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Achiral, Chiral, and Protein Separations with Molecular Micelles Using Chromatographic Techniques

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ACHIRAL, CHIRAL, AND PROTEIN SEPARATIONS WITH MOLECULAR MICELLES USING CHROMATOGRAPHIC TECHNIQUES

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LIST OF ABBREVIATIONS

Abbreviation	Name
5-IAF	5-Iodoacetamidofluorescein
BENZ	benzoin
BGE	background electrolyte
BME	benzoin methyl ether
BNA	1,1´-binaphthyl-2,2´-diamine
BNP	1,1´-bi-2-naphthyl-2,2´-dihydrogen phosphate
BOH	1,1´-bi-2-naphthol
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CIEF	capillary isoelectric focusing
CITP	capillary isotachophoresis
CZE	capillary zone electrophoresis
CMC	critical micelle concentration
COUM	coumachlor
CPL	4-chlorophenol
CZE	capillary zone electrophoresis
DCC	dicyclohexylcarbodiimide
EOF	electroosmotic flow
GEMBE	gradient elution moving boundary electrophoresis
GC	gas chromatography
HCl	hydrochloric acid

HPLC	high performance liquid chromatography
MBE	moving boundary electrophoresis
MEKC	micellar electrokinetic chromatography
LZP	lorazepam
MS	mass spectrometry
NaOH	sodium hydroxide
NHS	*N*-hydroxysuccinimide
OT-CEC	open-tubular capillary electrochromatography
OXP	oxazepam
PC-CEC	packed column capillary electrochromatography
PDADMAC	poly (diallyldimethylammonium chloride)
PEM	polyelectrolyte multilayer coatings
pI	isoelectric point
poly-L-glutamic acid-lysine	poly (L-glutamic acid-lysine hydrobromide)
poly-L-lysine	poly (L-lysine hydrobromide)
poly-L-lysine-serine	poly (L-lysine-serine hydrobromide)
poly-L-ornithine	poly (L-ornithine hydrobromide)
Poly-L-SULA	poly (sodium *N*-undecanoyl-L-leucylalanate)
Poly-L-SULV	poly (sodium *N*-undecanoyl-L-leucylvalinate)
Poly-SUS	poly (sodium *N*- undecylenic sulfate)
PSS	poly (styrene sulfonate)
RSD	relative standard deviation
SDS	sodium dodecyl sulfate
SFC	supercritical chromatography

SiO^-	deprotonated silanol groups
THF	tetrahydrofuran
TLC	thin layer chromatography
TRIS	tris(hydroxymethyl)aminomethane
TZP	temazepam

ABSTRACT

In this dissertation, several chromatographic techniques were developed for separation of proteins as well as achiral and chiral compounds. Firstly, polyelectrolyte multilayer (PEM) coatings were used to separate chiral analytes and proteins of pharmaceutical and biomedical interests. Coating polymers used in PEM coatings are instrumental in analyte separation. Therefore, the effect of changing four different cationic polymers, (poly-L-lysine, poly-L-ornithine, poly-L-lysine-serine, and poly-L-glutamic acid-lysine) and three anionic polymers (sodium poly (*N*-undecanoyl-L-leucyl-alaninate) (poly-L-SULA), sodium poly (*N*-undecanoyl-L-leucyl-valinate) (poly-L-SULV) and sodium poly (undecylenic sulfate) (poly-SUS)) were investigated. The simultaneous effects of cationic polymer concentration, number of bilayers, temperature, applied voltage, and pH of the background electrolyte on the separation on these analytes were analyzed using a Box Behnken experimental design. In addition, the influence of NaCl on the column reproducibility was investigated. Secondly, mixed mode separation using a combination of micellar electrokinetic chromatography (MEKC) and PEM coatings was used for the separation of achiral and chiral analytes. In this study, it was observed that achiral and separations using MEKC and PEM coatings individually resulted in partial resolution of 8 very similar aryl ketones and 5 chiral compounds when the molecular micelles (sodium poly (*N*-undecanoyl-L-glycinate) (poly-SUG)) and poly-L-SULV were used. However, when mixed mode separation was introduced, baseline resolution was achieved for all analytes. In the last study of this dissertation, PEM coatings were constructed using molecular micelles in open tubular capillary electrochromatography (OT-CEC) and gradient elution moving boundary electrophoresis (GEMBE) for protein separations. In OT-CEC, proteins were detected using both ultra violet (UV) and laser induced fluorescence (LIF) detection, while only LIF detection was used with the GEMBE technique. The effects of bilayer number, type of molecular micelle

as well as pH of the background electrolyte on the separation of 6 acidic proteins were analyzed using ultra violet (UV) detection in OT-CEC**.** In addition, internal diameter and the effective length of the capillary were studied to investigate their influence on protein separations with LIF detection. High resolution protein separations were achieved using PEM coatings, therefore, these conditions were also used for protein separations with the GEMBE technique.

CHAPTER 1

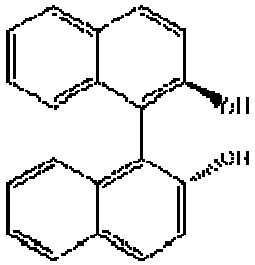
INTRODUCTION

1.1 Chirality

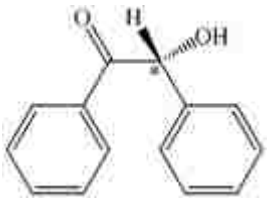
A chiral molecule has a non-superimposable mirror image and all forms are known as enantiomers or optical isomers. While chiral molecules, also known as optically active molecules, have the ability to rotate the plane of polarized light in equal but opposite directions, the molecules will have identical physical properties in an achiral environment [1]. Optically active molecules that rotate plane polarized light to the left are called levorotatory (L) (-) and those that rotate plane polarized light to the right are termed dextrorotatory (D) (+) [2, 3]. Scientist, Louis Pasteur, was the first to demonstrate chirality when he separated chiral crystals of sodium ammonium tartrate using a hand lens and a pair of tweezers [4, 5]. Pasteur reported that each crystal presented levorotatory or dextrorotatory behavior when placed under polarized light [5].

A chiral molecule is any molecule that contains either a tetrahedral carbon atom, with four different groups at each bond, or a plane/axis of asymmetry, as illustrated in Figure 1.1 [7]. The stereochemical configuration of an asymmetric carbon is denoted by either R (right or rectus) or S (left or sinister). When the priority of each bonded group is in a clockwise direction, it is called R configuration. Conversely, if the priority is in a counterclockwise direction, it is denoted as S [2, 3, 6]. When there are equal amounts of each enantiomer in a mixture, they do not rotate plane polarized light. Such mixed are called racemic mixtures and are described as optically inactive and are denoted by (±). This phenomenon occurs as a result of continuous rotation of molecules in each direction [8]. As one molecule rotates in the clockwise direction, another rotates in the counterclockwise direction. Chiral enantiomers have the same physical properties, such as melting and boiling points. However, enantiomers of the same drug can have

different effects in the human body since the body can metabolize these isomers through different pathways. One enantiomer of a drug may have therapeutic effects, however, the other enantiomer may have detrimental or undesired effects.



BNP
(asymmetric plane)



Benzoin
(asymmetric atom)

Figure 1.1 Examples of two different types of chiral molecules

One widely known example that illustrates the importance of the separation of chiral drugs is the thalidomide tragedy [7]. In 1956, thalidomide was given to pregnant women in the racemic form to help lessen symptoms associated with morning sickness. However, it was later discovered that only the (R)-(+)-enantiomer was a sedative while, the (S)-(-) enantiomer caused severe birth defects in thousands of babies [3, 16]. There are many other drugs that exhibit different pharmacokinetic behavior, and as a result, the United States Food and Drug Administration has recommended that the pharmacological and toxicological activity of each isomer be investigated and documented. Therefore, interest in methods to separate chiral analytes has grown to address these problems [1, 3, 9, 10].

