S_o \approx 0.02 \) at pH 10. The observed sieving coefficient data for \( \alpha \)-lactalbumin (bottom panel) look somewhat different due to the low isoelectric point for this protein (pI = 4.6). In this case, transmission of \( \alpha \)-lactalbumin decreases with increasing pH for all of the membranes since the pH is always above the pI, with the greatest retention at pH 7 and 10 again seen with the taurine-modified membrane.
Figure 5.9  Observed sieving coefficients for cytochrome c (top panel), α-lactalbumin (middle panel), and myoglobin (bottom panel) as a function of solution pH through an unmodified UltracelTM 30 kDa membrane and a series of charge-modified membranes in 10 mM solution ionic strength solutions.
The experimental data for the observed sieving coefficients of cytochrome c, myoglobin, and α-lactalbumin from Figure 5.9 have been re-plotted in Figure 5.10 as a function of the product of the dimensionless surface charge densities of the protein and membrane:

\[ \sigma_s = \frac{F r_p q_s}{\varepsilon_o \varepsilon_r RT} \quad (5.5) \]

\[ \sigma_p = \frac{F r_p q_p}{\varepsilon_o \varepsilon_r RT} \quad (5.6) \]

where \( \varepsilon_o \) is the permittivity of free space, \( \varepsilon_r \) is the dielectric constant of the solution, \( F \) is Faraday’s constant, \( R \) is the ideal gas constant, \( T \) is the absolute temperature, \( r_p \) is the pore radius, and \( q_s \) and \( q_p \) are the dimensional surface charge densities of the protein (solute) and pore. This form was suggested based on a theoretical analysis of the effects of electrostatic interactions on the partitioning of a charged sphere in a charged cylindrical pore (Smith and Deen, 1980) as described in Chapter 2. The protein charge for cytochrome c was evaluated from the measured protein electrophoretic mobility; values for myoglobin and α-lactalbumin were calculated from their respective amino acid composition as described in Chapter 2. The dimensional surface charge density of the protein, \( q_s \), was then calculated as:

\[ q_s = \frac{e z}{4 \pi r_s^2} \quad (5.7) \]

where \( e \) is the electron charge \((1.609 \times 10^{-19} \text{ C})\), \( z \) is the protein charge, and \( r_s \) is the protein radius. Filled symbols show data using the dimensionless membrane surface charge density evaluated from the apparent zeta potential data at pH 7 and pH 10 (Table
5.3) using Equation (4.4). The open symbols are for data at other pH with the membrane surface charge density evaluated from the pKₐ values of the corresponding Trizma analog using the relationship between the apparent zeta potential and fractional ligand charge in Figure 5.7. The results for the acidic, basic, and neutral proteins through the three zwitterionic membranes (modified with taurine, homotaurine, and glycine) for pH values between 4 and 11 collapse to essentially a single curve when plotted in this manner. The sieving coefficient decreases with increasing positive values of \( \sigma_s \sigma_p \) due to the strong repulsive electrostatic interactions under these conditions. In contrast, the sieving coefficient increases rapidly as \( \sigma_s \sigma_p \) drops below zero due to the attractive electrostatic interaction between the oppositely charged protein and membrane.
Figure 5.10  Observed sieving coefficients as a function of the charge interaction parameter (product of the dimensionless surface charge densities for the protein and membrane) for the zwitterionic membranes. Filled symbols represent the membrane charge evaluated from the measured apparent zeta potential at pH 7 and pH 10. Open symbols represent the predicted membrane charge based on the pKₐ values for the Trizma analogs as described in the text.

5.3.4 Fouling Studies

Fouling characteristics of the membranes modified with the zwitterionic ligands were examined under both static exposure and dynamic flow conditions. Results for the membrane permeability before and after overnight adsorption of the membrane in BSA...
solution are summarized in Table 5.4. There was essentially no change in the permeability of the zwitterionic (taurine-modified) membrane, with similar results obtained with both the unmodified Ultracel™ and the sulfonic acid-modified membranes. In contrast, there was more than a 60% decline in permeability for the Biomax™ membranes, reflecting the much more hydrophobic character of the polyethersulfone surface relative to that of the cellulosic membranes.

Table 5.4  Permeability values of the membranes measured before and after overnight protein adsorption. Permeability was measured using a 5 mM KCl solution buffered with 1 mM Bis-Tris at pH 7.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Clean membrane Permeability, (m)</th>
<th>Pre-adsorbed Permeability, (m)</th>
<th>% Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurine (100 kDa)</td>
<td>$2.0 \times 10^{-12}$</td>
<td>$2.0 \times 10^{-12}$</td>
<td>1.5</td>
</tr>
<tr>
<td>Sulfonic acid (100 kDa)</td>
<td>$2.7 \times 10^{-12}$</td>
<td>$2.9 \times 10^{-12}$</td>
<td>-7.5</td>
</tr>
<tr>
<td>Ultracel™ (30 kDa)</td>
<td>$0.78 \times 10^{-12}$</td>
<td>$0.77 \times 10^{-12}$</td>
<td>1.3</td>
</tr>
<tr>
<td>Ultracel™ (100 kDa)</td>
<td>$3.5 \times 10^{-12}$</td>
<td>$3.5 \times 10^{-12}$</td>
<td>1.0</td>
</tr>
<tr>
<td>Biomax™ (30 kDa)</td>
<td>$1.5 \times 10^{-12}$</td>
<td>$0.48 \times 10^{-12}$</td>
<td>67</td>
</tr>
<tr>
<td>Biomax™ (100 kDa)</td>
<td>$2.7 \times 10^{-12}$</td>
<td>$1.1 \times 10^{-12}$</td>
<td>61</td>
</tr>
</tbody>
</table>
Ultrafiltration experiments were performed to explore the protein fouling characteristics of the membranes under dynamic flow conditions. Typical experimental data for the normalized filtrate flux (top panel) and filtrate concentration (bottom panel) for the ultrafiltration of a 1 g/L BSA solution at pH 3.5 through an unmodified, taurine-modified (zwitterionic), and sulfonic acid-modified version of the 100 kDa Ultracele™ membranes at a constant pressure of 96 kPa (14 psi) are shown in Figure 5.11. The net BSA charge, as determined by Menon and Zydney (1998) using capillary electrophoresis, was approximately +17 at pH 3.5. Thus, there should be a strong electrostatic attraction between the negatively charged membrane and the positively charged protein under the experimental conditions used in this experiment. The flux data for each membrane have been normalized by the buffer flux evaluated immediately prior to the protein ultrafiltration at the same transmembrane pressure. The initial flux obtained with the protein solution was always lower than that of the buffer due to a combination of rapid fouling and concentration polarization effects associated with the osmotic pressure of the more concentrated solution of retained protein that accumulates above the filtering membrane. The taurine-modified membrane had the highest initial flux, while the unmodified Ultracele™ membrane had the lowest initial flux, with these differences due primarily to the different degrees of protein fouling; concentration polarization effects should be similar (and small) due to the same level of rigorous stirring employed in all experiments.

The corresponding data for the protein concentration in the filtrate solution are shown in the bottom panel. The initial filtrate concentration was greatest for the
zwitterionic modified membrane and gradually decreased by about an order of magnitude over the course of the 1 hr ultrafiltration. The measured bulk concentration in the stirred cell at the end of the filtration experiment was about 4-fold larger than the initial concentration due to the partial retention of BSA during the ultrafiltration. The final sieving coefficients for the unmodified, taurine-modified, and sulfonic acid-modified membranes were all approximately equal to 0.02 (compared to the initial value of 0.5 – 0.7), reflecting the significant amount of fouling that occurred in these experiments.
Figure 5.11 Filtrate flux (top panel) and filtrate concentration (bottom panel) for ultrafiltration of a 1 g/L BSA solution at pH 3.5 and 5 mM ionic strength through unmodified, taurine-modified, and sulfonic acid-modified 100 kDa Ultracel™ membranes at a constant pressure of 97 kPa (14 psi).
The results from the different fouling experiments are summarized in Table 5.5, which shows the permeability values both before and after the ultrafiltration experiments, with the permeability values before the ultrafiltration obtained after overnight static adsorption in the protein solution. The greatest reduction in permeability was seen with the Biomax™ polyethersulfone membranes. The final permeability after the ultrafiltration was around 50% smaller than that before the ultrafiltration and about 5-fold smaller than that for the clean membrane. In contrast, the permeability declined by only 12% for the taurine-modified membrane, with a total drop in permeability of only 14% compared to the clean membrane. This behavior is considerably better than that for either the sulfonic acid or unmodified 100 kDa UltraCel™ membranes. However, at least some of this difference may be due to the smaller initial permeability of the taurine-modified membrane; the permeability for a 30 kDa unmodified UltraCel™ membrane decreased by only 7% after the BSA ultrafiltration.
Table 5.5 The measured apparent zeta potential at pH 7, along with permeability data for different membranes measured before and after ultrafiltration of BSA.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Apparent zeta potential ( \zeta_{app} ) (mV)</th>
<th>Pre-filtration permeability (m)</th>
<th>Post-filtration permeability (m)</th>
<th>% Permeability Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurine (100 kDa)</td>
<td>-6</td>
<td>( 2.0 \times 10^{-12} )</td>
<td>( 1.7 \times 10^{-12} )</td>
<td>12</td>
</tr>
<tr>
<td>Sulfonic acid (100 kDa)</td>
<td>-10</td>
<td>( 2.9 \times 10^{-12} )</td>
<td>( 2.1 \times 10^{-12} )</td>
<td>29</td>
</tr>
<tr>
<td>Ultracel(^{TM}) (100 kDa)</td>
<td>-4</td>
<td>( 3.5 \times 10^{-12} )</td>
<td>( 2.6 \times 10^{-12} )</td>
<td>26</td>
</tr>
<tr>
<td>Ultracel(^{TM}) (30 kDa)</td>
<td>-1.6</td>
<td>( 0.77 \times 10^{-12} )</td>
<td>( 0.72 \times 10^{-12} )</td>
<td>7</td>
</tr>
<tr>
<td>Biomax(^{TM}) (30 kDa)</td>
<td>-13.2*</td>
<td>( 0.48 \times 10^{-12} )</td>
<td>( 0.28 \times 10^{-12} )</td>
<td>41</td>
</tr>
<tr>
<td>Biomax(^{TM}) (100 kDa)</td>
<td>-12.9*</td>
<td>( 1.1 \times 10^{-12} )</td>
<td>( 0.50 \times 10^{-12} )</td>
<td>53</td>
</tr>
</tbody>
</table>

*Apparent zeta potential data for the Biomax\(^{TM}\) membranes adapted from Burns and Zydney (2000), and Ebesrold and Zydney (2004b)

The results from a series of sequential ultrafiltration experiments using the taurine-modified, sulfonic acid-modified, and un-modified 100 kDa membranes are summarized in Table 5.6. Membranes were cleaned between cycles by flushing at least 25 L/m2 of a 0.1 M NaOH solution through the membranes. The data are reported in terms of several fouling ratios (Shi et al., 2011). The total flux loss due to fouling is:

\[
r_f = 1 - \frac{J_p}{J_i}
\]  \hspace{1cm} (5.8)

where \( J_p \) is the protein solution flux at the end of the filtration and \( J_i \) is the buffer flux evaluated prior to the protein filtration. The extent of reversible \( (r_r) \) and irreversible \( (r_{ir}) \) fouling were determined as

\[
r_r = \frac{J_f - J_p}{J_i}
\]  \hspace{1cm} (5.9)

\[
r_{ir} = \frac{J_f}{J_i}
\]  \hspace{1cm} (5.10)
where $J_f$ is the buffer flux determined right after the protein filtration. And the flux recovery ratio is $FRR = J_f/J_i$. The zwitterionic membrane had the lowest irreversible fouling with a final flux recovery of $FRR = 95.4\%$. This is significantly better than either the sulfonic acid ($FRR = 86.5\%$) or unmodified ($FRR = 81.8\%$) membranes.

Table 5.6  BSA fouling of unmodified and charge modified 100 kDa UltraselTM membranes during three-cycle ultrafiltration experiments.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>First Cycle</th>
<th>Second Cycle</th>
<th>Third Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r_t$</td>
<td>$r_r$</td>
<td>$r_{ir}$</td>
</tr>
<tr>
<td>Taurine</td>
<td>0.81</td>
<td>0.78</td>
<td>0.03</td>
</tr>
<tr>
<td>Sulfonic acid</td>
<td>0.86</td>
<td>0.70</td>
<td>0.16</td>
</tr>
<tr>
<td>Unmodified</td>
<td>0.88</td>
<td>0.77</td>
<td>0.11</td>
</tr>
</tbody>
</table>

5.4  Conclusions

The data for protein transport through semipermeable zwitterionic membranes provide the first quantitative results for the effects of different zwitterionic ligands on the protein sieving coefficient. Protein transmission through the zwitterionic membranes approached a value of 100% at pH below the protein isoelectric point due to the attractive electrostatic interactions between the negatively-charged membrane and the positively-charged protein under these conditions. The sieving coefficient decreased sharply at pH
above the protein isoelectric point; this was true for acidic (α-lactalbumin), neutral (myoglobin), and basic (cytochrome c) proteins. For example, the sieving coefficient for cytochrome c through the taurine-modified membrane in a 10 mM ionic strength solution decreased by more than an order of magnitude as the solution pH was increased from 10 to 11.

The sieving coefficient data for the 3 similarly-sized proteins collapse to a single curve when plotted as a function of the product of the dimensionless surface charge densities of the protein and membrane, consistent with available theoretical analyses of the effects of electrostatic interactions on protein transmission through semipermeable membranes. The very similar results obtained with zwitterionic membranes produced using ligands having terminal sulfonic and carboxylic acid groups suggest that the detailed chemistry of the charged ligand has minimal effect on protein transmission, beyond that associated with the difference in apparent zeta potential arising from the different degree of ionization of the zwitterions.