In order to achieve chiral separation, enantiomers must be placed in a chiral environment. This is generally achieved through the use of a chiral selector or a chiral discriminating agent capable of differentially interacting with the individual enantiomers. There are two separation methods in which chiral separation can be achieved: indirect and direct. In the indirect separation method, the chiral selector interacts with the enantiomers by forming a covalent bond, whereas in direct separations a complex is formed between the isomers and the chiral selector

and no covalent bond is formed. Each case results in the formation of two non-superimposable isomeric compounds [2]. For the studies presented in this dissertation, the direct separation method is employed.

Although numerous reports have described the separation of enantiomers using a variety of chiral selectors, the mechanism involved in chiral discrimination has yet to be fully understood. Separation scientists view the "three point rule" as the basis of chiral discrimination [11, 12]. The "three point rule" describes three simultaneous interactions between one enantiomer and the chiral selector and at most, two interactions between the other enantiomer and the chiral selector. These interactions may be hydrophobic interactions between the polymer core and the chiral analyte, electrostatic interactions, or hydrogen bonding between the analyte and head group of the polymer. In addition, several other interactions may occur due to ion-dipole bonds, Van der Waals forces and π-π interactions. The differences in these interactions results in the resolution of two isomers. Each enantiomer interacts with the chiral selector in a unique way because of spatial restraints. The mechanism supporting the three point rule is illustrated in Figure 1.2.

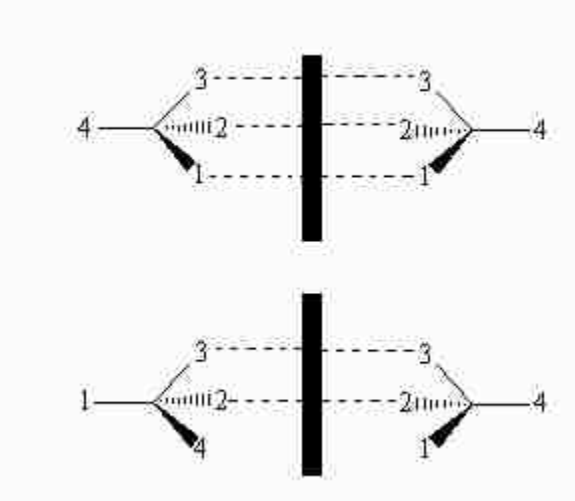
1.2. Chiral Selectors

Several chiral selectors have been used in capillary electrophoresis (CE) separations, including bile acids [13], crown ethers [14, 15], polysaccharides [16], proteins [17, 18], cyclodextrins [19, 20], and molecular micelles [21, 22, 23]. Though each chiral selector previously mentioned has many advantages, this dissertation reports the use of molecular micelles for each chromatographic technique used.

1.2.1 Surfactants and Micelles

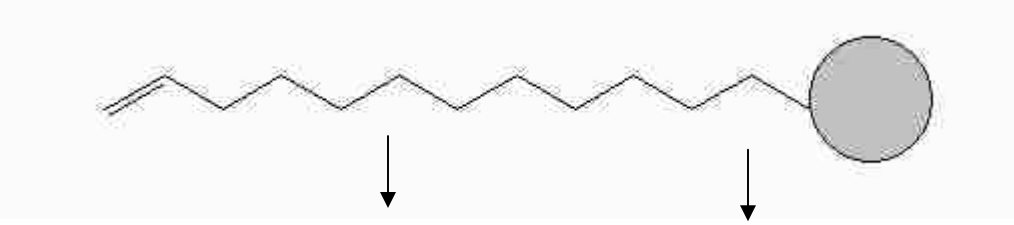
Surfactants, also referred to as amphiphiles, consist of a hydrophilic polar head group and a long hydrophobic hydrocarbon tail (Figure 1.3). If the head group of the surfactant is

positively charged, it is said to be cationic, conversely, an anionic surfactant contains a negatively charged head group. When the surfactant molecule has both positive and negative charges, it is termed zwitterionic, while, surfactants with no charge are called nonionic [24, 25].



Enantiomers **Chiral Selector**

Figure 1.2 Three-point interaction rule

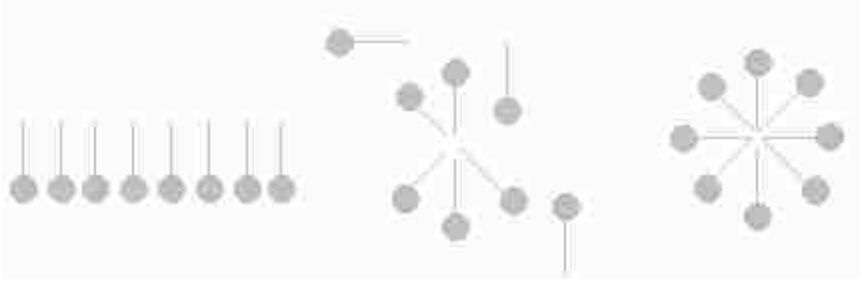


Hydrophobic Tail Group Hydrophilic Head Group

Figure 1.3 Structure of a surfactant molecule

When low concentrations of surfactant molecules are placed in an aqueous environment, hydrophobic tail groups position themselves inwards while the hydrophilic head groups are positioned outwards to interact with the aqueous surroundings (Figure 1.4A). At higher concentrations, the surfactant molecules begin to aggregate (Figure 1.4B). As the surfactant concentration increases to a concentration known as the critical micelle concentration (CMC), these molecules form organized assemblies known as micelles (Figure 1.4C). The hydrophobic

tail is turned to the inside of the micelle, therefore, having limited solvent accessibility and the hydrophilic head groups create the outer surface of the micelle.



(A) (B) (C)

Figure 1.4 Illustration of different stages of surfactant molecules in an aqueous environment. (A) Low concentration of surfactant molecules; (B) Higher concentration of surfactant molecules; (C) CMC

The CMC of each surfactant is different and can be determined using several methods however, the most commonly used is surface tension measurements. This technique, first introduced by Du-Nouy in 1919, involves increasing the surfactant monomer concentration until there is no change in the surface tension of the solution [26]. At the CMC, the number of surfactant molecules that are aggregated (aggregation number, n) are commonly between 50 and 100 [26].