All of the zwitterionic membranes had significant negative values for the apparent zeta potential at pH 7 even though the zwitterionic ligands (taurine, homotaurine, and glycine) were electrically neutral at this pH. This was a direct result of the shift in the pKₐ of the amine functionality associated with the conversion of the primary amine in the ligand into a secondary amine upon chemical modification of the membrane (Figures 5.1 and 5.2). This shift in pKₐ was quantified using data for a series of Trizma analogs (TES, TAPS, and tricine) that have the same basic structure as the cellulosic membranes modified with the different zwitterions (taurine, homotaurine, and glycine). The apparent
zeta potential of the zwitterionic membranes was very well correlated with the predicted fractional charge of the Trizma analog. This correlation not only provides additional insights into the surface charge characteristics of these zwitterionic membranes, it also provides a simple approach for estimating the apparent zeta potential at different pH based on the pKₐ values of the chemical groups on the surface of the membrane. The pH-dependent charge of the zwitterionic membranes results in a strong pH dependence for the protein transmission, a phenomenon that could potentially be exploited in applications requiring pH responsive transport.

The data obtained during the protein sieving experiments demonstrated that there was no evidence of any fouling of the cellulose membranes modified with the different zwitterionic ligands during protein ultrafiltration, even under conditions where the protein and membrane were oppositely charged. This is consistent with previous studies which have demonstrated the very low fouling behavior of zwitterionic ultrafiltration membranes. Ultrafiltration experiments using bovine serum albumin (BSA) as a model protein also showed little fouling of the taurine-modified membrane, with a flux recovery of 95.4% after 3 repeat ultrafiltration cycles. However, it should be noted that this membrane also had a lower permeability than either the unmodified or sulfonic acid modified membranes, and this could have contributed to the smaller degree of flux decline.

The very low fouling characteristics of these zwitterionic membranes, coupled with the high degree of protein retention at pH > pI arising from the strong electrostatic repulsion between the like-charged protein and membrane, could be very attractive for
the separation of high value proteins using these surface-modified ultrafiltration membranes.
Chapter 6

Effect of Surface Charge Distribution on Protein Transport through

Semipermeable Ultrafiltration Membranes


6.1 Introduction

It is well-established that protein transport through semipermeable ultrafiltration membranes can be strongly affected by electrostatic interactions between the charged membrane and charged protein. For example, Pujar and Zydney (1994) showed that a reduction in solution ionic strength from 0.15 to 0.0015 M caused more than a two orders of magnitude reduction in the transmission of bovine serum albumin (BSA) through a negatively-charged polyethersulfone membrane due to the increased electrostatic exclusion of the negatively-charged BSA from the membrane pores. Protein transport is also a strong function of solution pH, with maximum transmission typically obtained near the protein isoelectric point, i.e., at the pH where the protein has zero net charge (Burns and Zydney, 1999). Several studies have shown that direct charge-charge interactions can be exploited using charge-modified membranes, with protein transmission being
significantly reduced under conditions where the protein and membrane have like polarity (Mehta and Zydney, 2006; van Reis at al., 1999).

Experimental results for electrostatic interactions in membrane systems are typically analyzed in terms of the equilibrium partition coefficient for the protein between the bulk solution and the fluid space within the pore. Most studies have employed the theoretical framework developed by Smith and Deen (1980) for the free energy of interaction of a charged sphere in a charged cylindrical pore. Pujar and Zydney (1997) subsequently extended this analysis to account for the effects of charge regulation, the shift in the protein charge that occurs because of the change in local electrostatic potential as the charged sphere enters the pore. These models all treat the protein as a sphere with uniform surface charge characteristics, completely neglecting the possible effects of the surface charge distribution on the magnitude of the electrostatic interactions.

The importance of protein charge distribution has been clearly demonstrated in ion exchange chromatography in which the presence of charge "patches" significantly affects protein adsorption to the charged resin (Chung et al., 2010; Yao and Lenhoff, 2004; Yamamoto and Ishihara, 2000; Janson and Ryden, 1998; Regnier, 1987; Kopaciewicz et al., 1983). For example, Kopaciewicz et al. (1983) measured the retention of a variety of proteins at their respective isoelectric point using strong ion exchange resins. Protein retention could be quite significant even at the isoelectric point, which the authors attributed to local variations in the electrical potential over the surface of the protein. DePhillips and Lenhoff (2001) examined the isocratic retention of lysozyme, $\alpha$-chymotrypsinogen, and cytochrome c on a number of cation-exchange
chromatography columns. The stronger retention of lysozyme compared to that of cytochrome c at pH 7 was attributed to differences in both the surface charge distribution and amino acid composition, in particular the number of arginine residues and the ratio of arginines to lysines (Yao and Lenhoff, 2005; DePhillips and Lenhoff, 2001).

In a recent study, Chung et al. (2010) examined the retention of lysozyme charge ladders, made by partial acetylation of lysozyme, on a cation exchange column. Some lysozyme variants with different net charge co-eluted from the cation exchange column, and in some cases more positively charged variants showed weaker retention than variants with less positive charge when using a negatively-charged resin. The authors attributed these differences in protein retention to the effects of the surface charge distribution, and more specifically the presence of charge patches, on protein retention during cation exchange chromatography.

Chen et al. (2011) investigated the role of surface “charge patches” on the binding of a cationic nanoparticle to the proteins bovine serum albumin (BSA) and β-lactoglobulin (BLG). The much higher binding observed for BLG versus BSA, particularly at pH below the protein isoelectric point, was attributed to differences in their surface charge distribution and the role of a specific negative charge patch in determining nanoparticle binding to BLG.

The overall objective of the studies presented in this Chapter was to examine the possible effects of a protein charge distribution on electrostatic interactions during protein ultrafiltration. Data were obtained with cytochrome c and lysozyme, two proteins that have similar size and net charge but different amino acid composition and surface
charge distribution. Limited data were also obtained with peracetylated cytochrome c formed by reaction of cytochrome c with acetic anhydride to block all free lysine groups. Ultrafiltration experiments were performed using Ultracei™ composite regenerated cellulose membranes, both as received (nearly neutral) and charge-modified by chemical attachment of a quaternary amine functionality. The ultrafiltration data were compared with the measured protein retention on a strong cation exchange resin to provide additional insights into the role of net charge / charge distribution on protein partitioning and binding.

6.2 Materials and Methods

6.2.1 Protein Solutions

Buffered salt solutions were prepared as described in Chapter 3 by dissolving appropriate amounts of Trizma® Base (tris[hydroxymethyl]aminomethane, Sigma T-1503), sodium tetraborate decahydrate (Sigma, S9640), and NaCl (BDH Chemicals, BDH0286) in deionized water obtained from a NANOpure® Diamond water purification system (Barnstead Thermolyne Corporation, Dubuque, IA) with a resistivity greater than 18 MΩ-cm. Cytochrome c (horse heart) and lysozyme (chicken egg white) were obtained from Sigma Chemical (St. Louis, MO). Catalog numbers and key physical properties are summarized in Table 6.1. The protein isoelectric points, amino acid composition, and calculated dipole moments were taken from literature data (Mehta and Zydney, 2008; DePhillips and Lenhoff, 2001; Lohrmann et al., 2005; Yao and Lenhoff, 2005; Malmgren et al., 1978). Protein solutions were prepared by slowly dissolving the
protein powder in the desired buffer, with the resulting solution filtered through a 0.22 μm Acrodisc® syringe filter (Pall Corp.) to remove any protein aggregates immediately prior to use. Protein solutions were used within 12 h of preparation to minimize the likelihood of protein aggregation or denaturation.

Table 6.1  Physico-chemical properties of proteins

<table>
<thead>
<tr>
<th>Property</th>
<th>Cytochrome c</th>
<th>Lysozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalog number</td>
<td>C 7752</td>
<td>L 6876</td>
</tr>
<tr>
<td>Molecular Weight (Da)</td>
<td>12384</td>
<td>14307</td>
</tr>
<tr>
<td>Equivalent Radius (nm)</td>
<td>1.53</td>
<td>1.60</td>
</tr>
<tr>
<td>Isoelectric Point</td>
<td>10 - 10.5</td>
<td>11</td>
</tr>
<tr>
<td>Number of arginine residues</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>Number of lysine residues</td>
<td>19</td>
<td>6</td>
</tr>
<tr>
<td>Dipole moment (Debye)</td>
<td>325</td>
<td>72</td>
</tr>
</tbody>
</table>

* a Radius of sphere of equivalent volume

Protein concentrations in the binary mixture were determined spectrophotometrically using the basic approach described by Ghosh (2001). The absorbance was measured at both 260 and 410 nm using a SPECTRAmax Plus 384 UV-vis spectrophotometer (MD Corp., Sunnyvale, CA). The cytochrome c concentration was evaluated assuming that the absorbance at 410 nm was only due to cytochrome c, with the lysozyme concentration then determined from the total absorbance at 260 nm by
subtracting off the contribution associated with the known concentration of cytochrome c. Actual concentrations were evaluated by comparison of the absorbance with that of known protein standards.

Peracetylated cytochrome c was formed by reaction of cytochrome c with acetic anhydride to block all free lysine groups, following the procedure described by Yang et al. (2003) with slight modifications. A 100 µM cytochrome c solution in 0.1 M HEPES buffer (Fluka biochemika, 83264) was prepared and incubated at pH 8.3 for two hours. The reaction was performed at pH 8.2, in an ice bath by adding 20 equivalents (per mole of protein lysine groups) of 2 M acetic anhydride (Sigma, 242845) solution in 1,4-dioxane (Sigma, D-9553) dropwise to the protein solution. The resulting solution was incubated overnight at 4 °C to ensure complete reaction. To remove dioxane and any residual reactants, the peracetylated cytochrome c solution was diafiltered with 4 diavolumes of deionized water in an Amicon stirred cell (Millipore Corp., Bedford, MA) using a 10 kDa Ultracel™ membrane. Peracetylated cytochrome c was then incubated in 0.1 M LiOH (Sigma, 545856) solution for 1 hr at 4 °C to de-esterify the tyrosine groups. Finally, the peracetylated solution was diafiltered with 4 diavolumes of deionized water.

Concentrations of cytochrome c and its peracetylated variant were determined spectrophotometrically, with absorbance measured at 410 and 390 nm for native and peracetylated cytochrome c, respectively, based on the absorbance maxima for these proteins. Actual concentrations were evaluated by comparison of the measured absorbance with that of known protein standards.
6.2.2 Capillary Electrophoresis

The net protein charge was calculated from the electrophoretic mobility determined using a G1600A High-Performance Capillary Electrophoresis instrument (Agilent Technologies, Palo Alto, CA) as described in Chapter 3. Detection was by UV absorbance at 214 nm. Negatively-charged fused silica capillaries (Agilent Technologies, Catalog Number G1600-62211, Palo Alto, CA) were used at pH above the protein isoelectric point using an applied voltage of 25 kV. Positively-charged eCAP™ Amine capillaries (Beckman Coulter, Inc., Catalog Number 477431, Fullerton, CA) were used at pH below the protein isoelectric point. Both capillaries had 50 µm inner diameter with lengths of 80.5 and 65 cm, respectively. Data were obtained with a running buffer containing 10 mM NaCl with 1 mM sodium acetate (for pH 4-6), 1 mM Tris (for pH 7-8), or 1 mM sodium tetraborate (for pH 9-11.5). Mesityl oxide was used as a neutral marker. The capillaries were initially conditioned by washing with 0.1 M NaOH for 10 min followed by the running buffer for an additional 10 min. The eCAP™ Amine capillary was regenerated between runs using amine regenerator solution (Beckman Coulter, Inc., Catalog Number 477433). Injection of 15-30 nL samples was done by application of a 3.5 kPa pressure for 25 s. Electropherograms were recorded and analyzed using 3D-CE ChemStation Software (version A.09.03, Agilent Technologies, Palo Alto, CA).

Additional details on the experimental procedures and data analysis are available elsewhere (Menon and Zydney, 1998; Ebersold and Zydney, 2004).
6.2.3 Streaming Potential Measurement

The membrane pore charge characteristics were evaluated from streaming potential measurements following the procedure presented by Burns and Zydney (2000) as described in Chapter 3. Flow was directed through the membrane pores to obtain a measure of the effective charge on the surface of the pores. The apparent zeta potential ($\zeta_{app}$) was evaluated from the slope of the voltage (streaming potential) as a function of pressure using the Helmholtz–Smoluchowski equation (Equation (3.1)).

6.2.4 Ultrafiltration

Ultrafiltration experiments were performed using Ultracel™ composite regenerated cellulose membranes with 30 kDa nominal molecular weight cut-off (Millipore Corp., Bedford, MA). Positively charged versions of the Ultracel™ membranes were generated by chemical attachment of a quaternary amine functionality to the free hydroxyl groups using a proprietary solution chemistry developed by Millipore as described in Chapter 3. The extent of charge modification could be controlled by varying the reaction time.

Ultrafiltration experiments were performed in an Amicon 8010 stirred cell with effective membrane area of 4.1 cm$^2$ (Millipore Corp., Bedford, MA) as described in Chapter 3. The stirred cell was connected to an air-pressurized acrylic solution reservoir, with the filtrate flux controlled by adjusting the pressure. The stirring speed was set to 600 rpm using a Strobotac Type 1531-AB strobe light (General- Radio Co., Concord, MA). The membrane hydraulic permeability ($L_p$) was evaluated by measuring the filtrate
flux as a function of pressure using a 500 mM NaCl solution buffered with 1 mM Tris at pH 9. The high salt concentration minimized the effect of counter-electroosmosis. The permeability was evaluated from the slope of the data using Equation (2.6).

Each membrane disk was soaked overnight at 4 °C in the appropriate protein solution to minimize any initial transients associated with protein adsorption on and within the membrane pores. The membrane permeability was re-evaluated after this initial adsorption and then again after the ultrafiltration experiment. The hydraulic permeability decreased by about 10% after the overnight protein adsorption, with an average reduction of approximately 3% after the protein filtration.

Ultrafiltration experiments were performed using a binary mixture of 3 g/L lysozyme and 1 g/L cytochrome c. The higher concentration of lysozyme was used to improve the accuracy of the protein concentration measurements (the extinction coefficient of cytochrome c at 410 nm is considerably greater than that of lysozyme at 260 nm). Data were obtained at a transmembrane pressure of approximately 10 kPa corresponding to a filtrate flux of about 4 – 7 µm/s, with the filtrate flow rate evaluated by timed collection using a digital balance (Model AG104, Mettler Toledo, Columbus, OH) with an accuracy of 0.1 mg. Data were obtained at low ultrafiltration velocities to minimize the effects of concentration polarization and/ or fouling. Protein samples were collected from the filtrate and bulk solutions as described in section 3.4.3. The observed sieving coefficient was evaluated as

\[ S_o = \frac{C_f}{C_b} \]

(6.1)
where $C_f$ and $C_b$ are the protein concentration in the filtrate and bulk solutions, respectively. The stirred cell was then carefully emptied, flushed with at least 25 L/m$^2$ of 0.01 M buffered NaCl solution, and refilled with a fresh protein solution at a new pH. All experiments were performed at room temperature (22 ± 3 °C).

### 6.2.5 Ion exchange chromatography

Cation exchange chromatography was performed using an AKTAexplorer 100 chromatography system with SP Sepharose Fast Flow (SP FF) resin (GE Healthcare) as described in Chapter 3. Chromatography experiments were performed using a binary mixture of 2 g/L lysozyme and 2 g/L cytochrome c. Protein detection was at 214 nm using the UV-visible detector provided as part of the AKTAexplorer system.

The system was first equilibrated by flushing the column with 8 column volumes (CV) of 10 mM NaCl solution buffered with 10 mM sodium tetraborate or Tris at the desired pH using a flow rate of 0.9 mL/min (9.5 CV/hr). A 500 µL sample containing the binary protein mixture was manually injected and the column was washed with 2 CV of the equilibration buffer. The protein was then eluted using a 10 CV linear gradient between the equilibration buffer and a 500 mM NaCl/10 mM sodium tetraborate or Tris buffer at the same pH. The column was then rinsed with 5 CV of the final elution buffer followed by 5 CV of the equilibration buffer. The column was cleaned with 1 M NaOH when necessary.
6.3 Results and Discussions

6.3.1 Membrane Surface Charge Characteristics

The effective surface charge characteristics of the membranes were evaluated from streaming potential measurements obtained with the fluid flow directed through the membrane pores. The apparent zeta potential was evaluated directly from the slope of the streaming potential versus pressure data using the Helmholtz - Smoluchowski equation (Equation 3.1). The membrane pore charge density \( q_p \) was calculated from the apparent zeta potential \( \zeta_{\text{app}} \) using the Helmholtz – Smoluchowski equation (Equation 4.4).

Equation (4.4) neglects the effects of surface conductance and a thick double layer, thus the calculated values of \( q_p \) represent an effective surface charge density of the membrane pores. The dimensionless surface charge density \( \sigma_p \) was then calculated using Equation (2.15).

Figure 6.1 shows experimental data for the apparent zeta potential (left axis) and the effective surface charge density (right axis) as a function of solution pH for both the un-modified and the positively charged version of the Ultracei™ membranes. The surface charge of the un-modified membrane is slightly negative for pH > 3, with this small negative charge arising from the preferential binding of negatively-charged anions from the bulk electrolyte (Burns and Zydney, 2000). The surface charge density for the modified membrane is approximately 1.2 mC/m\(^2\) between pH 4 and 8, corresponding to an apparent zeta potential of approximately 5.3 mV, consistent with the attachment of the quaternary amine functionality. The small reduction in surface charge density above pH
8 is likely due to anion or hydroxyl binding / association; the pKₐ of the quaternary amine is greater than pH 12 and should thus remain positively-charged even at high pH.

![Graph showing apparent zeta potential and effective surface charge density vs. solution pH.](image)

**Figure 6.1** Apparent zeta potential (left axis) and effective surface charge density (right axis) for the unmodified and positively-charged Ultracel™ membranes determined from the measured streaming potential using 10 mM buffered KCl solutions.

**6.3.2 Ultrafiltration Experiments – Lysozyme and Cytochrome c**

Figure 6.2 shows typical experimental data for the observed sieving coefficients of lysozyme and cytochrome c through an unmodified Ultracel™ 30 kDa membrane as a function of solution pH. The data were obtained for a binary protein feed (3 g/L...
lysozyme and 1 g/L cytochrome c) using a single membrane at constant flux of $5.2 \pm 0.05 \mu m/s$. The observed sieving coefficient ($S_o$) was evaluated at each pH from Equation (6.1). Note that the observed sieving coefficients are essentially equal to the actual sieving coefficients since concentration polarization effects were minimal at the low filtrate flux used in these experiments. This was confirmed experimentally by evaluating the sieving coefficient over a range of filtrate flux, with the $S_o$ values varying by less than 10 % for filtrate flux between 3 and 9 µm/s. The filled symbols in Figure 6.2 represent data obtained with increasing pH (beginning at pH 7 and moving to pH 11.3) while the open symbols represent data obtained by sequentially decreasing the pH back to pH = 7. The results for the two pH cycles are nearly identical, which is consistent with the lack of any measurable change in the measured hydraulic permeability of the Ultracef™ membrane over the course of the experiment. The sieving coefficients of lysozyme and cytochrome c were relatively independent of pH below the isoelectric point, with very similar results below pH 9 ($S_o$ values within 15 %), consistent with the similar size and net charge of the proteins under these conditions. The sieving coefficients of both proteins decreased at pH above the isoelectric point due to the electrostatic repulsion between the negatively-charged protein and the negatively-charged membrane. There were significant differences in the sieving behavior for the two proteins at higher pH. The sieving coefficient of cytochrome c decreases sharply above pH 10 while lysozyme shows a small decrease in $S_o$ under these conditions. The net result is that the sieving coefficient of lysozyme is nearly four times larger than that of cytochrome c at pH 11.
Figure 6.2  Observed sieving coefficients for lysozyme and cytochrome c through an unmodified UltracelTM membrane as a function of pH using a 10 mM buffered NaCl solution. Filled symbols represent data with increasing pH while open symbols represent data with decreasing pH.

In order to understand the pH effects seen in Figure 6.2, it is helpful to evaluate the net protein charge as a function of solution pH. The electrophoretic mobilities ($\mu_E$) of lysozyme and cytochrome c were evaluated from the migration times in capillary electrophoresis as:

$$\mu_E = \frac{(L_d / t_p) - (L_d / t_n)}{(V / L_e)}$$  \hspace{1cm} (6.2)
where $L_d$ is the path length to the detector, $L_t$ is the total capillary length, $t_n$ and $t_p$ are the migration times of the neutral marker and protein, and $V$ is the applied voltage.

The results are shown in the upper panel of Figure 6.3; the solid curves are simple spline fits to the data. Data at pH $\leq 10.5$ were obtained using the positively-charged eCAP™ Amine capillaries while those at pH $> 10.5$ were obtained with the negatively-charged fused silica capillaries to avoid protein adsorption to the oppositely-charged capillary. It was not possible to evaluate the mobility very close to the protein isoelectric point due to protein adsorption to both the positive and negative capillaries under these conditions. The mobilities for lysozyme and cytochrome c show very similar pH dependence, with the curve for cytochrome c shifted slightly to the left corresponding to a somewhat lower isoelectric point.

Polynomial interpolation of the data around $\mu E = 0$ gives a pI for cytochrome c of 10.4 ± 0.1 and a pI for lysozyme of 11.0 ± 0.1. The measured pI values for lysozyme and cytochrome c are both in good agreement with the values reported in the literature (Table 6.1).
Figure 6.3  Protein electrophoretic mobility (top panel) and net protein charge (bottom panel) evaluated by capillary electrophoresis as a function of solution pH. Solid curves are simple spline fits to the data.
The mobility data were used to calculate the effective protein charge as described in Section 2.4.2:

\[ z = \frac{6 \pi \mu a (1 + \kappa a) \mu_E}{e f_H} \]  

(6.3)

where \( a \) is the protein radius, \( \kappa^{-1} \) is the Debye length (3 nm for the 10 mM ionic strength solutions used in the capillary electrophoresis experiments), \( e \) is the electron charge \((1.602 \times 10^{-19} \text{ C})\), and \( f_H \) is Henry's function (Henry, 1931), which accounts for the finite double layer thickness as described in Chapter 2. Equation (6.3) assumes that the protein is a uniformly charged hard-sphere with hydrodynamic mobility given by the Stokes drag law. The calculated values of the protein charge are shown in the lower panel of Figure 6.3. The charge on cytochrome c varies from 6.9 at pH 4 to -5.3 at pH 12 due to the change in ionization of the various acidic and basic amino acid residues.

In order to enhance the magnitude of the electrostatic interactions, a series of ultrafiltration experiments was performed using a positively-charged version of the Ultracel\textsuperscript{TM} 30 kDa membrane generated by covalent coupling of quaternary amine groups to the base cellulose. The unmodified Ultracel\textsuperscript{TM} 30 kDa membrane had a permeability of \( 6.6 \times 10^{-13} \text{ m} \); this decreased to \( 5.9 \times 10^{-13} \text{ m} \) after charging for 90 min. The apparent zeta potential for this charge-modified membrane was 5.3 mV at pH 7 compared to a value of -1.6 mV for the un-modified membrane under the same conditions (Figure 6.1). Limited data obtained with membranes pre-adsorbed with protein showed only small changes in the zeta potential, consistent with the absence of any significant protein adsorption to the very hydrophilic cellulose membranes. The small negative charge on
the base cellulose is probably due to the preferential adsorption of negative ions from the bulk electrolyte solution (Burns and Zydney, 2000).

Figure 6.4 shows the observed sieving coefficients for lysozyme and cytochrome c through the positively-charged Ultracel™ 30 kDa membrane. The sieving coefficients for both proteins are substantially smaller than the values seen with the un-modified membrane, particularly at low pH where the proteins have a large positive charge. This behavior is a direct result of the strong electrostatic repulsion between the positively-charged proteins and the positively-charged membrane. The $S_o$ values for lysozyme and cytochrome c are similar at low pH, but show distinct differences between pH 9 and 12. The observed sieving coefficient for cytochrome c goes through a distinct maximum at a pH near the protein isoelectric point before decreasing by about a factor of 4 as the pH increases from 10.5 to 11.7. In contrast, the sieving coefficient for lysozyme increases monotonically with increasing pH up to pH 11.7. The net result is that lysozyme transmission is approximately 6-fold greater than that for cytochrome c at pH 11.7.
Figure 6.4  Observed sieving coefficients for lysozyme and cytochrome c through a positively-charged Ultracel™ membrane as a function of pH. Solid curves are simple spline fits to the data.

The observed sieving coefficient data for the positively-charged membrane have been replotted in Figure 6.5 as a function of the net protein charge evaluated from the electrophoretic mobility. The data for both proteins appear to collapse to a single curve when plotted in this manner, with a maximum in the observed sieving coefficient when \( z \approx 0 \). The observed sieving coefficients decrease sharply for \( z > 0 \), reflecting the strong electrostatic repulsion between the positively-charged proteins and the positively-charged membrane. The more gradual reduction in \( S_o \) for \( z < 0 \) is due to the weak attraction
between the protein and membrane, although the net interaction is "repulsive" due to the energetic penalty associated with the distortion of the electrical double layer around the charged protein. This behavior is discussed in more detail by Burns and Zydney (2001).

**Figure 6.5** Observed sieving coefficients for lysozyme and cytochrome c through a positively-charged Ultracel™ membrane as a function of the net protein charge determined from the measured electrophoretic mobility.
6.3.3 Ultrafiltration Experiments – Peracetylated Cytochrome c

A more rigorous approach to investigate the possible effects of a surface charge distribution on protein sieving is to examine the behavior of an un-modified and a "peracetylated" version of a given protein. The peracetylated protein was formed by saturation reaction of the native cytochrome c with acetic anhydride to convert all free amine groups on the lysine residues to the corresponding amide as described in Section 6.2.1. Figure 6.6 compares the sieving behavior of a native and peracetylated version of cytochrome c; the isoelectric point of the peracetylated protein was 3.7 ± 0.1 compared to 10.4 ± 0.1 for the native cytochrome c (both determined by capillary electrophoresis), consistent with the elimination of all 19 amine groups. In both cases data were obtained using an unmodified Ultracel™ 30 kDa composite regenerated cellulose membrane. The observed sieving coefficients for the peracetylated protein are shifted to markedly lower pH, consistent with the shift in isoelectric point, with the $S_0$ values for both proteins passing through a maximum at a pH near the corresponding value of the pI.
Figure 6.6  Observed sieving coefficients for native and peracetylated cytochrome c through an un-modified Ultracel™ membrane as a function of pH. Solid curves are simple spline fits to the data.

The sieving coefficients for the native and peracetylated protein are re-plotted as a function of the net protein charge (determined by capillary electrophoresis) in Figure 6.7. The data for the two proteins are brought into much better agreement when plotted as a function of the net protein charge, suggesting that the detailed charge distribution has a relatively small effect on the sieving coefficient. The small differences in sieving coefficient seen in Figure 6.7 are likely due primarily to differences in the surface charge density of the membranes used for the two experiments. In this case, the un-modified
Ultracel\textsuperscript{TM} membrane has a greater negative charge at high pH (i.e., for the data with the native cytochrome c), leading to greater electrostatic repulsion at a given value of $z$ for pH > pI (i.e., for $z < 0$).

![Graph showing observed sieving coefficients for native and peracetylated cytochrome c through an unmodified Ultracel\textsuperscript{TM} membrane as a function of the net protein charge determined from the measured electrophoretic mobility.]

**Figure 6.7** Observed sieving coefficients for native and peracetylated cytochrome c through an unmodified Ultracel\textsuperscript{TM} membrane as a function of the net protein charge determined from the measured electrophoretic mobility.