1.2.2 Molecular Micelles

When surfactants are polymerized at concentrations at or above the CMC, the aggregates are termed molecular micelles. The resulting polymer, thought to resemble a conventional micelle, is preferred over the unpolymerized micelles for several reasons. Molecular micelles have no CMC and, thus, can be used at very low concentrations. Furthermore, the covalent bonds formed during the polymerization process eliminate the dynamic equilibrium between monomer and micelle. As a result, experimental parameters such as pH and concentration of added organic solvent, known to disrupt the formation of conventional micelles, do not destroy molecular micelles [22, 23, 27]. The structures of a conventional micelle and a molecular micelle

are illustrated in Figure 1.5. Molecular micelles can be used for achiral, chiral, and protein separations, which will be discussed in subsequent chapters of this dissertation. The structure of a commonly used molecular micelle, sodium poly(*N*-undecanoyl-L-leucyl-valinate) (poly-L-SULV) (Figure 1.6) consists of an amino acid polar head group and a hydrophobic hydrocarbon tail. The hydrophobic core has the ability to solubilize non-polar analytes, a good characteristic for separation science.

conventional micelle **molecular micelle**

Figure 1.5 Representation of structures of micelles

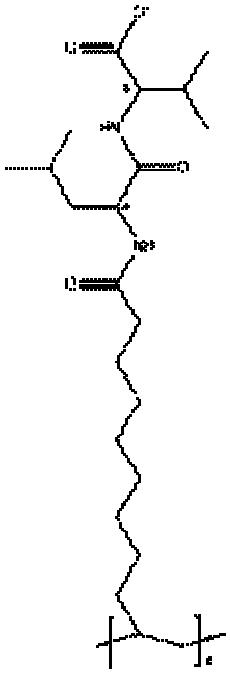


Figure 1.6 Representative molecular structure of poly-L-SULV

1.3 Proteins

Proteins are large globular structures found in all living cells that are primarily comprised of amino acids. Proteins are responsible for approximately 50% of the body's dry weight and are the major component in all of human and animal tissues. The primary structure of a protein may consist of a linear sequence of amino acids along a protein chain which contains disulfide (-S-S) bridges as demonstrated in Figure 1.7. The unique properties of a protein are due to the amino acid arrangement, if one amino acid is changed, the overall characteristics of the protein will be different [28]. Characteristics such as solubility can influence blood flow and result in health complications in humans and animals [28].

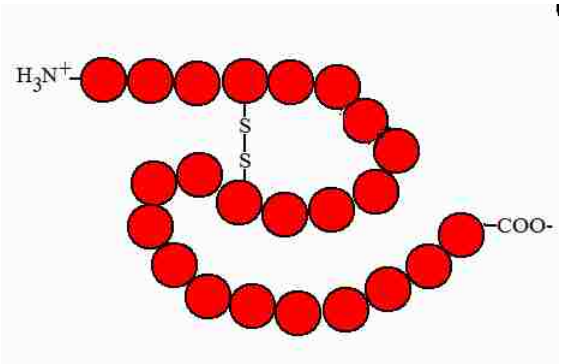


Figure 1.7 Structure of a protein

Proteins are biomolecules of great interest as biomarkers for diseases, markers for stage development of organisms, and as food additives [29]. Cytochrome c, a protein biomarker for disease, is an indictor of apoptosis. Ribonuclease A reveals the presence of kidney disease and α-chymotrypsinogen A is a major protein found in cancerous cells in the prostate. Another protein, lysozyme, found in saliva, is also a biomarker for intestinal inflammation [29]. A structural representation of lysozyme is illustrated in Figure 1.8. These proteins are separated as test mixtures in Chapter 3. Many studies have been dedicated to the separation and identification of milk proteins such as α-lactalbumin, β-lactoglobulin A, β-lactoglobulin B, bovine serum albumin (BSA) because of immunogenic properties [30] These bovine whey proteins (discussed

in Chapter 5) are also widely used as test mixtures for many techniques to determine the quality of nutrients in processed milk [30].

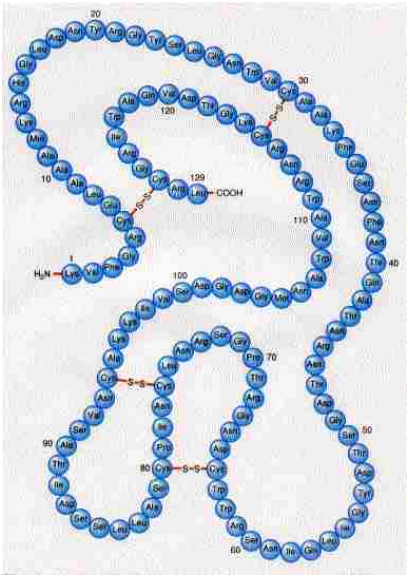


Figure 1.8 Structure of lysozyme [31]

1.3.1 Effective Net Charge of a Protein

The overall net charge of a protein is determined by its tertiary structure which is the sum of the amino acid charges, as well as the metal ions and cofactors. Gao et. al was one of the first to conduct experiments to determine the effective net charge of proteins using CE [32]. The authors determined the charge of a native protein by observing the migration time of the modified form of the same protein in CE. The effective net charge of a protein is also determined by its environment since the proteins associate with counterions in solution. In protein separations, the isoelectric point of a protein, commonly referred to as pI, is an important factor because it determines protein migration. At this point, the concentrations of both cationic and anionic forms of the protein components are equal [33]. When the pI of the protein is greater than the pH of the background electrolyte, the protein is positively charged under those experimental conditions. However, when the opposite is true and the pI of the protein is less

than the pH, its charge is negative. Table 1.1 summarizes the molecular weights and pIs of the proteins investigated in Chapters 3 and 5.

Table 1.1 Proteins Investigated

Proteins	Molecular Weight (Da)	pI
α -chymotrypsinogen A	25, 656	9.10
Lysozyme	14, 300	10.7
Ribonuclease A	13, 700	9.45
Cytochrome c	12, 327	10.0
Albumin	66, 000	4.90
Deoxyribonuclease I	31, 000	6.70
β- lactoglobulin A	18, 276	5.10
β- lactoglobulin B	18, 276	5.20
Myoglobin	17, 000	7.20
α- lactalbumin	14, 200	4.50

1.4 Analytical Separation Techniques

Several analytical techniques, such as high performance liquid chromatography (HPLC), thin layer chromatography (TLC), gas chromatography (GC), supercritical chromatography (SFC), and CE have been used to separate chiral compounds [3, 34, 35]. GC is only applicable for the separation of volatile compounds, while HPLC has been one of the most popular methods to separate chiral drugs. The advantages of HPLC include its ability to be used on the preparative scale, as well as its low detection limit, and high injection volume. However, CE has emerged as one of the leading separation techniques since high separation efficiencies are achieved with relatively low consumption of analyte and chiral selector. In addition, CE has the added advantages of a relatively simple method development and short analysis times [35, 36].

1.4.1 Capillary Electrophoresis

There are many modes of CE and each will be briefly described. Capillary zone electrophoresis (CZE), the simplest and most commonly used mode, performs separations based on differences in the charge to size ratio as well as the differences in electrophoretic mobilities of analytes [3, 35]. Capillary isoelectric focusing (CIEF) is used to separate proteins and peptides based on their pI. Capillary isotachophoresis (CITP) uses a combination of two electrolytes for analytes to migrate at the same velocity. Capillary gel electrophoresis (CGE) uses a porous gel matrix to separate analytes according to their charge and size [35, 36]. Micellar electrokinetic capillary electrochromatography (MEKC) and capillary electrochromatography (CEC) are hybrid techniques that combine the benefits of electrophoresis and chromatography to separate charged and neutral analytes [35 - 37]. MEKC and CEC are the chosen modes of separation for the studies presented in this dissertation.

CE affords high resolution separation of both small and large molecules such as achiral and chiral drugs, pesticides, dyes, vitamins and inorganic acids, among others [36]. The separation mechanism is based on differences in the electrophoretic mobilities of analytes when an electric field is applied. Jorgenson and Lukacs [38] were among the first to achieve high resolution separations of small analytes in narrow bore fused silica capillaries. CE demonstrates several advantages when compared to other separation techniques. These advantages include a simple automated instrumentation, which results in high efficiencies and analyte resolution with small sample and reagent consumption. In addition, various modes of CE offer different analyte selectivity.