### 6.3.4 Ion Exchange Chromatography

As discussed in the Introduction, several previous studies have demonstrated that the surface charge distribution and amino acid composition can both have a strong affect on protein binding in ion exchange chromatography. This phenomenon was examined
experimentally by measuring the elution of lysozyme and cytochrome c from a strong cation exchange resin in the presence of a linear salt gradient. Figure 6.8 shows a typical elution profile at pH 11 after injection of a mixture containing 2 g/L cytochrome c and 2 g/L lysozyme using a Sepharose FastFlow (SP FF) cation exchange resin. The cytochrome c was rapidly eluted consistent with the net negative charge on cytochrome c at this pH. In contrast, lysozyme showed significant retention on the negatively-charged resin even though the net protein charge was approximately zero at pH 11. This type of strong binding near the protein isoelectric point has previously been attributed to the presence of localized positively-charged regions (“charge patches”) on the lysozyme surface (Yamamoto and Ishihara, 2000; Kopaciewicz et al., 1983).
Figure 6.8  Protein elution from the SP FF cation exchange resin at pH 11 using a linear salt gradient.

Figure 6.9 shows experimental data for a series of chromatographic experiments performed with lysozyme and cytochrome c using the SP FF cation exchange resin. Each experiment was performed at a distinct value of the mobile phase pH, with the data plotted as a function of the net protein charge determined from electrophoretic mobility measurements at the same pH. Cytochrome c was eluted immediately (retention volume of 4.9 mL) at pH above its isoelectric point ($z < 0$), consistent with the absence of any significant binding between the negatively-charged protein and the negatively-charged resin. The retention volume increased significantly as the protein charge increased, with the retention volume for cytochrome c being slightly larger than that for lysozyme at $z$
greater than about 3. The largest difference in binding characteristics occurred around the isoelectric point. The retention volume for lysozyme began to increase at $z \approx -2$ even though the protein and resin were both negatively-charged under these conditions. The retention volume for lysozyme increased to 31.3 mL at $z = 0$, which is more than 5 times the value for cytochrome c ($V = 6$ mL at $z = 0$). This dramatic difference in protein binding is a direct result of the different surface charge distributions and amino acid compositions for the lysozyme and cytochrome c as discussed previously by DePhillips and Lenhoff (2001) and Yao et al. (2005).

Figure 6.9 Retention volume for lysozyme and cytochrome c on the SP FF resin as a function of net protein charge determined from the measured electrophoretic mobility.
The retention volume data for lysozyme and cytochrome c were used to evaluate the retention factor:

\[ k' = \frac{V_R - V_0}{V_0} \]

(6.4)

where \( V_R \) is the measured retention volume and \( V_0 \) is the retention volume for an unbound solute, with the latter evaluated as \( V_0 = 4.1 \) mL from experiments performed with cytochrome c at pH 11 using 500 mM sodium chloride as the elution buffer, i.e., under conditions in which electrostatic interactions should be negligible. The retention factor is a measure of the time the protein resides in the stationary phase relative to the time it spends in the mobile phase. The retention factor is thus related to the free energy of interaction between the protein molecule and the charged sites within the porous ion exchange resin (Yao and Lenhoff, 2004). The calculated values of the retention factor at each pH are plotted as a function of the observed sieving coefficient at the corresponding pH value in Figure 6.10. Low pH values correspond to high retention factors and small sieving coefficients, reflecting the strong electrostatic attraction between the positively-charged protein and the negatively-charged cation exchanger and the strong repulsion between the positively-charged protein and the positively-charged version of the Ultracel™ membranes. The results for lysozyme and cytochrome c are very similar at \( S_o < 0.02 \), conditions that correspond to very strong electrostatic interactions. However, there are significant differences in the data for the two proteins for \( S_o > 0.02 \), corresponding to pH values near the protein isoelectric point. For example, the sieving coefficients for cytochrome c at pH 10 and 10.5 are essentially identical (\( S_o \approx 0.17 \)), but the retention factors at these pH vary by more than a factor of 14 (\( k' = 7.1 \) at pH 10 and \( k' \)
= 0.5 at pH 10.5). The large effect of solution pH on protein binding near the isoelectric point reflects the large change in electrostatic interactions and the significant impact of the surface charge distribution on the binding characteristics in ion exchange chromatography. The results in Figure 6.10 clearly demonstrate that the surface charge distribution has a fundamentally different effect on protein binding in IEX and protein sieving in UF.

**Figure 6.10** Retention factor for lysozyme and cytochrome c on the SP FF resin as a function of the observed sieving coefficients determined at the same pH using the positively-charged Ultracei™ membrane.
6.3.5 Partitioning Model

In order to obtain additional insights into the electrostatic interactions in ultrafiltration, the sieving coefficient data were analyzed using the theoretical model developed by Smith and Deen (1980) for the partitioning of a charged sphere in an infinitely long cylindrical pore as described in Chapter 2. The actual sieving coefficient ($S_a$), defined as the ratio of the protein concentration in the filtrate solution to that in the solution immediately upstream of the membrane, can be expressed as (Deen, 1987):

$$S_a = (1 - \lambda)^2 K_c \exp\left(\frac{-\psi_E}{k_B T}\right)$$

(6.5)

where the term $(1-\lambda)^2$ describes the steric (hard-sphere) exclusion of the sphere from the region within one solute radius of the pore wall (with $\lambda$ equal to the ratio of the solute radius to the pore radius), $K_c$ is the hindrance factor associated with convection, and

$$\left(\frac{\psi_E}{k_B T}\right)$$

is the electrostatic energy of interaction as described in Chapter 2:

$$\frac{\psi_E}{k_B T} = (A_s \sigma_s^2 + A_{sp} \sigma_s \sigma_p + A_p \sigma_p^2) / A_{den}$$

(6.6)

where $A_s, A_{sp}, A_p$ and $A_{den}$ are functions of the solution ionic strength and pore size (equations for the different coefficients are given in Section 2.3.2) and $\sigma_s$ and $\sigma_p$ are the dimensionless surface charge densities of the solute (protein) and pore. The three terms in Equation (6.6) represent the energy of interaction associated with the distortion of the electrical double layer around the solute (protein), direct charge-charge interactions
between the solute and the pore, and the distortion of the electrical double layer adjacent
to the pore wall, respectively.

The experimental data for the observed sieving coefficients of cytochrome c and
lysozyme are plotted as a function of the product of the dimensionless protein and
membrane surface charge densities in Figure 6.11. The y-axis is the scaled sieving
coefficient, which was calculated as the ratio of the sieving coefficient at a given pH to
that at the protein isoelectric point. This normalization should eliminate the steric
interactions described by the $(1-\lambda)^2 K_c$ term in Equation (6.5). The protein charge was
evaluated directly from the electrophoretic mobility data using Equation (6.3), which
implicitly assumes that the charge groups are uniformly distributed over the surface of an
effective sphere of radius a. The dimensionless protein surface charge density ($\sigma_p$) was
then calculated as:

$$\sigma_s = \frac{z e^2 r_p}{4 \pi a^2 e_0 e_k B T}$$

(6.7)

where $r_p$ is the membrane pore radius and $k_B$ is the Boltzman constant. The
dimensionless membrane surface charge density was calculated from streaming potential
data as described in the section 6.3.1. The results for lysozyme and cytochrome c for the
positively-charged membrane collapse to nearly a single curve when plotted in this
manner even though these proteins have different surface charge distributions. The data
are also highly linear when plotted on a semi-log scale ($r^2 > 0.94$ for both lysozyme and
cytochrome c), which is consistent with predictions of the partitioning model assuming
that the electrostatic interactions are dominated by the direct charge-charge interactions (the second term on the right-hand side of Equation 6.6).

![Figure 6.11](image)

**Figure 6.11** Scaled sieving coefficients for lysozyme and cytochrome c as a function of the dimensionless charge interaction parameter. Protein charge density determined from capillary electrophoresis; membrane charge density determined from streaming potential measurements.

The solid lines in Figure 6.11 are the linear regression fits (on a semi-log plot) to the experimental data for each protein separately. The slopes for these lines were used to calculate the best fit values of the coefficient $A_{sp}/A_{den} = 4.3$ for lysozyme and 5.2 for
cytochrome c. The ratio \( A_{sp} / A_{den} \) is a function of the solution ionic strength and pore size with specific equations given in Chapter 2. These best fit values of \( A_{sp} / A_{den} \) were then used to evaluate the effective pore radius of the positively-charged Ultracel\textsuperscript{TM} membrane giving \( r_p = 3.4 \) nm and \( 3.2 \) nm, respectively. Note that this analysis neglects the effects of a pore size distribution and assumes that the protein and membrane both have constant and uniform surface charge. These values of the pore radius were somewhat larger than the values that are calculated directly from the protein sieving coefficients at the isoelectric point, i.e., under conditions where the electrostatic interactions are negligible. For example, the sieving coefficient for cytochrome c at pH 10.5 (\( S_a = 0.12 \)) and for lysozyme at pH 11 (\( S_a = 0.09 \)) both yield \( r_p = 2.2 \) nm using Equation (6.5) and the expression for \( K_c \) for a sphere in a cylindrical pore (Zeman and Zydney, 1996).

It was also possible to obtain an independent estimate of the pore radius from the measured hydraulic permeability of the positively-charged Ultracel\textsuperscript{TM} membrane, \( L_p = 5.2 \times 10^{-13} \) m, using the Hagen-Poiseuille equation:

\[
L_p = \frac{\varepsilon r_p^2}{8\delta_m} \tag{6.8}
\]

where \( \varepsilon \) is the membrane porosity and \( \delta_m \) is the membrane thickness. The pore radius calculated from Equation (6.8) is between 2 and 5 \( \text{nm} \) assuming \( \frac{\varepsilon}{\delta_m} = 0.2 \) to 1 \( \mu\text{m}^{-1} \), which corresponds to a skin thickness of the asymmetric membrane between 0.5 – 2.5 \( \mu\text{m} \) based on a membrane porosity of \( \varepsilon = 0.5 \) (Mehta and Zydney, 2005). The good agreement between these different estimates of the pore radius provides further
confirmation of the use of the electrostatic partitioning model given by Equation (6.6) to describe protein sieving in ultrafiltration.

6.4 Conclusions

The results presented in this Chapter provide the first experimental investigation into the possible effects of a surface charge distribution on the magnitude of the electrostatic interactions that occur during protein ultrafiltration. Ultrafiltration and ion exchange chromatography data were obtained over a range pH using cytochrome c and lysozyme as model proteins that have similar size and net charge but different amino acid composition and surface charge distribution. The sieving data for both proteins collapsed to a single curve when plotted as a function of the net protein charge (evaluated from the electrophoretic mobility), with a maximum in the sieving coefficient obtained at \( z \approx 0 \) for the positively-charged membrane. In contrast, the retention factor for lysozyme and cytochrome c obtained during cation exchange chromatography showed very distinct differences, particularly at pH values around the isoelectric point. Previous studies have clearly demonstrated that these differences arise from the effects of the protein surface charge distribution and amino acid distribution on protein binding, an effect that was not seen in the ultrafiltration data.

Use of "peracetylated" version of cytochrome c provided an opportunity to investigate the possible effects of a surface charge distribution on protein sieving with "identical" proteins having different surface charge distribution. The sieving data for the native and “peracetylated” version of cytochrome c collapse to essentially a single curve
when plotted as a function of the net protein charge suggesting that the detailed charge distribution has a relatively small effect on protein transport through ultrafiltration membranes.

The experimental results for the protein sieving coefficient were analyzed in terms of the normalized sieving coefficient, defined as the ratio of the protein sieving coefficient at a given pH to that at the protein isoelectric point. The data for lysozyme and cytochrome c both collapse to a straight line when plotted as a function of the product of the protein and membrane surface charge densities, consistent with predictions of an electrostatic partitioning model developed for a uniformly charged sphere in a charged cylindrical pore. In addition, the calculated values of the pore radii determined from the slope of the data were consistent with independent estimates of the pore radius evaluated from the membrane hydraulic permeability and the sieving coefficient of the uncharged protein, providing further evidence that protein transmission in ultrafiltration is determined almost entirely by the average surface charge density of the protein.

In contrast to the sieving data, the measured retention volumes for lysozyme and cytochrome c were very different, particularly near the protein isoelectric point; it was not possible to analyze these data simply using the net protein charge. The very different behavior seen in ultrafiltration and ion exchange chromatography is due to the different physical phenomena governing the performance of these processes. The retention factor in ion exchange chromatography provides a measure of the strength of the binding interaction, where the protein binds to the resin through only a small number of charged sites on the protein surface. These binding interactions are strongly affected by the local
charge distribution and the specific nature of the charge sites (e.g., arginine versus lysine amino acids). In contrast, the protein sieving coefficient in ultrafiltration is determined by the equilibrium partition coefficient between the bulk solution and the liquid space within the pore. The magnitude of the partition coefficient is determined by the sum of the interactions between all of the charge groups on the protein and all of the charges on the pore surface. In addition, the rotational diffusion coefficients for the proteins examined in this Chapter are fairly large, e.g., $D_{rot} = 17 \times 10^6 \text{ s}^{-1}$ for lysozyme at pH 4.2 in a 0.1 M acetate buffer (Dubin et al. 1971). Thus, on average the protein rotates more than 40 times every 0.001 s (compared to the residence time in the pore of approximately 0.05 s), effectively averaging the electrostatic interactions over the entire surface of the protein. In addition, the characteristic time for relaxation of the double layer, which can be estimated as $1/(\kappa^2 D)$ where $\kappa^{-1}$ is the Debye length and $D$ is the ion diffusivity, is 3 orders of magnitude less than the characteristic time for rotation indicating that the distribution of ions in the double layer is always relaxed, i.e. at equilibrium. The net result is that the protein sieving coefficient through these oppositely charged membranes is determined almost entirely by the net surface charge density of the protein, irrespective of the detailed distribution of charge groups over the protein surface.
Chapter 7

Use of Protein Charge Ladders to Study Protein Transport through Charge-Modified Membranes

7.1 Introduction

The experimental data presented in previous chapters clearly demonstrate the importance of electrostatic interactions in improving the performance of ultrafiltration membranes, particularly for protein concentration and buffer exchange. For example, the results demonstrated that positively-charged membranes, produced with a number of novel ligands, can provide very high filtrate flux (permeability) while still providing high levels of retention of a positively-charged protein product due to the strong electrostatic repulsion between the charged protein and membrane.