The simple instrumentation of CE (Figure 1.9) includes silica capillary, photodiode detector, UV lamp, sample and buffer reservoirs, a cathode and anode, high voltage power supply, and a computer for recording data. For CE separations, both ends of the narrow bore

fused silica capillary are placed in the inlet and outlet reservoirs, which also contain the electrodes connected to the high voltage power supply. After the capillary is filled with buffer, the inlet is then placed into the sample reservoir to inject the analyte. In order to initiate sample migration, the inlet reservoir replaces the sample reservoir and an electric field is applied. The sample ions then move through the capillary past the detection window. At this point, the data in the form of electropherograms are recorded and displayed on the computer.

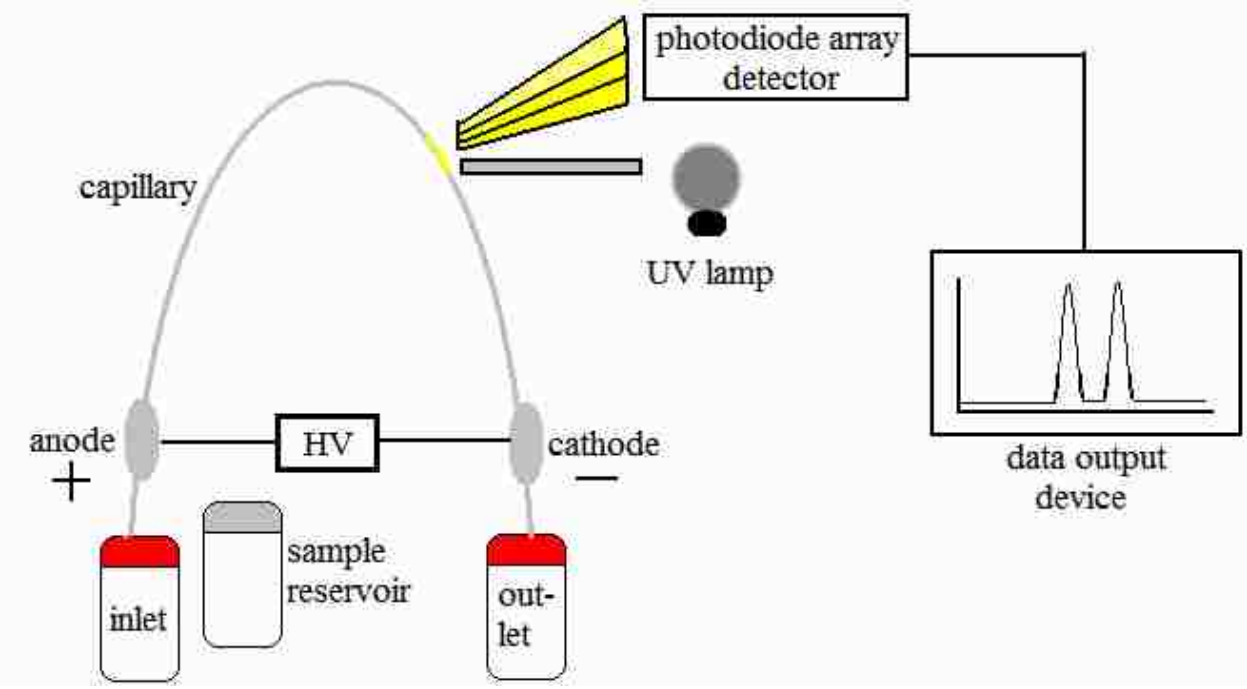


Figure 1.9 Schematic diagram of CE instrumentation

The movement of both charged and neutral analytes through the capillary is due to the electroosmotic flow (EOF). The EOF is originated from the electrical double layer that is formed when the negatively charged inner capillary wall (SiO^-) and the positive ions (counterions) of the background electrolyte interface. The positively charged ions (cations) are held adjacent to the inner capillary wall by electrostatic interactions forming the stern layer. Not all ions are held at the walls, some are able to move throughout the capillary, forming the diffuse layer. The electrical double layer is illustrated in Figure 1.10. For separations to occur, an

electric field is applied, which results in the cations of the diffuse layer to be drawn towards the cathode causing movement of the bulk solution.

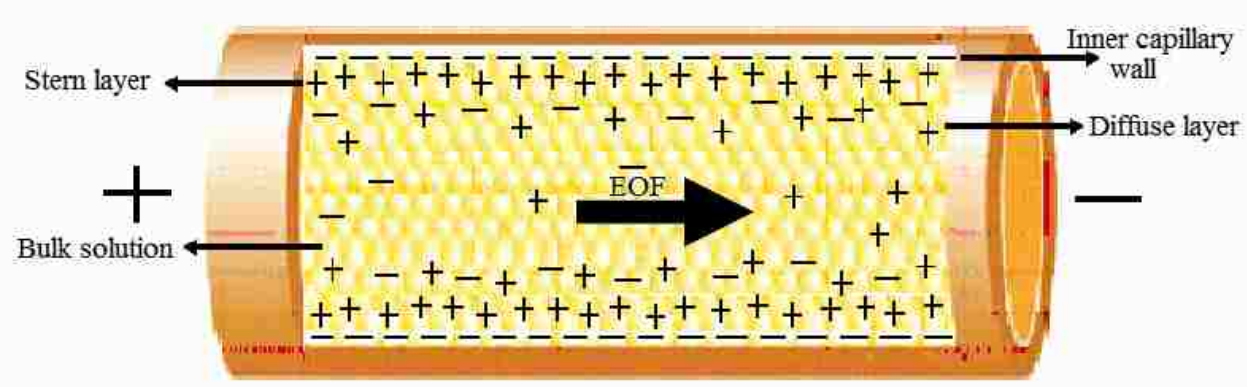


Figure 1.10 Electrical double layer

The EOF is described by the following equations:

$$_{EOF} = (\varepsilon \zeta / \eta) E \qquad 1.1$$

$$\mu_{EOF} = (\varepsilon \zeta / \eta) \qquad 1.2$$

where, υ is the velocity of the EOF, ε is the dielectric constant of the BGE, ζ is the zeta potential (potential across the layers depending on thickness of diffuse layer and the surface charge), η is the solvent viscosity, E is the applied electric field, and μ is the mobility of the EOF. At low pH values, the EOF is suppressed by the protonation of the SiO^- groups which, results in a decreased ζ. Conversely, at high pH values the opposite is true.

In CZE, charged analytes migrate through the capillary at a velocity based on electrophoretic mobility, as well as the charge to size ratio, while the migration of neutral analytes depends on the EOF. Overall, the EOF causes all analytes (charged or neutral) to migrate in the same direction. This relationship is given by the equation:

$$\mu_{app} = \mu_e + \mu_{EOF} \qquad 1.3$$

where, μ_{app} is apparent mobility of the analyte, μ_e is the electrophoretic mobility of the analyte, and μ_{EOF} is the electrophoretic mobility of the BGE. The charge of an ion determines the size

of its electrophoretic mobility and order in which it migrates through the capillary. The first species to elute are the cations since their electrophoretic mobility is in the same direction as the EOF. Next, the neutral species elutes with the EOF. It should be noted that neutral species are not separated using CZE. Finally, the anionic species migrate toward the cathode. Anions are the last to elute because their electrophoretic mobility is in the opposite direction to the EOF. The magnitude of the EOF is greater than the electrophoretic mobility of the anionic species. Furthermore, larger species have a greater electrophoretic mobility than smaller species of the same charge. A schematic of the elution order of charged and neutral species in CZE is illustrated in Figure 1.11.