A number of recent studies have also demonstrated that electrostatic effects can be exploited to achieve high resolution protein separations through the use of high performance tangential flow filtration (HPTFF), with the charged membranes providing high retention of like-charged proteins while allowing relatively uncharged solutes to pass into the permeate (van Reis and Zydney, 2007; Zydney, 2009; van Reis et al., 1999). Examples include the separation of an antigen binding fragment from E. coli host cell proteins (Lebreton et al., 2008), a monoclonal antibody from mammalian cell host
proteins (van Reis and Zydney, 2007), and a model pegylated protein from the unreacted protein and polyethylene glycol (Molek and Zydney, 2007), among others.

Menon and Zydney (2001) demonstrated that protein charge ladders provided a novel method for studying the separation characteristics of ultrafiltration membranes. Protein charge ladders consist of an array of derivatives of a given protein differing primarily in their surface charge. The individual “rungs” on the ladder can be analyzed using capillary electrophoresis. Protein charge ladders were originally developed by Whitesides and co-workers to study the role of electrostatic interactions in a variety of biophysical processes (Gao et al., 1996; Colton et al., 1997; Gitlin et al., 2006). Menon and Zydney (2001) used these charge ladders to evaluate the effects of electrostatic interactions on protein transport through polyethersulfone ultrafiltration membranes.

The objective of the work described in this Chapter was to examine the separation characteristics of several of the positively-charged, negatively-charged, and zwitterionic membranes developed in previous Chapters of this thesis using protein charge ladders. Protein charge ladders were synthesized by chemical modification of lysozyme, myoglobin, and α-lactalbumin by reaction with either acetic anhydride or succinic anhydride. Ultrafiltration data were analyzed in terms of the net charge of the individual rungs in the protein charge ladder as determined from the measured electrophoretic mobility. Results were also compared with available theoretical models to obtain additional insights into the effects of electrostatic interactions on protein transport.
7.2 Materials and Methods

7.2.1 Buffer Solutions

Buffered salt solutions were prepared as described in Chapter 3 by dissolving appropriate amounts of Trizma® Base (tris[hydroxymethyl]aminomethane, Sigma T-1503), glycine (Sigma G-7403), and KCl (BDH Chemicals, BDH0258) in deionized water obtained from a NANOpure® Diamond water purification system (Barnstead Thermolyne Corporation, Dubuque, IA) with a resistivity greater than 18 MΩ-cm. Glycine/tris buffer with ionic strength of 10 mM was prepared by dissolving 0.192 mol of glycine and 0.025 mol of Trizma® Base in 1 L of deionized water. Higher ionic strength solutions were prepared by dissolving appropriate amounts of KCl in glycine/tris solution.

7.2.2 Protein charge ladders

Myoglobin, α-lactalbumin, and lysozyme were obtained from Sigma Chemical (St. Louis, MO). Catalog numbers and key physical properties are summarized in Table 7.1. Myoglobin charge ladders were synthesized by reaction of one or more of the lysine ε-amino groups on the protein with succinic anhydride (Figure 7.1a) following the procedure described by Ebersold and Zydney (2004a) with slight modifications. The succinylation reaction converts the natural (positively-charged) amine group into an amide attached to a negatively-charged carboxylic acid. These variants have essentially the same size and conformation as the native myoglobin, differing in total molecular weight by less than 2%. A 3 g/L myoglobin solution in deionized water was prepared
and cooled to 3-5 °C in an ice bath to minimize protein denaturation during the chemical modification. The solution pH was adjusted to 11 and kept constant during the reaction by addition of 0.1 M KOH as required. Five equivalents (per gram mole of protein) of the 0.1 M succinic anhydride (Aldrich Chemical Co., Milwaukee, WI) solution in 1,4-dioxane (Sigma, D-9553) were added drop-wise to the myoglobin solution. An additional 5 equivalents of the 0.1 M succinic anhydride solution were added after 30 min. The reaction was allowed to continue for an additional 30 min, giving a total reaction time of 1 hr. The pH was then lowered to 7 by adding 0.1 M HCl to quench the reaction. The resulting solution was immediately diafiltered with 4 diavolumes of chilled deionized water to remove the dioxane and any residual reactants using an Amicon stirred cell (Millipore Corp., Bedford, MA) with a 10 kD Ultracelest membrane as described in Chapter 3. The resulting protein solution was concentrated and then mixed with an appropriate volume of tris/glycine buffer at the pH and ionic strength of interest, resulting in a final protein concentration of approximately 9 g/L.

Lysozyme and α-lactalbumin charge ladders were synthesized by reaction of one or more of the lysine ε-amino groups with acetic anhydride (Figure 7.1b) following the procedure described by Chung et al. (2010). A 10 g/L protein solution was prepared in DI water, with the solution pH brought to pH 12 using 1 M NaOH solution. The reaction was performed with about 4 equivalents (per gram mole of protein) of 0.1 M acetic anhydride in 1,4-dioxane. The reaction was quenched after 5 min by lowering the pH to 7.0 using a 1 M HCl solution, followed by diafiltration of the protein solution with DI
water to remove any residual reactants. The amide resulting from the acylation exists in the –NH-form and thus makes no contribution to the net protein charge.

All protein solutions were pre-filtered through a 0.22 μm Acrodisc® syringe filter (Pall Corp.) to remove any protein aggregates immediately prior to use. Protein solutions were used within 12 h of preparation to minimize the likelihood of protein aggregation or denaturation.

![Diagram of acylation reaction](image)

**Figure 7.1** Schematic representation of the acylation reaction using (a) succinic anhydride and (b) acetic anhydride (adapted from Ebersold and Zydney (2004 a,b)).
Table 7.1 Physicochemical properties of proteins

<table>
<thead>
<tr>
<th>Property</th>
<th>Myoglobin</th>
<th>Lysozyme</th>
<th>α-lactalbumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalog number</td>
<td>M 0630</td>
<td>L 6876</td>
<td>L 5385</td>
</tr>
<tr>
<td>Molecular Weight (Da)</td>
<td>17600</td>
<td>14307</td>
<td>14200</td>
</tr>
<tr>
<td>Equivalent Radius (nm)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.7</td>
<td>1.60</td>
<td>1.60</td>
</tr>
<tr>
<td>Isoelectric Point</td>
<td>10.4</td>
<td>11</td>
<td>4.6</td>
</tr>
<tr>
<td>Number of lysine residues</td>
<td>19</td>
<td>6</td>
<td>12</td>
</tr>
</tbody>
</table>

<sup>a</sup> Radius of sphere of equivalent volume

7.2.3 Capillary Electrophoresis

The concentration and net charge for each rung of the charge ladder were determined by capillary zone electrophoresis using a G1600A High-Performance Capillary Electrophoresis instrument (Agilent Technologies, Palo Alto, CA) as described in Chapter 3. Detection was by UV absorbance at 214 nm. Negatively-charged fused silica capillaries (Agilent Technologies, Catalog Numbers G1600-62211, G1600-61211, Palo Alto, CA) were used for analysis of the myoglobin and α-lactalbumin charge ladders using an applied voltage of 25 kV. Positively-charged eCAP™ Amine capillaries (Beckman Coulter, Inc., Catalog Number 477431, Fullerton, CA) were used for the positively charged ladders made from lysozyme. Both capillaries had 50 μm inner diameter with lengths of 80.5 and 65 cm, respectively. Data were obtained with a 10 mM tris/glycine running buffer at the pH of interest. Mesityl oxide (Fluka, 63940) was used
as a neutral marker. The capillaries were initially conditioned by washing with 0.1 M NaOH for 10 min followed by the running buffer for an additional 10 min. The eCAP™ Amine capillary was regenerated between runs using amine regenerator solution (Beckman Coulter, Inc., Catalog Number 477433). Injection of 15-30 nL samples was done by application of a 3.5 kPa pressure for 25 s. Electropherograms were recorded and analyzed using 3D-CE ChemStation Software (version A.09.03, Agilent Technologies, Palo Alto, CA). The net protein charge for each variant was determined directly from the electrophoretic mobility as discussed in Chapters 2, and 6.

### 7.2.4 Membrane Preparation

Ultrafiltration experiments were performed using Ultracel™ composite regenerated cellulose membranes with 30 kDa nominal molecular weight cut-off (Millipore Corp., Bedford, MA). Zwitterionic ultrafiltration membranes were generated by covalent attachment of either taurine or glycine to a base cellulose membrane using the reaction chemistry developed by Riordan et al. (2009) as discussed in Chapter 5. Positively charged versions of the Ultracel™ membranes were generated by chemical attachment of a quaternary amine functionality to the free hydroxyl groups using a proprietary solution chemistry developed by Millipore as described in Chapter 3. A positively charged version of the Ultracel™ membrane with three secondary amines and a terminal primary amine was generated by reaction with diaminobutane using a sequential reaction/activation scheme as discussed in Chapter 4. A negatively charged version of the Ultracel™ membrane was generated by chemical attachment of a small
sulfonic acid group to the free hydroxyl as described in Chapter 3, with the extent of charge modification controlled by varying the reaction time. Membranes were soaked overnight at 4 °C in the appropriate protein charge ladder solution prior to performing the ultrafiltration experiments to minimize any initial transients associated with protein adsorption on and within the membrane pores.

7.2.5 Streaming Potential Measurement

The membrane charge characteristics were evaluated from streaming potential measurements following the procedure presented by Burns and Zydney (2000) as described in Chapter 3. Flow was directed through the membrane pores to obtain a measure of the effective charge on the surface of the pores. The apparent zeta potential ($\zeta_{app}$) was evaluated from the slope of the voltage (streaming potential) as a function of pressure using Equation (3.1).

7.2.6 Ultrafiltration

Ultrafiltration experiments were performed in an Amicon 8010 stirred cell with effective membrane area of 4.1 cm$^2$ (Millipore Corp., Bedford, MA) as described in Chapter 3. Filtration experiments were performed at either constant pressure, which was maintained by air pressurization of the stirred cell, or at constant filtrate flux, which was maintained by a Masterflex 7523-20 peristaltic pump (Cole-Parmer, Vernon Hills, IL) connected to the filtrate line (downstream of the membrane). The feed reservoir was air-pressurized during constant flux experiments so that a positive
gauge pressure of approximately 10 kPa (corresponding to 1.5 psi) was maintained throughout the system. The stirring speed was set at 600 rpm. Protein samples were collected from the filtrate and bulk solutions as described in Chapter 3. Data were obtained at low ultrafiltration velocity (5 µm/s) to minimize the effects of concentration polarization and/or fouling. The protein concentration for each rung of the charge ladder in the filtrate and bulk samples was determined by integrating the area under each peak obtained by capillary electrophoresis. Overlapping peaks were simply split at the minimum. All experiments were performed at room temperature (22 ± 3 °C).

The membrane hydraulic permeability ($L_p$) was evaluated by measuring the filtrate flux as a function of pressure using a 500 mM KCl solution buffered with 20 mM Tris-Glycine at pH 8. The high salt concentration minimized the effects of counter-electroosmosis. The permeability was evaluated from the slope of the data using Equation (2.6).

7.3 Results and Discussions

7.3.1 Membrane Surface Charge Characteristics

The effective surface charge characteristics of the membranes were evaluated from streaming potential measurements obtained with the fluid flow directed through the membrane pores. The apparent zeta potential was evaluated directly from the slope of the streaming potential versus pressure data using the Helmholtz-Smoluchowski equation (Equation (3.1)), with the results summarized in Table 7.2. The greatest positive apparent zeta potential was obtained for the membrane modified with 1,4
diaminobutane. This was the membrane that had the best permeability – selectivity tradeoff for conventional ultrafiltration as discussed in Chapter 4. The largest negative value was obtained with the sulfonic acid ligand, followed by the membranes modified with the zwitterionic ligands taurine and glycine.

Table 7.2  Apparent zeta potentials (ζ<sub>app</sub>) for the unmodified and charge-modified 30 kDa Ultrace<sub>TM</sub> membranes evaluated from streaming potential measurements at pH 7 in 10 mM KCl.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Zeta Potential, ζ&lt;sub&gt;app&lt;/sub&gt; (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified</td>
<td>-1.6</td>
</tr>
<tr>
<td>Taurine</td>
<td>-3.9</td>
</tr>
<tr>
<td>Glycine</td>
<td>-2.3</td>
</tr>
<tr>
<td>Sulfonic</td>
<td>-5.5</td>
</tr>
<tr>
<td>Quaternary amine</td>
<td>3.5</td>
</tr>
<tr>
<td>Diaminobutane</td>
<td>6.2</td>
</tr>
</tbody>
</table>

7.3.2 Charge Ladder Characterization

Figure 7.2 (top panel) shows a typical capillary electropherogram for a 5 g/L solution of the lysozyme charge ladder in a 10 mM ionic strength tris/glycine buffer at pH 8. The first peak represents the neutral marker, while the last peak represents the unmodified lysozyme; the peaks in between correspond to lysozyme variants with decreasing number of modifications. At pH 8, all of the lysozyme variants were positively charged and migrated back against the bulk electroosmotic flow so that they
passed the detector after the neutral marker. A corresponding electropherogram for a 5 g/L solution of the α-lactalbumin charge ladder is shown in the lower panel of Figure 7.2. In this case, the data were obtained using a negatively-charged silica capillary by applying positive polarity (opposite to that for the lysozyme). Thus, the first peak represents the unmodified α-lactalbumin (least negatively charged) with the subsequent peaks corresponding to the more negatively charged α-lactalbumin variants.
Figure 7.2  Typical capillary electropherograms for the lysozyme (top panel) and $\alpha$-lactalbumin (bottom panel) charge ladders in a 10 mM ionic strength tris/glycine buffer solution at pH 8.
The net charge for each of the protein variants was evaluated from the
electrophoretic mobility, which was determined from the migration times of the neutral
marker and the specific protein variant (based on the location of the peak maximum) as
discussed in Chapter 6. Figure 7.3 shows the calculated net charge for the first 7 peaks of
the lysozyme (pH 8), α-lactalbumin (pH 8), and myoglobin (pH 7.5) charge ladders in 10
mM tris-glycine buffer solution. The solid lines simply connect the data points. Data for
lysozyme were obtained using the positively-charged eCAP™ Amine capillaries while
those for myoglobin and α-lactalbumin were obtained with the negatively-charged fused
silica capillaries to avoid protein adsorption to the oppositely-charged capillary. Peak
number 0 corresponds to the native protein, with the subsequent peak numbers
corresponding to variants with increasing number of modifications, i.e., with increasing
number of chemically-reacted amine groups. The net charge for the myoglobin charge
ladder shows the strongest dependence on the number of modifications since that charge
ladder was generated by reaction with succinic anhydride (conversion of a positive amine
into a negative carboxylic acid) while the charge ladders for lysozyme and α-lactalbumin
were generated by reaction with acetic anhydride (conversion of a positive amine to a
neutral amide).
The data for all 3 charge ladders show a much smaller change in net charge than predicted based on the simple conversion of the free amine groups to an amide or carboxylic acid. For example, the net charge on α-lactalbumin decreases by 2.5 electronic charges as one goes from the unmodified protein (Z = -3.5) to the protein with 6 reacted amine groups (Z = -6), which is much less than the expected reduction of 6 electronic charges. This behavior has been discussed in detail by Menon and Zydney (2000) and is due to the effects of charge regulation. The elimination of each positive
amine group increases the net negative charge on the protein, which in turn causes an increase in the local H⁺ concentration at the protein surface due to the electrostatic attraction between the positive H⁺ and the negative protein. This decreases the local pH at the protein surface (pH = -\log[H⁺]), leading to protonation of some of the ionizable groups on the protein, partially offsetting the effect of the lysine modification.