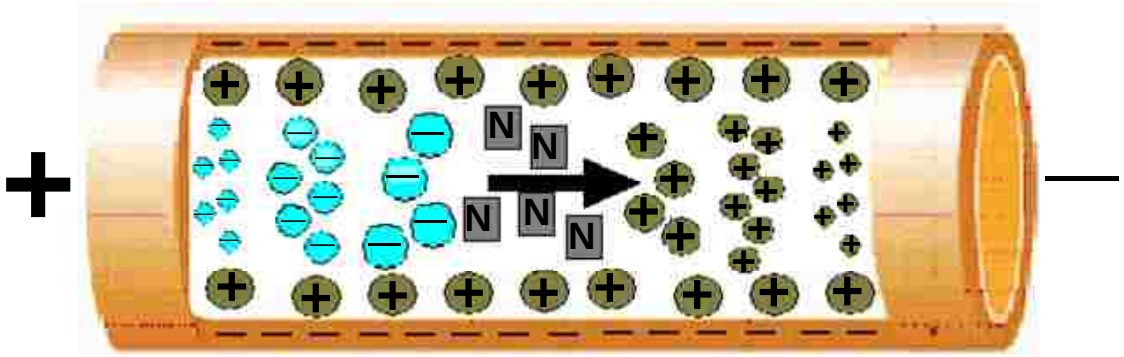


Figure 1.11 Analyte migration (cationic, neutral, anionic) in CZE

1.4.2 Micellar Electrokinetic Chromatography

Unlike CZE, MEKC can be used to separate charged as well as neutral analytes. Terabe was the first to introduce MEKC in the 1980s when he placed surfactants in the BGE (pseudostationary phase) at concentrations higher than the CMC to ensure micelle formation [39, 40]. The authors reported the separation of 14 phenols using sodium dodecyl sulfate (SDS) micelles. In MEKC, the separation mechanism is based on differences in the analytes' electrophoretic mobility and the partitioning of analytes in the micellar pseudostationary phase. For neutral species, only the analyte partitioning is applicable for separations. Other interactions between the analyte and the pseudostationary phase include hydrophobic, ionic, dispersive and

electrostatic interactions as well as hydrogen bonding. A schematic representation of the MEKC technique is shown in Figure 1.12. Conventional micelles [41, 42, 43] have been used for several separations in MEKC, however, the dynamic equilibrium between the surfactant monomers and micelles results in thermodynamic instability [44]. Molecular micelles can be used to overcome these limitations and are commonly used as the pseudostationary phase in MEKC. Molecular micelles eliminate the dynamic equilibrium between monomer and micelle and results in more stable, rigid structures with controlled sizes. These rigid structures are useful when organic modifiers are added to the BGE for enhanced separations. Traditionally, organic modifiers are known to disrupt the micellar configuration in conventional micelles. Other advantages of molecular micelles over conventional micelles include their use at low concentrations which results in minimal Joule heating. Molecular micelles were described briefly in Section 1.2.2.

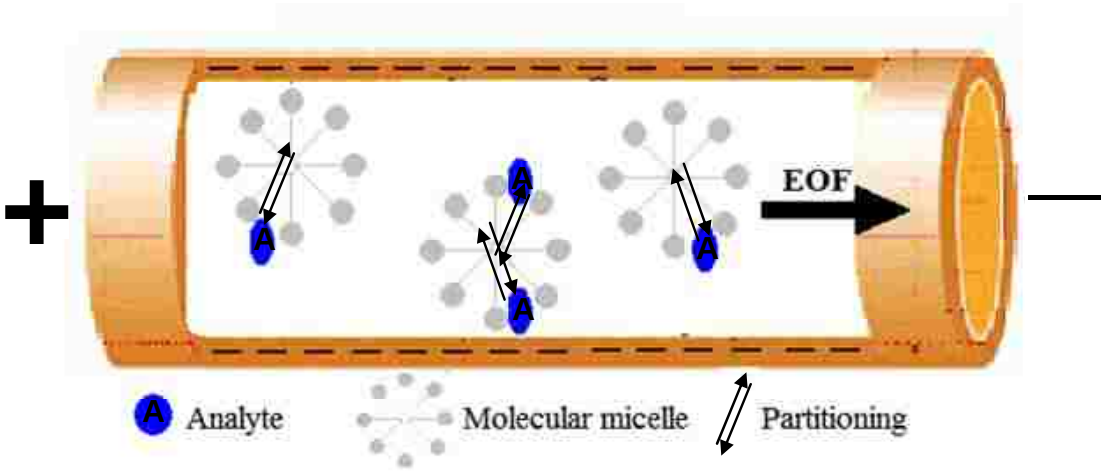


Figure 1.12 Illustration of the MEKC technique

Separation of charged and neutral analytes can be influenced by the BGE concentration, pH of BGE, operating temperature, applied voltage, micelle size and charge, BGE modifiers and molecular micelle concentration. These experimental parameters are important to the separation and migration of analytes in the capillary using MEKC. For example, when an anionic

molecular micelle is used, there are increased interactions between the analyte and micelle; therefore the migration time of the analyte through the capillary is increased. This phenomenon can be explained because the electrophoretic mobility of an anionic micelle is in the opposite direction of the EOF. The magnitude of the EOF is greater than the electrophorectic mobility of the anionic micelle, therefore, the micelles and analyte migrate towards the cathode.

The major goal in separation science is the resolution of the analyte of interest. Baseline resolution has a value of 1.5 which is dependent on the efficiency and migration time, as well as the selectivity of the separation technique. The effectiveness of the column in separating the analyte is determined by the equation 1.4:

$$K = c_s/c_m \qquad 1.4$$

where K is the partition coefficient, c_s is the molar concentration of the analyte in the pseudostationary phase and c_m is the analyte concentration in the mobile phase. The capacity factor is the ratio of the molar concentration of the analyte in the pseudostationary phase to the molar concentration of the analyte in the mobile phase. This relationship is given by equation 1.5:

$$k' = \frac{(t_r - t_o)}{t_o(1-t_r/t_m)} = K\,(V_s/V_m) \qquad 1.5$$

where k′ is the capacity factor, t_r and t_o are the retention time of the analyte and neutral marker, t_m is the retention time of the micelle, K is the partition coefficient, V_s is the volume of the micellar phase and V_m is the volume of the mobile phase. Less hydrophobic analytes have little interaction with the pseudostationary phase and are the first to elute while more hydrophobic analytes interact longer and elute last. The selectivity, α, is the ratio at which these two types of analytes are retained by the column given by equation 1.6

$$\alpha = k'_2/\ k'_1 \qquad 1.6$$

where k'_1 is the first peak to elution and k'_2 is the second. A selectivity value of 1 indicates no separation. For optimum separations, the peaks are sharp and symmetrical in shape. The mathematical representation of peak shape is described in terms of peak efficiency or the number of theoretical plates, N:

$$N = 5.54 \, (t_n / w_{1/2})^2 \qquad 1.7$$

where t_n is the elution time for peak n, and $w_{1/2}$ is the peak width at half height. Optimizing the capacity factor, k′, the selectivity, α, and the peak efficiency, N will all improve the resolution between the analyte of interest. Resolution, R_s, is given by equation 1.8:

$$R_s = \frac{(N^{1/2})}{4} \frac{(\alpha - 1)}{\alpha} \frac{(k'_2)}{k'_2 + 1} \frac{(1 - (t_o / t_m))}{1 - (t_o / t_m) \, k'_1} \qquad 1.8$$

The symbol representations are the same as listed in earlier equations. A simplified version of equation 1.8 used in experimental calculations is given by equation 1.9.

$$Rs = \frac{2(t_2 - t_1)}{w_1 + w_2} \qquad 1.9$$

where w_1 and w_2 are the width at baseline of the first and second peaks.