7.3.3 Negatively-Charged Membranes - Myoglobin Charge Ladder

The effect of solution ionic strength on the observed sieving coefficients of several “rungs” of the myoglobin charge ladder (i.e., individual peaks in the capillary electropherogram) is shown in Figure 7.4 for a sulfonic acid modified membrane with an effective charge of approximately -0.0013 C/m² (corresponding to a zeta potential of -5.5 mV). The net charge for each protein variant (shown in parentheses in the legend) was determined from the electrophoretic mobility as described previously. The observed sieving coefficients (S₀) should provide a measure of the intrinsic membrane properties since concentration polarization effects are minimal at the low filtrate flux (5 μm/s) and high stirring speed used in these experiments. Protein transmission decreases with decreasing ionic strength and with increasing negative protein charge as expected. The sieving coefficients vary by more than 3 orders of magnitude from S₀ = 0.54 for the least charged variant in the highest ionic strength solution to less than 0.001 at the lowest ionic strength; the S₀ values for variant 3 were below the detectable limit in the 50, 20 and 5 mM ionic strength solutions. The electrostatic interactions are most pronounced at low
ionic strength, but there is still a 7-fold variation in the myoglobin sieving coefficient at 100 mM ionic strength between the variants with \( Z = -2.6 \) and \( Z = -5.7 \).

**Figure 7.4** Effect of solution ionic strength on the observed sieving coefficients of several myoglobin charge variants through a negatively charged 30 kDa Ultragel™ membrane (\( \zeta_{\text{app}} = -5.5 \) mV) at pH 7.5. Data points plotted at \( S_o = 0.001 \) correspond to filtrate concentrations below the detection limit (and thus \( S_o \leq 0.001 \)). Solid curves are spline fits to the data.

### 7.3.4 Positively-Charged Membranes – Lysozyme Charge Ladder

Figure 7.5 shows typical experimental data for the observed sieving coefficients of the various elements of the lysozyme charge ladder at pH 8 in a 10 mM ionic strength solution for positively-charged versions of the 30 kDa Ultragel™ membrane made by reaction with either the quaternary amine or diaminobutane ligand. The data are plotted
as a function of the net protein charge determined from the electrophoretic mobility data. Thus, the results for each membrane represent data obtained in a single ultrafiltration experiment, with the $S_o$ values for the different species corresponding to the different peaks in the capillary electropherograms. Also shown for comparison are the sieving coefficients obtained using an unmodified 30 kDa Ultracel™ membrane. The observed sieving coefficients for the unmodified membrane were nearly independent of the protein charge with values around $S_o = 0.8$. In contrast, the sieving coefficients for the positively-charged membranes decrease sharply with increasing protein charge. This strong reduction in the sieving coefficient is a direct result of the strong electrostatic repulsion between the positively-charged membranes and the positively-charged proteins, an effect which is totally absent for the unmodified (slightly negatively-charged) membrane.

The greatest protein retention was obtained with the membrane formed by sequential reaction with 1,4 diaminobutane, which has 3 secondary amines along the length of the ligand and one terminal primary amine. This was also the membrane that had the greatest surface charge density ($\zeta_{\text{app}} = 6.2 \text{ mV}$) and the lowest permeability (and thus the smallest effective pore size), with the small pore size arising from the greater size of the ligand generated by sequential reaction with the diaminobutane.

The results for both of the positively-charged membranes show a sharp decline in the sieving coefficient above some critical protein charge, with $S_o$ decreasing from $S_o > 0.5$ to a value of less than 0.01. This critical charge was between $Z = 3.1$ and 3.7 for the
quaternary amine-modified membrane and between $Z = 1.9$ and 2.5 for the membrane modified with 1,4 diaminobutane.

**Figure 7.5** Observed sieving coefficients as a function of net protein charge for the ultrafiltration of lysozyme charge ladders in a 10 mM ionic strength solution at pH 8 through an unmodified 30 kDa Ultracel™ membrane and positively-charged versions generated using the diaminobutane and quaternary amine ligands. Measured values for the apparent zeta potential of each membrane are shown on the graph. Data points plotted at $S_o = 0.01$ correspond to filtrate concentrations below the detection limit of the CE instrument. Solid lines are shown connecting the data points.

The experimental data for the observed sieving coefficients of different lysozyme variants from Figure 7.5 have been re-plotted in Figure 7.6 as a function of the product of
the dimensionless surface charge densities of the protein and membrane as described in Chapter 6. This form was suggested in Chapter 6 based on a theoretical analysis of the effects of electrostatic interactions on the partitioning of a charged sphere in a charged cylindrical pore (Smith and Deen, 1980) as described in Chapter 2. The surface charge densities for different protein variants were evaluated from the measured protein electrophoretic mobility at the bulk pH used in these experiments (pH 8) (Figure 7.3); the dimensionless membrane surface charge density was evaluated from the apparent zeta potential data at pH 7 (Table 7.2) using Equation (4.4) as described in Chapter 6. The results for the lysozyme variants through the positively-charged membranes (modified with quaternary amine and diaminobutane) collapse to nearly a single curve when plotted in this manner. The sieving coefficient decreases with increasing $\sigma_s\sigma_p$ due to the strong repulsive electrostatic interactions under these conditions. The larger values of the sieving coefficients for the quaternary amine-modified membrane at small values of $\sigma_s\sigma_p$ is likely due to the smaller pore size of the membrane generated with the diaminobutane ligand.
Figure 7.6  Observed sieving coefficients as a function of the charge interaction parameter (product of the dimensionless surface charge densities for the protein variant and membrane) for the ultrafiltration of lysozyme charge ladders in a 10 mM ionic strength solution at pH 8.

The sharp decline in the sieving coefficients in Figure 7.6 occurs at a surprisingly large positive value of $\sigma_s \sigma_p$ – the theoretical model presented in Chapter 6 would suggest that this transition would occur at $\sigma_s \sigma_p \approx 0$. One possible explanation for this behavior is the "pH shift" that occurs between the bulk solution and the pore pH due to the presence of the positively charged ligands on the membrane pore surface. In this case, the amine ligands would repel the $\text{H}^+$ ions leading to an increase in the pH within the pore relative
to that in the bulk solution. This shift in pH would cause the deprotonation of some charge sites on the lysozyme, causing the protein to become less positively-charged than expected based on the bulk pH. This change in pore pH and its impact on the protein transmission during ultrafiltration has been discussed in more detail by Ebersold and Zydney (2004b).

The magnitude of the pH shift can be estimated as:

$$pH_{pore} = -\log_{10}[H^+_{pore}] = pH_{bulk} + \frac{1}{2.303} \left( \frac{F \zeta_{app}}{RT} \right)$$ (7.1)

assuming a Boltzmann distribution with the average electrostatic potential in the pore equal to the apparent zeta potential determined from the streaming potential measurements. This neglects the detailed variation of the electrostatic potential and $H^+$ concentration with radial position within the pore along with all of the other approximations inherent in the use of the Helmholtz-Smoluchowski equation to evaluate the apparent zeta potential. The calculated pH in the pore for the membrane modified with the quaternary amine ligand is 8.06, which is only 0.06 pH units larger than that in the bulk. This small pH shift would cause the net charge on variant 3 to decrease from 3.86 to 3.82, where the protein charge was calculated directly from the amino acid composition accounting for the removal of 3 positively-charged amine groups as discussed in Chapter 2. This small change in protein charge would be unable to explain the sharp reduction in the observed sieving coefficients observed between Variant 3 and 2 in Figure 7.6.
In order for the sharp reduction in sieving coefficient to occur at the point where the protein charge changes sign, the pH in the pore would have to shift from 8 to 10.5 for the membrane modified with the quaternary amine ligand and to 9.8 for the membrane modified with the diaminobutane. However, there is no available evidence for such a large pH shift, suggesting that there may be some other physical explanation for the behavior seen in Figure 7.6. Additional experimental data, covering a wider range of membrane charge and with better resolution to allow quantification of the small sieving coefficients for the more highly charged species, would be needed to explore this behavior more quantitatively.

The effect of membrane charge on the performance of the positively-charged membranes can be seen more clearly in Figure 7.7, which shows the selectivity between variant 6 and variant 3, defined as the ratio of the $S_o$ values for these two lysozyme variants (van Reis and Saksena, 1997), as a function of the apparent zeta potential of the membrane. The selectivity of the unmodified membrane is equal to one; the sieving coefficients of the two lysozyme peaks are identical for the unmodified Ultracel™ membrane due to the absence of any significant electrostatic interactions. The selectivity increases with increasing membrane zeta potential, attaining a value of nearly 50-fold for the membrane modified with the 1,4-diaminobutane even though the two lysozyme variants differ by only 3 charge groups (corresponding to a difference in net protein charge of approximately 2 electron charges). This increase in selectivity is a direct result of the greater electrostatic repulsion of the more positively-charged lysozyme variant by the more positively-charged membrane.
Figure 7.7  Selectivity between lysozyme variants 6 and 3 as a function of the apparent zeta potential of the membrane.

7.3.5  Zwitterionic Membranes

Experimental data for the observed sieving coefficients of α-lactalbumin charge ladders through 30 kDa Ultracel™ membranes modified with the zwitterionic ligands glycine and taurine are shown in Figure 7.8 along with corresponding data for an unmodified membrane. The data were obtained during ultrafiltration of 5 g/L solutions of the α-lactalbumin charge ladder at pH 8 (top panel) and pH 7 (bottom panel) at a constant flux of 5 µm/s using a stirring speed of 600 rpm. The greatest protein
transmission is seen with the unmodified membrane, with the observed sieving coefficients remaining nearly constant independent of the protein charge. In contrast, the α-lactalbumin variants were almost completely retained by the taurine-modified membrane at pH 8 ($S_o < 0.01$) due to the strong electrostatic repulsion between the negatively charged membrane and the negatively charged protein under these conditions. The results with the glycine-modified membrane are somewhat different. The weakly charged species show high transmission, but the sieving coefficient then declines sharply for the more negatively-charged species due to the increase in electrostatic repulsion. Similar behavior was observed at pH 7 (bottom panel), but with lower retention of the weakly charged variants through the membranes modified with the zwitterionic ligands. This effect is most pronounced for the taurine-modified membrane, with the sieving coefficient equal to around 0.3 for the more weakly charged species at pH 7 compared to $S_o < 0.01$ at pH 8.
Figure 7.8  Observed sieving coefficients as a function of protein charge for the ultrafiltration of $\alpha$-lactalbumin charge ladders through an unmodified, a taurine-modified, and a glycine-modified membrane. Data obtained using a 10 mM ionic strength solution at pH 8 (top panel) and pH 7 (bottom panel). Measured values for the membrane apparent zeta potential are shown for each membrane. Results plotted at $S_o = 0.01$ correspond to filtrate concentrations below the detection limit of the CE instrument (thus $S_o \leq 0.01$). Solid lines simply connect the data points.
In order to obtain additional insights into the sharp decline in the sieving coefficients seen in Figure 7.8, the shift in pH within the membrane pores due to partitioning of the H\(^+\) ions was analyzed as discussed in section 7.3.4. In this case, the zwitterionic ligands have a net negative charge which would attract the H\(^+\) ions and cause a decrease in the pH within the pore relative to that in the bulk solution. This shift in pH would cause the \(\alpha\)-lactalbumin protein to become less negatively-charged than predicted based on the bulk pH. Protein charge calculations from the amino acid composition data shows that a shift in the pore pH from 7 to 4.4 for the taurine-modified membrane and to 4.3 for the glycine-modified membrane would cause the protein charge to change sign, which would be expected to cause a sharp decline in the sieving coefficient. However, the magnitude of the pH shift required to cause a change in the sign of the protein charge is much larger than that predicted by Equation (7.1), similar to the results seen in the last section for the positively-charged amine membranes. Additional data would again be required to develop a more fundamental understanding of this behavior.

The effect of membrane charge on the performance of the zwitterionic membranes can be seen more clearly in Figure 7.9, which shows the selectivity between variant 0 (the native protein) and variant 3 of the \(\alpha\)-lactalbumin charge ladder as an explicit function of the apparent zeta potential of the zwitterionic membranes. The selectivity increases with increasing negative charge of the membranes due to the increase in the electrostatic repulsion of the more negatively-charged variant, similar to the results seen with the lysozyme variants using the positively-charged membranes.
7.4 Conclusions

A number of previous studies have demonstrated that electrostatic interactions can be exploited for highly selective protein separations by using electrically charged ultrafiltration membranes and by appropriately adjusting the solution pH and ionic strength. However, there have been relatively few quantitative studies examining the effects of different charged ligands having different molecular structures on the
separation characteristics of these charged membranes. The experimental results obtained in this Chapter using protein charge ladders provide important insights into the nature of the electrostatic interactions for a number of different charge-modified composite regenerated cellulose membranes including the potential for using these membranes for high-resolution protein separations.