Wang and Warner [45] were among the first to synthesize and demonstrate the use of sodium poly (undecenyl-L-valinate) (poly-L-SUV) for the separation of chiral analytes. Since then, MEKC with molecular micelles has been used to separate a number of achiral and chiral analytes [3, 21-23, 46-48, 67, 78, 79]. Williams et al. [46] used multivariate analysis for optimization in MEKC to separate the two (2) chiral binaphthyl derivatives (±)-1,1'-bi-2-naphthyl-2,2'-dihydrogen phosphate (BNP) and (±)1,1'-bi-2-naphthol (BOH) using poly-L-SULV. In addition, the chiral compounds, benzoin, hydrobenzoin, coumachlor, warfarin, lorazepam, and temazepam were also separated. Furthermore, four achiral analytes were separated using MEKC with sodium poly(*N*-undecylenic sulfate)(poly-SUS) [46]. Numerous other achiral and chiral analytes have been separated using MEKC by other separation scientists.

Akbay et al. [47] employed MEKC to separate seven benzodiazepines and seven alkyl phenyl ketones using pseudostationary phases comprised of the polymers of sodium 10-undecenoyl-L-leucinate (SUL) and SUS [47]. Rizvi et al. [48] used three sulfate head group bearing chiral surfactants and three carboxylate head group bearing chiral surfactants to separate basic and neutral chiral compounds. The authors revealed that various surfactants showed superiority in different environments [48]. MEKC experiments are reported in Chapter 4 of this dissertation.

1.4.3 Capillary Electrochromatography

Like MEKC, capillary electrochromatography (CEC) is a hybrid technique of CE and HPLC that can be used to separate both charged and neutral analytes. Advantages of this technique include high selectivity and peak efficiency. Unlike MEKC, CEC involves the incorporation of a stationary phase within the capillary. The separation mechanism is based on differences in the electrophoretic mobilities of the analytes, as well as the analyte partitioning into the stationary phase. In the case of neutral species, partitioning is the only mechanism of separation. CEC is similar to CZE in terms of EOF generation and high peak efficiencies, however, it is widely known that the separation of basic analytes, such as proteins, has been a problem for CZE users. Protein separations carried out in an unmodified silica capillary exhibit poor reproducibilities. Adsorption of proteins to the capillary wall may be due to electrostatic interactions, hydrogen bonding, hydrophobic patches, and biospecfic sites. As a result, several problems including peak tailing, unstable base lines, irreproducibility from run to run, low peak efficiency and irreversible sample adsorption may occur [49, 50]. For chiral separations in CEC, selectivity can be enhanced by altering the stationary phase to support chiral interactions.

CEC is a versatile technique that has attracted the interest of separation scientists in recent years as an alternative to CZE. The CEC technique was first introduced by Pretorius et al. [51] in 1974, when hydrodynamic pumping was replaced with electrokinetic pumping. Next,

Jorgenson and Lukacs used CEC to separate 2-methylanthracene and perylene in a packed CEC column [52]. As in MEKC, CEC separations are based on analyte interaction with the micellar phase for both charged and neutral analytes. When the species is charged, the analytes' electrophoretic mobility is also a factor in separation [53]. The capacity factor, k'_{CEC}, has been expressed in several ways [54-56], however, one commonly used mathematical expression is given in equation 1.10:

$$k'_{CEC} = \frac{k' - (\mu_e / \mu_{eof})}{1 + (\mu_e / \mu_{eof})} \qquad 1.10$$

This equation combines both the electrophoretic and chromatographic mechanisms of CEC. k' is the retention resulting from the chromatography factor, μ_e is the electrophoretic mobility of the analyte and, μ_{eof} is the magnitude of the EOF. When neutral species are being investigated, μ_e is zero.

There are three main modes of CEC: packed column CEC (PC-CEC), monolithic columns and open tubular-CEC (OT-CEC) (Figure 1.13). In PC-CEC, the stationary phase is prepared by packing the capillary, usually 50 -100μm internal diameter (ID) with a silica-based packing material, i.e. octadecyl silica beads. Some expertise is required to successfully pack these columns because of the small inner diameter. For column packing, the ends of the capillary are burned to prepare a frit. Then, the packing material, i.e. the stationary phase is pumped into the column with high pressure. Finally, another frit and a detection window are prepared [55]. There are several problems associated with using PC-CEC, such as the formation of bubbles within the column and the difficulties in maintaining stable frits. These problems can negatively affect chromatographic separations [35, 53, 54].

Monolithic CEC columns are prepared by *in situ* polymerization of organic species or sol-gel materials. After preparation, monolith columns consist of a uniform macroporous

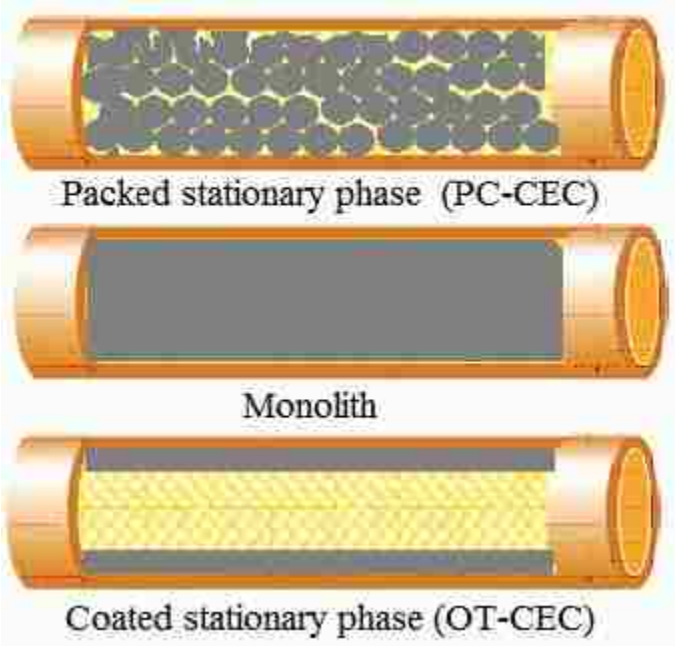


Figure 1.13 Schematic of the 3 types of CEC stationary phases

stationary phase [57, 58]. These stationary phases may be constructed using organic materials, silica sol gel, and immobilized particles. Free radical or UV initiation are commonly used to polymerize monomers of the organic porous monolith columns. Hydrolysis and polycondensation reactions form porous networks of silica gel when sol-gel monoliths are prepared. Immobilized particle monoliths are constructed using a method similar to PC-CEC. First, the organic polymers or sol-gels are packed into the capillary and retained using frits, which are burnt into the end of the capillary. Then, the packing material is polymerized by pumping organic based monomers mixed with porogenic solvent through the capillary. Monolith columns offer some advantages over PC-CEC since the stationary phase can be tailored to alter analyte selectivity. In addition, column preparation is simpler.

Preparation of OT-CEC involves coating polymers onto the inner wall of the capillary, thus alleviating the problems with packing and unstable frits as in PC-CEC. In OT-CEC, the stationary phase is adsorbed to the capillary wall and the mobile phase, which flows through the column, is driven by the EOF. Therefore, there is no pressure drop within the capillary and the

EOF produces a flat flow profile. The coating must be stable in order to achieve successful chromatographic separations. The stationary phase can be prepared adsorption, where the stationary phase can either be dynamically or physically adsorbed to the capillary wall to shield the negatively charged silanol groups with a layer of the coating material [54, 59]. In a dynamically coated capillary, the adsorption of the coating material to the capillary wall is relatively weak, and the coating material is placed in the running buffer to ensure the coating remains stable. On the other hand, if the adsorption of the coating polymer is strong, that is, it is physically adsorbed to the wall, then the addition of the coating material to the running buffer is not necessary [54, 59]. Covalent bonding and/or crosslinking is another method of forming a stationary phase for CEC separations. This approach offers a long capillary lifetime but it is laborious and time consuming to prepare [60]. There are some disadvantages associated with the use of CEC, such as a low phase ratio and decreased surface area, however, the formation of porous silica layers [61] and chemical bonding after etching [62] and sol gel [63] are all techniques used to increase the phase ratio, loading capacity, and the surface area of the capillary [64].

Advantages of using OT-CEC include coating small ID columns, which results in high peak efficiencies. Furthermore, when high voltages are applied, joule heating is minimal. However, coating small ID capillaries results in low sample capacity, which may compromise analyte resolution. Also, covering the silanol groups on the inner capillary wall may result in a low EOF. Pesek et al. increased the surface area by etching and then chemically modifying a 20μm ID capillary by the silanization/hydrosilation method to separate lysozyme and cytochrome c [65]. Constantin and Freitag developed novel stationary phases for use in OT-CEC where various C_n (n = 6, 8, 16) polymers were used as the stationary phase in order to separate different charged biological molecules [66].

1.4.3.1 Polyelectrolyte Multilayer Coatings

Dynamic coatings are adsorbed to the capillary wall by electrostatic interactions and hydrogen bonding. One widely used coating constructed by a physical adsorption process is a polyelectrolyte multilayer (PEM) coating. PEM coatings are formed from multiple electrostatic interactions and ion exchange that result in a stable coating [23, 67]. A PEM coating is constructed by alternately exposing the hydrophilic inner wall of a silica capillary first to cationic and then anionic polymers. The combination of each polymer is called a bilayer (Figure 1.14). Decher et al. [68, 69] were among the first to prepare PEM coatings by using oppositely charged polymers. The authors suggested that deposited polymers primed the surface for polymers of the opposite charge. Dubas and Schlenoff [70] formulated a mathematical interpretation of this phenomenon given by equation 1. 11:

$$Pol^- M^+_{(m)} + Pol^+ A^-_{(aq)} \leftrightarrow Pol^- Pol^+_{(m)} + M^+_{(aq)} + A^-_{(aq)} \qquad 1.11$$

where Pol^+ and Pol^- are the charged segments of the polymers, M^+ and A^- represent the salt counterions, and m is the surface region. The charges on a polymer are balanced by the oppositely charged polymer or by salt ions present within the multilayer. Intrinsic compensation occurs when the positive charges on one polymer is counterbalanced by the negative charges on another polymer. Extrinsic compensation results from the balancing of polymer charges by additive salt ions. The net charge of the multilayer is determined by the charge of the outermost polymer.

PEM coatings are simply constructed by using multiple rinse functions of the CE instrument, however, its internal structure is not fully understood. Dubas and Schlenoff [70] have reported that the multilayers are interpenetrating, stratified structures. Furthermore, each layer may penetrate up to four layers from its original deposition [71]. The addition of salt to the coating polyelectrolytes may influence the morphology and surface roughness of PEM coatings.

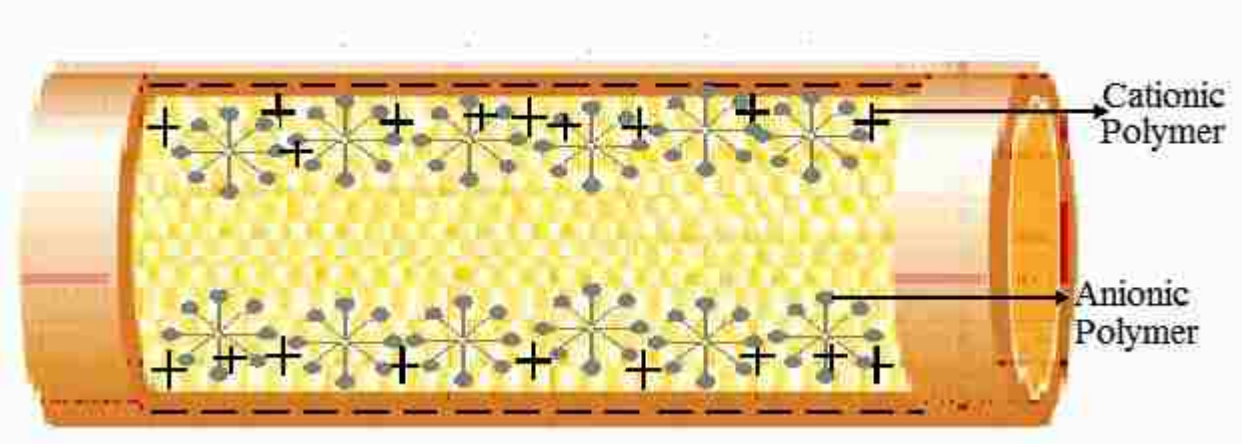


Figure 1.14 Schematic representation of a PEM coating

Salt additives have also been reported to increase PEM thickness, which results in increased analyte resolution and peak efficiency. The thickness of PEM layers can also be increased by increasing the concentrations of the coating polymers, increasing the number of bilayers, and increasing the rinse time of the polymer deposition.

1.4.3.2 PEM Coatings Used in OT-CEC

In the past, CE separations using MEKC resulted in two main problems; large amounts of chiral selector were required to achieve separation and MEKC could not be coupled to a mass spectrometer (MS). The presence of the chiral selector in the running buffer has the tendency to foul the ionization source [67]. PEM coatings have been viewed as a great alternative to MEKC because they are constructed using a simple rinse procedure requiring less chiral selector than in MEKC separations. Also, PEM coated separations have the ability to be coupled with the MS detector (CE/MS) [67]. PEM coatings are amendable to successful separations of numerous compounds and offer several advantages over other stationary phases because a wide range of coating polymers of different structural and chemical properties can be used [72]. These polymers include those that are commercially available or polymers that are synthesized in

research laboratories, e.g. our molecular micelles [21, 23, 45, 46]. Polymers used in PEM coatings may affect the resolution, selectivity, and retention time of the analytes because the interactions between the PEM coating and analyte may vary.

The outermost layer of the PEM coating can be altered to reduce analyte adsorption and enhance separation. The charge of the outer layer also determines the direction and magnitude of the EOF. The EOF is normal (anode to cathode) when the last layer of the PEM coating is negatively charged and the EOF is reversed (cathode to anode) when the last layer is positively charged.

1.4.3.2.1 Protein Separations with PEM Coatings

Katayama et al. first demonstrated PEMs using OT-CEC in 1998 when acidic proteins and amino acids were separated using multilayers of polybrene and dextran sulfate [73, 74]. Although the authors referred to the coating as successive multiple ionic-polymer layer (SMIL) coating, the structure and formation was very similar to PEM coatings. This stable coating was independent of pH and reduced or eliminated protein adsorption to the capillary wall, a problem in conventional CE. Protein separations are achieved by two mechanisms, the coating suppresses protein adsorption to the capillary wall by electrostatic repulsion of like charges between proteins and polymers as well as protein interactions with the stationary phase. Proteins have been successfully separated using PEM coated columns and the mechanisms of separation are based on differences in protein-PEM interactions [75]. The overall charge of the last layer of PEM coatings and the net charge of proteins play an important role in protein adsorption and interaction with PEM coatings. Salloum and Schlenoff [75] have investigated protein adsorption onto PEM coatings as well as the influence of surface charge, ionic strength, and thickness of the PEM coating on protein-PEM interactions. Results from that study indicated that the last layer of PEM coatings determines how proteins adsorb and interact with the coating. Furthermore, the

net surface charge of PEM coatings can be tailored so as to allow or retard protein adsorption. Protein adsorption on oppositely-charged surfaces is due to electrostatic interactions, whereas adsorption on like-charged surfaces can be attributed to non-electrostatic interactions [75]. Salloum and Schlenoff [75] have also demonstrated that proteins can be adsorbed and interact within the multilayers of PEM coatings if proteins and PEM coatings are of opposite charge. However, when proteins are of the same charge as PEM coatings, interactions occur at the surface layer regardless of PEM coating thickness. PEM coatings used in this dissertation employed protein-PEM interactions at the surface of the multilayer.