Protein transmission through the charge-modified membranes was strongly affected by membrane and protein charge as well as the solution conditions (ionic strength and pH). Protein transmission through the positively-charged, negatively-charged, and zwitterionic ultrafiltration membranes was highly correlated with the apparent zeta potential of the membrane, with the greatest protein retention obtained with the membrane that had the largest apparent zeta potential (under conditions where the protein and membrane had the same polarity). There was a sharp decline in transmission of the individual elements of the lysozyme charge ladders when the variant charge exceeded a certain value. This transition occurred at a small but clearly positive value of the product of the protein and membrane surface charge densities. This behavior might be due to a shift in the pH within the membrane pores due to partitioning of the H\(^+\) ions associated with electrostatic interactions, although the magnitude of the pH shift required to explain the experimental results was significantly greater than that predicted using a simple electrostatic model. The reason for this discrepancy is unclear.

The selectivity of the charge-modified membranes for the separation of any two protein variants increases with increasing membrane zeta potential, consistent with the greater electrostatic repulsion between the variant and the charged membrane. These
results could have important implications for the separation of protein variants in large-scale protein production, which is one of the more challenging purification problems in downstream processing. Such variants can arise during the production of recombinant proteins from a variety of post-translational modifications, including deamidation of asparagine, isomerization of aspartic acid, carboxylation of glutamic acid, and oxidation of methionine (Ebersold and Zydney, 2004a). These product variants can have different biological activity and immunologic response (Doyle and Mamula, 2001; Mamula et al., 1999); thus, the concentration of these variants needs to be reduced to acceptable levels in the final product formulation. Although ion exchange chromatography has been widely used for the separation of protein variants (Weitzhandler et al., 1998; Herve et al., 2001), these chromatographic processes can be expensive and throughput and scale-up can be challenging. The very high selectivity obtained with the charge-modified membranes in this Chapter demonstrates that these membranes could potentially be used for the separation of these protein variants, although additional studies would be required to demonstrate the commercial potential of this approach.
Chapter 8

Conclusions and Recommendations

8.1 Introduction

The production of high value recombinant proteins in the biotechnology industry requires robust, cost-effective, and high-resolution purification methods that can provide high yield and purification. Membrane processes have significant potential for meeting these needs since these processes can provide high throughput protein purification in a format that is much easier to scale-up than chromatographic separations. Although ultrafiltration was originally viewed as a purely size-based separation process, it is now well established that protein transmission is strongly affected by electrostatic interactions between the charged protein and charged membranes. Electrostatic interactions can be exploited to develop high performance ultrafiltration membranes with significantly greater protein retention (or selectivity) for a given value of the permeability than conventional UF membranes. This thesis provides a comprehensive study of the effects of electrostatic interactions on the transport and separation characteristics of a number of electrically charged ultrafiltration membranes with different charge functionalities, providing a framework for the design of high performance charge-modified membranes for bioprocessing application. The following subsections summarize the key
experimental and theoretical results from the different parts of this thesis. Recommendations for future work are also discussed.

8.2  Effect of Ligand Chemistry on the Performance of Charge-Modified Ultrafiltration Membranes

Previous studies of electrostatic interactions during protein ultrafiltration have generally assumed that the membrane surface charge density and pore size are the only membrane properties that determine the overall performance, without any consideration of the detailed molecular structure of the charged ligands. The data presented in Chapter 4 provide one of the first quantitative experimental studies of the possible effects of the detailed molecular structure of a series of charged ligands containing different numbers of primary, secondary, and quaternary amines on the performance characteristics of charge-modified ultrafiltration membranes. A series of charged composite regenerated cellulose ultrafiltration membranes was generated by covalent attachment of ligands having similar physical size but with different numbers of amine groups. Ultrafiltration experiments were performed over a range of solution ionic strength using cytochrome c as a model protein. The results clearly demonstrate that the positively-charged membranes provide significantly greater retention of the positively charged cytochrome c at low ionic strength due to the strong electrostatic exclusion under these conditions. Protein transmission was well correlated with the apparent zeta potential of the membrane, determined from streaming potential measurements obtained with flow through the membrane pores, without any additional contribution from the number of
amine groups along the length of the membrane or the presence of a quaternary versus primary amine. For example, the observed sieving coefficient in a 2 mM ionic strength solution decreased from $S_o = 0.1$ for the membrane modified with pentaethylenehexamine ($\zeta_{app} = 4.4$ mV with 6 amine groups) to a value of only $S_o = 0.02$ for the membrane modified by sequential reaction with 1,4 diaminobutane ($\zeta_{app} = 6.2$ mV with 4 amine groups). There was a corresponding increase in the electrostatic selectivity with increasing apparent zeta potential, with the more heavily charged membrane providing nearly 40-fold selectivity for the separation of cytochrome c from a neutral dextran with similar hydrodynamic radius.

The very similar results obtained with membranes having a terminal quaternary amine and a terminal primary amine suggest that the detailed chemistry of the charged ligand may have minimal effect on the membrane performance, beyond that associated with the difference in apparent zeta potential. However, the relationship between the measured zeta potential and the underlying ligand structure remains unclear; there was no obvious reason why the membrane with 4 amine groups had a greater positive zeta potential than the membrane modified with pentaethylenehexamine that had 6 amine groups. This is discussed in more detail in the Recommendations section of this Chapter.

8.3 Protein Transport through Zwitterionic Ultrafiltration Membranes

Several recent studies have demonstrated that zwitterionic ultrafiltration membranes show very low fouling behavior due to the highly hydrated structure of the zwitterions. The results presented in Chapter 5 provide the first quantitative studies on
protein transport / retention through a series of zwitterionic ultrafiltration membranes generated by covalent attachment of small zwitterionic ligands to a base cellulose membrane. Ultrafiltration experiments were performed using basic (cytochrome c), neutral (myoglobin), and acidic (α-lactalbumin) proteins over a range of solution pH.

Protein transport through the zwitterionic membranes was a strong function of solution conditions due to the electrostatic interactions between the charged proteins and the zwitterionic ligands. For example, the sieving coefficient for cytochrome c through the taurine-modified membrane in a 10 mM ionic strength solution decreased by more than an order of magnitude as the solution pH was increased from 10 to 11 due to the electrostatic repulsion between the negatively-charged membrane and the negatively-charged protein at pH above the protein isoelectric point.

The protein sieving coefficient was well correlated with the product of the surface charge densities of the protein and membrane. The sieving coefficient data for the three similarly-sized proteins collapse to a single curve when plotted as a function of the product of the dimensionless surface charge densities of the protein and membrane, consistent with available theoretical analyses of the effects of electrostatic interactions on protein transmission through semipermeable membranes. The very similar results obtained with zwitterionic membranes produced using ligands having terminal sulfonic and carboxylic acid groups suggest that the detailed chemistry of the charged ligand has minimal effect on protein transmission, beyond that associated with the difference in apparent zeta potential arising from the different degree of ionization of the zwitterions.
The apparent zeta potential of the zwitterionic membranes was very well correlated with the predicted fractional charge of the Trizma analog; this compound has the same basic structure as the zwitterionic ligand but with the primary amine replaced by a secondary amine bound to three hydroxymethyl groups. This correlation not only provides additional insights into the surface charge characteristics of these zwitterionic membranes, it also provides a simple approach for estimating the apparent zeta potential at different pH based on the $\text{pK}_a$ values of the chemical groups on the surface of the membrane. The pH-dependent charge of the zwitterionic membranes results in a strong pH dependence for the protein transmission, a phenomenon that could potentially be exploited in applications requiring pH responsive transport.

The data obtained during the protein sieving experiments showed no evidence of any fouling of the zwitterionic membranes during protein ultrafiltration, even under conditions where the protein and membrane were oppositely charged. This is consistent with previous studies demonstrating the very low fouling behavior of zwitterionic ultrafiltration membranes. Ultrafiltration experiments using bovine serum albumin (BSA) as a model protein also showed little fouling of the taurine-modified membrane, with a flux recovery of 95.4% after 3 repeat ultrafiltration cycles. However, it should be noted that this membrane also had a lower permeability than either the unmodified or sulfonic acid modified membranes, and this could have contributed to the smaller degree of flux decline.

The very low fouling characteristics of the zwitterionic membranes, coupled with the high degree of protein retention at pH > pI arising from the strong electrostatic
repulsion between the like-charged protein and membrane, demonstrate the potential of using zwitterionic ligands to generate high performance ultrafiltration membranes for bioprocessing applications.

8.4 Effects of Protein Surface Charge Distribution on Electrostatic Interactions during Protein Ultrafiltration

Most studies of protein ultrafiltration have implicitly assumed that the protein is a hard sphere with uniform surface charge, consistent with the theoretical development for the free energy of interaction of a charged sphere in a charged cylindrical pore as discussed in Section 2.3.2. However, this completely neglects the possible effects that a surface charge distribution might have on the magnitude of the electrostatic interactions, a phenomenon that is well known to have a significant effect on the extent of protein binding to charged resins in ion exchange chromatography. The results presented in Chapter 6 provide the first experimental investigation into the possible effects of a protein surface charge distribution on the magnitude of the electrostatic interactions that occur during protein ultrafiltration. Ultrafiltration and ion exchange chromatography data were obtained over a range of pH using cytochrome c and lysozyme as model proteins that have similar size and net charge but different amino acid composition and surface charge distribution. The sieving data for both proteins collapse to a single curve when plotted as a function of the net protein charge (evaluated from the electrophoretic mobility), with a maximum in the sieving coefficient obtained at $z \approx 0$ for the positively-charged membrane. In contrast, the retention factor for lysozyme and cytochrome c
obtained during cation exchange chromatography showed very distinct differences, particularly at pH values around the isoelectric point.

Additional insights into the possible effects of the protein surface charge distribution were obtained using a "peracetylated" version of cytochrome c formed by converting all lysine amino groups into the corresponding amide; this provided an "identical" protein but with different surface charge distribution. The sieving data for the native and “peracetylated” version of cytochrome c collapsed to essentially a single curve when plotted as a function of the net protein charge suggesting that the detailed charge distribution has a relatively small effect on protein transport through these ultrafiltration membranes.

The experimental results for the protein sieving coefficient were analyzed in terms of the normalized sieving coefficient, defined as the ratio of the protein sieving coefficient at a given pH to that at the protein isoelectric point. The data for lysozyme and cytochrome c both collapse to a straight line when plotted as a function of the product of the protein and membrane surface charge densities, consistent with predictions of an electrostatic partitioning model developed for a uniformly charged sphere in a charged cylindrical pore. In addition, the calculated values of the pore radii determined from the slope of the data were consistent with independent estimates of the pore radius evaluated from the membrane hydraulic permeability and the sieving coefficient of the uncharged protein, providing further evidence that protein transmission in ultrafiltration is determined almost entirely by the average surface charge density of the protein, irrespective of the detailed distribution of charge groups over the protein surface.
8.5 Separation Characteristics of Charge - Modified Ultrafiltration Membranes

A number of previous studies have demonstrated that electrostatic interactions can be exploited for highly selective protein separations by appropriately adjusting the solution pH and ionic strength and by using appropriately charged ultrafiltration membranes. However, there have been relatively few quantitative studies examining the effects of different charged ligands having different molecular structures on the separation characteristics of these charged membranes. The experimental results obtained in Chapter 7 using protein charge ladders provide important insights into the nature of the electrostatic interactions for a number of different charge-modified composite regenerated cellulose membranes including the potential for using these membranes for high-resolution protein separations. Filtration experiments were performed using protein charge ladders, consisting of a set of covalently modified derivatives of a single protein that have similar size but different net charge. Ultrafiltration data were analyzed in terms of the net charge of the individual rungs in the protein charge ladder as determined from the measured electrophoretic mobility.

Protein transmission through the charge-modified membranes was strongly affected by membrane and protein charge as well as the solution conditions (ionic strength and pH). Protein transmission through the positively-charged, negatively-charged, and zwitterionic ultrafiltration membranes was highly correlated with the apparent zeta potential of the membrane, with the greatest protein retention obtained with the membrane that had the largest apparent zeta potential (under conditions where the protein and membrane had the same polarity). There was a sharp decline in transmission
of the individual elements of the lysozyme charge ladders when the variant charge exceeded a certain value. This transition occurred at a small but clearly positive value of the product of the protein and membrane surface charge densities. This behavior might be due to a shift in the pH within the membrane pores due to partitioning of the H+ ions associated with electrostatic interactions, although the magnitude of the pH shift required to explain the experimental results was significantly greater than that predicted using a simple electrostatic model.

The selectivity of the charge-modified membranes for the separation of any two protein variants increased with increasing membrane zeta potential, consistent with the greater electrostatic repulsion between the variant and the charged membrane. These results could have important implications for the separation of protein variants in large-scale protein production, which is one of the more challenging purification problems in downstream processing. The very high selectivity obtained with the charge-modified membranes in this Chapter demonstrates that these membranes could potentially be used for the separation of these protein variants.

8.6 Recommendations

The results presented in this thesis provide significant fundamental insights into the effects of electrostatic interactions on protein transport during ultrafiltration. This information can be utilized in developing the next generation high performance charged ultrafiltration membranes / processes. However, there are a number of important areas which could benefit from additional experimental and theoretical investigations.
The experimental studies described in this thesis focused on the effects of electrostatic interactions on protein partitioning, with data obtained in a small stirred cell at relatively low filtration velocities to minimize the concentration polarization effects. However, commercial devices are typically operated using tangential flow filtration modules and at significantly higher filtrate flux where concentration polarization effects are much more significant, influencing both protein retention and filtrate flux. Future experimental studies should be performed using linearly scalable tangential flow filtration modules so that the results can be more directly extrapolated to predict the behavior of large-scale commercial membrane systems. Electrostatic interactions can also affect the bulk mass transfer of proteins due to the intermolecular protein – protein interactions, which increase the protein diffusivity, as well as direct protein-membrane interactions, which can change the rate of protein transport away from the membrane and back into the bulk solution. Thus, additional experimental investigations of these bulk mass transfer effects would be required for the design of effective protein separation processes suitable for industrial applications.

Most of the experimental studies conducted in this thesis were focused on the behavior of ultrafiltration membranes for protein concentration and buffer exchange using model proteins. It would be very desirable to extend this work to examine multi-component protein mixtures used in biotechnology industry, e.g., monoclonal antibodies, due to the growing clinical and commercial market of this type of proteins. These studies would provide a more complete description of the factors governing protein separations using charged ultrafiltration membranes.
The results presented in Chapter 4 clearly demonstrate the potential of developing a range of novel ultrafiltration membranes using different ligands having different numbers of charged groups. Protein transmission was well correlated with the apparent zeta potential of different membranes. However, the underlying factors causing the differences in apparent zeta potential for the membranes made with the different charged ligands is unclear. The zeta potential for the membrane with 4 amine groups (formed by sequential reaction with 1,4-diaminobutane) was greater than that for the membrane with only two amine groups (formed by reaction with 1,10-diaminodecane) as expected. However, the apparent zeta potential for the membrane with three secondary amines and a terminal quaternary amine was only 3.4 mV, even though this membrane actually had a higher degree of modification than the membrane with 4 amine groups (formed by sequential reaction with 1,4-diaminobutane) based on the XPS spectra. One possibility is that the quaternary amine group is so strongly associated with a negative counterion that the membrane behaves as if it is more weakly charged, both in terms of its apparent zeta potential and protein retention. In addition, the apparent zeta potential only provides a very crude measure of the membrane surface charge. More quantitative analysis accounting for the detailed effects of the ligand molecular structure on fluid flow, ion distributions, and electrostatic interactions would be needed to fully understand the relationship between the ligand structure, apparent zeta potential, and ultimate membrane performance. It might also be possible to obtain additional insights into the effects of ligand chemistry on the zeta potential using molecular dynamics / structure simulations. Theoretical calculations could be performed to evaluate the electrostatic potential around
each ligand, and to visualize contour maps (equipotential surfaces) at different distances from the charged ligand.

The very similar results obtained with membranes having a terminal quaternary amine and a terminal primary amine in Chapter 4 suggest that the detailed chemistry of the charged ligand may have minimal effect on the membrane performance, beyond that associated with the difference in apparent zeta potential. However, this behavior might be very different at higher pH where the primary amine would become de-protonated (and thus uncharged) while the quaternary amine would be expected to retain its charge due to the very high pK$_a$ value. Further studies would be needed to determine whether the apparent membrane zeta potential remains the critical parameter describing the surface charge, and the strength of the electrostatic interactions during ultrafiltration, under these conditions.

The experimental data presented in Chapter 5 demonstrate the importance of electrostatic interactions on the rate of protein transport through the membranes modified with the taurine, homotaurine, and glycine with the secondary amine group from the ligand attached to the membrane surface. It would be interesting to extend these studies by performing experiments using membranes modified with the zwitterionic ligands oriented the other way, i.e., with the amine group facing toward the solution and the sulfonic acid (e.g., in the ligand taurine) or the carboxylic acid (e.g., in glycine) attached to the membrane surface. It might well be possible to estimate the effective charge, and thus correlate the sieving coefficients, using the pK$_a$ values of the appropriate ligand analogs to account for the actual linkage chemistry.
Additional experiments could also be performed using “mosaic” membranes, generated by covalent attachment of different negatively-charged and positively-charged ligands to the cellulose membrane. This would make it possible to determine the importance of the charge distribution over the membrane surface on the magnitude of the electrostatic interactions during protein ultrafiltration. For example, two membranes with similar apparent zeta potential may have different behavior depending upon the distribution of the number of negatively-charged and positively-charged groups. It might also be possible to fine tune the extent of modification in order to generate a zwitterionic membrane (with zero surface charge density), and to compare the sieving behavior of these novel membranes with those modified with ligands having both functionalities on a single ligand as discussed in Chapter 5.

Limited experimental data were obtained in Chapter 5 on fouling characteristics of the charge-modified membranes using BSA as a model protein. The zwitterionic membranes showed minimal fouling over a wide range of filtration conditions, including conditions where the protein and membrane had opposite net charge. It would be interesting to extend these experiments to more heavily fouling solutions, including protein mixtures, ionic surfactants, and DNA solutions. These experimental studies should be conducted with membranes having different surface charge, functionality groups, and pore-size distribution characteristics to examine the fouling behavior in more detail.

The experimental results obtained in Chapter 7 using protein charge ladders demonstrated the potential of using charge-modified membranes for high-resolution
protein separations, although additional studies would be required to demonstrate the commercial potential of these membranes in specific applications. Protein transmission through the charge-modified membranes was strongly affected by the membrane and protein charge as well as the solution conditions (both ionic strength and pH). There was a sharp decline in transmission of the individual elements of the lysozyme charge ladders through the positively-charged membrane when the variant charge exceeded a certain value. This transition occurred at a small but clearly positive value of the product of the protein and membrane surface charge densities. This behavior might be due to a shift in the pH within the membrane pores due to partitioning of the H$^+$ ions associated with electrostatic interactions, although the magnitude of the pH shift required to explain the experimental results was significantly greater than that predicted using a simple electrostatic model. Additional experimental data, covering a wider range of membrane charge and with better resolution to allow quantification of the small sieving coefficients for the more highly charged species, would be needed to explore this behavior more quantitatively.

The results presented in this thesis clearly demonstrate that electrically charged membranes can have much better performance characteristics (higher permeability and higher selectivity) than commercially available (essentially uncharged) membranes due to the strong electrostatic exclusion of the like charged protein. However, these electrically charged membranes require the use of relatively low ionic strength solutions (typically less than 20 mM ionic strength) to obtain high resolution; electrostatic shielding at higher ionic strengths largely eliminates the repulsion needed to obtain high protein retention. It
would be interesting to examine the performance characteristics of a series of membranes modified with some of the “salt tolerant” ligands that have recently been developed for ion exchange chromatography (e.g., agmatine and Tris (2-aminoethyl) amine (TAEA)). For example, Riordan et al. (2009) demonstrated that these ligands could provide a much higher virus log reduction value (LRV) at high salt concentrations (up to 150 mM) compared to more conventional quaternary amine ligands in anion exchange membrane adsorbers. There is currently no data on the possible use of these ligands in developing high performance charged membranes.
REFERENCES


Roy R.N., Robinson R.A., Bates R.G., Thermodynamics of the two dissociation steps of N-tris (hydroxymethyl) methylglycine (Tricine) in water from 5 to 50 degrees, Journal of the American Chemical Society 95 (1973) 8231.


van Reis R., Charged filtration membranes and uses therefor. 2006: U.S. patent 7,001,550


Appendix A

Amino Acid Composition of Proteins

As discussed in Chapter 2, protein surface charge density is determined by the dissociation of the various ionizable amino acid residues on the surface of the protein. The number and pK$_a$ values of the various amino acids present in the proteins used in this thesis are presented in this section.

Table A.1  Number ($n_i$) and pK$_{int}^i$ values of charged amino acids in cytochrome c (Mehta and Zydney, 2006)

<table>
<thead>
<tr>
<th>Type</th>
<th>Number ($n_i$)</th>
<th>pK$_{int}^i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-Amino</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>His</td>
<td>3</td>
<td>6.2</td>
</tr>
<tr>
<td>Arg</td>
<td>2</td>
<td>12.5</td>
</tr>
<tr>
<td>Lys</td>
<td>19</td>
<td>10.3</td>
</tr>
<tr>
<td>Glu</td>
<td>9</td>
<td>4.5</td>
</tr>
<tr>
<td>Asp</td>
<td>3</td>
<td>3.5</td>
</tr>
<tr>
<td>$\alpha$-carboxyl</td>
<td>1</td>
<td>2.19</td>
</tr>
<tr>
<td>Heme COOH</td>
<td>2</td>
<td>4.3</td>
</tr>
<tr>
<td>Tyr</td>
<td>4</td>
<td>10.3</td>
</tr>
</tbody>
</table>
**Table A.2** Number ($n_i$) and $pK_{int}^i$ values of charged amino acids in lysozyme (Sharma et al., 2003)

<table>
<thead>
<tr>
<th>Type</th>
<th>Number ($n_i$)</th>
<th>$pK_{int}^i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-Amino</td>
<td>1</td>
<td>7.5</td>
</tr>
<tr>
<td>His</td>
<td>1</td>
<td>6.3</td>
</tr>
<tr>
<td>Arg</td>
<td>11</td>
<td>12.5</td>
</tr>
<tr>
<td>Lys</td>
<td>6</td>
<td>10.5</td>
</tr>
<tr>
<td>Glu</td>
<td>2</td>
<td>4.4</td>
</tr>
<tr>
<td>Asp</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>$\alpha$-carboxyl</td>
<td>1</td>
<td>3.8</td>
</tr>
<tr>
<td>Tyr</td>
<td>3</td>
<td>9.6</td>
</tr>
</tbody>
</table>

**Table A.3** Number ($n_i$) and $pK_{int}^i$ values of charged amino acids in myoglobin (Ebersold and Zydny, 2004b)

<table>
<thead>
<tr>
<th>Type</th>
<th>Number ($n_i$)</th>
<th>$pK_{int}^i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-term Gly</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>His</td>
<td>7</td>
<td>6.6</td>
</tr>
<tr>
<td>Arg</td>
<td>2</td>
<td>12.5</td>
</tr>
<tr>
<td>Lys</td>
<td>19</td>
<td>10.3</td>
</tr>
<tr>
<td>Glu</td>
<td>13</td>
<td>4.3</td>
</tr>
<tr>
<td>Asp</td>
<td>8</td>
<td>4.3</td>
</tr>
<tr>
<td>C-term Gly</td>
<td>1</td>
<td>4.3</td>
</tr>
<tr>
<td>Heme COOH</td>
<td>2</td>
<td>4.3</td>
</tr>
<tr>
<td>Tyr</td>
<td>2</td>
<td>10.3</td>
</tr>
</tbody>
</table>
### Table A.4
Number ($n_i$) and $pK_{\text{int}}^i$ values of charged amino acids in α-lactalbumin (Molek, 2008)

<table>
<thead>
<tr>
<th>Type</th>
<th>Number ($n_i$)</th>
<th>$pK_{\text{int}}^i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-term</td>
<td>1</td>
<td>9.87</td>
</tr>
<tr>
<td>His</td>
<td>3</td>
<td>6.04</td>
</tr>
<tr>
<td>Arg</td>
<td>0</td>
<td>12.5</td>
</tr>
<tr>
<td>Lys</td>
<td>12</td>
<td>10.54</td>
</tr>
<tr>
<td>Glu</td>
<td>8</td>
<td>3.9</td>
</tr>
<tr>
<td>Asp</td>
<td>9</td>
<td>3.9</td>
</tr>
<tr>
<td>C-term</td>
<td>1</td>
<td>2.16</td>
</tr>
<tr>
<td>Tyr</td>
<td>4</td>
<td>10.3</td>
</tr>
</tbody>
</table>

It is important to note that the small differences in $pK_{\text{int}}^i$ values in Tables A.1 through A.4 reflect the range of $pK_a$ for the various amino acids based on the local environment within the protein. We used the values previously determined for each of the proteins since those numbers have been shown to properly describe the net charge data for that particular molecule. No attempt was made to evaluate a “consensus” set of $pK_{\text{int}}^i$ values that could be used for all four proteins.
Appendix B

Calculation of the Membrane Surface Charge Density

The surface charge density within the membrane pores was calculated from the dye binding assay and XPS results using an estimate of the internal membrane pore area:

\[
A_p = N_p \frac{2\pi r_p \delta_p}{r_p} = \frac{2eA \delta_p}{r_p}
\]

where the second expression is developed using the definition of the membrane porosity \(\varepsilon\). The average pore radius was estimated as 6.9 nm from the measured hydraulic permeability using Equation (4.2), with the pore length set equal to the thickness of the membrane skin layer \(\delta_p = 1 \mu m\).

The surface charge density based on the XPS data was calculated from the product of the ligand density (determined from the N signal in the XPS) and the net charge of the ligand as

\[
q_p = A \delta_p (1 - \varepsilon) \rho f N_{ligand} F
\]

where \(N_{ligand}\) is the total number of charge groups (amines) per ligand, \(MW_{glucose}\) is the molecular weight of each glucose monomer in cellulose, \(\rho = 1.5 \text{ g/cm}^3\) is the density of cellulose, and \(F\) is Faraday's constant. The parameter \(f\) is the fraction of glucose rings with an attached ligand as calculated via Equation (4.3).
A similar approach was used to calculate the charge density from the dye-binding results as:

\[ q_p = Q_m \left( \frac{V_m}{A_p} \right) \left( \frac{A_p}{A_p + A_m} \right) \]  

(B.3)

where the volumetric charge density was evaluated from Equation (4.6) and the total volume of the cellulosic part of the membrane disk is \( V_m = 1.64 \times 10^{-8} \text{ m}^3 \) and \( A_p \) and \( A_m \) are the internal surface area in the membrane skin and substructure, respectively:

\[ \frac{A_p}{A_m} = \left( \frac{\delta_p}{r_p} \right) \left( \frac{r_m}{\delta_m} \right) \]  

(B.4)

where \( r_m = 0.5 \mu \text{m} \) and \( \delta_m = 40 \mu \text{m} \) are the effective pore size and thickness of the cellulosic substructure beneath the skin. Note that Equation (B.4) assumes that the porosity is uniform throughout the cellulosic layer. The correction factor given by the area ratio in Equation (B.3) was about 0.63; this factor accounts for the fraction of the total internal surface area located within the membrane skin assuming that dye-binding occurs uniformly throughout the entire cellulose region of the membrane (including both the membrane skin and the cellulosic substructure that lies between the skin and the polyethylene support on which the membrane is cast).

The large difference in surface charge density calculated from the dye-binding and streaming potential data (Table 4.2) is surprising. One possible explanation is that dye-binding occurs preferentially in the cellulosic substructure, possibly due to steric or charge-charge interactions in the very small pores of the skin. However, in order to account for the very large difference in calculated surface charge densities, one would
need to assume that more than 99% of the dye is bound within the membrane substructure (instead of the skin), which seems highly unlikely given that these regions are both composed of the same base polymer (cellulose) and they have very similar pore surface areas.
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