Several studies involving separations of cationic proteins using a positively charged wall coating have been previously performed [29, 72, 75]. Wang and Dubin [72] have investigated the influence of an immobilized adsorbed coating using poly(dimethyldiallyammonium chloride) (PDADMAC) as the coating polymer for the separation of cationic proteins. Results indicated that high molecular weight polymers at high ionic strengths provided optimal coating conditions. In addition, the authors reported that the coating polymer formed loops and tails, which were important for the reversal of the EOF and provided a stationary phase for protein interactions as well as increased the stability and efficiency of the coated column [72]. Graul and Schlenoff [49] used PDADMAC and poly (styrene sulfonate) (PSS) as polyelectrolytes in PEM coatings for the separation of four basic proteins. Also, the authors investigated the influence of the number of bilayers on protein resolution and reported that the use of 6.5 bilayers improved protein resolution [49]. Protein separations using PEM coatings are demonstrated in Chapters 3 and 5 of this dissertation. Several commercially available cationic polymers are investigated for use in protein separations. The importance of bilayer number and PEM coating thickness are highlighted to illustrate their influence on protein separations. In addition, the introduction of PEM coatings constructed with molecular micelles is demonstrated for the first time.

1.4.3.2.2 Chiral Separations with PEM Coatings

Mayer and Schurig [76] were among the first to report chiral separations using OT-CEC with Chiralsil-Dex as the stationary phase. PEM coatings with chiral cationic and anionic polymers have been used to separate a number of chiral analytes. Rmaile and Schlenoff used the polymers, poly-L-lysine and poly-L-glutamic acid, among others, to resolve some chiral probes such as ascorbic acid and a chiral viologen [77]. In our laboratory, Kamande et al. used poly-L-lysine hydrobromide and poly-L-SULA to separate three binaphthyl derivatives and two β-blockers. These columns have remarkable reproducibility and stability for over 290 runs. In addition, the coupling of PEM coated columns to MS was reported for the first time [78]. It is not necessary for both polymers to be chiral in order to separate chiral analytes. For example, Kapnissi et al. optimized several experimental parameters using PEMs generated with achiral PDADMAC and chiral poly-L-SULV to resolve chiral analytes. In this study the authors created up to a 12 bilayer capillary using ionic liquids as additives [79]. Usually, PEM coated capillaries are stable and robust. For example Kapnissi et al. demonstrated a coating that was able to withstand over 200 runs with a relative standard deviation (RSD) of the EOF of less than 1% [67]. Chapters 2 and 4 illustrate chiral separations using PEM coatings.

1.4.4 Experimental Design

Experimental design uses statistical methods to design experiments that provide the optimum conditions for a desired result [80]. In CE, the optimization of separation parameters to achieve high resolution separations in a reasonable analysis time continues to be an active area of research [81, 82]. Examples of such parameters (design variables) include the choice and concentration of the cationic and anionic polymer, as well as the applied voltage, temperature, BGE pH, and the number of bilayers in the PEM coating. The conventional approach to optimizing a PEM coating can be a tedious process since only one parameter is usually changed

at a time. In recent studies, experimental design and multivariate analysis have been widely used to simultaneously optimize separation parameters, leading to higher resolution in relatively short analysis times [46, 83–88]. Previously in our research group, Williams et al. reported the use of experimental design and multivariate analysis for the optimization of separation parameters to predict the migration time, resolution, and resolution *per* unit time of several chiral and achiral analytes using MEKC [46]. Experimental design has also been used to optimize the separation conditions of two different stationary phases for the separation of four basic peptides in OT-CEC [83]. Yang *et al*. [84] have used experimental design, specifically central composite design, in CEC to simultaneously optimize the separation parameters of 11 nucleosides and nucleobases in *Cordyceps sinensis*, a traditional Chinese medicine.

Box Behnken experimental design uses the simultaneous variation of separation parameters at three levels [80]. For example, if temperature is chosen as a design variable, the three levels investigated may be 15˚C, 20˚C, 25˚C. Surface response plots demonstrate the relationship between the design variables and the desired result (response). In chapter 3 of this dissertation, a Box Behnken experimental design is used to optimize separation parameters using four different chiral cationic polymers and the anionic molecular micelle, poly-L-SULA for PEM coatings in the OT-CEC separation of four basic proteins: a-chymotrypsinogen, lysozyme, ribonuclease A, and cytochrome *c*. The design variables investigated included the type and concentration of polymers, applied voltage, temperature, BGE pH, as well as the number of bilayers.

1.5 Moving Boundary Electrophoresis

Tiselius et al. [89, 90] was the first to demonstrate moving boundary electrophoresis (MBE) in 1937. MBE is described as electrophoresis of a free solution in which all analyte ions move at the same rate through the separation channel to create a sharp moving boundary. These

manuscripts included the separation of colloids with the implementation of a new experimental setup called the Tiselius apparatus. The Tiselius apparatus includes a U-shaped cell filled with buffer solution and an inlet and outlet electrode. More recently, Harmon et al. [91] used Tiselius apparatus for electrophoretically mediated microanalysis (EMMA) to separate leucine aminopeptidase. The authors reported that the MBE technique provided greater sensitivity when compared to the original EMMA method. Gao et al. [92] used MBE for the development of frontal analysis continuous capillary electrophoresis (FACCE) to investigate the interactions of proteins to polyelectrolytes. The authors introduced the use of continuous sampling when a voltage is applied to eliminate the need for sample injection. Advantages of this technique were reported to be the integration of sample introduction and sample separation.

1.5.1 Gradient Elution Moving Boundary Electrophoresis

Controlling the bulk flow in CE is an important parameter to investigate in order to enhance separations. Bulk flow control can be accomplished through establishing hydrodynamic or electrokinetic gradients. Kok hydrodynamically controlled the bulk flow in a CE system to reduce zone broadening with off column detection [93]. Also, Culbertson et al. [94] demonstrated the use of a pressure induced counterflow to control the migration of different species in a separation channel. The authors reported increased peak efficiencies and analyte resolution due to higher analyte retention in the separation channel. Peak efficiencies can also be increased by applying an external voltage. Polson et al. [95] controlled the EOF in a microfluidic device by applying an external potential of $\leq$120 V, which resulted in peak efficiencies over 40 times of other published values.

Recently, Shackman et al. [96] implemented a new technique, gradient elution moving boundary electrophoresis (GEMBE). In this method, analytes are separated by applying a controlled hydrodynamic counterflow that is varied with time. GEMBE promotes high

resolution separations in short, narrow separation microchannels. GEMBE has the advantage of allowing continuous sample injection into the separation channel which eliminates conventional injection methods such as electrokinetic and hydrodynamic injections. Experiments using GEMBE are performed by carefully controlling a hydrodynamic counterflow, which allows analytes to enter the separation channel. Separations are achieved due to differences in the electrophoretic mobilities of the analytes. Therefore, only analytes with an electrophoretic mobility that is greater than the counterflow will enter the separation channel.

GEMBE instrumentation is illustrated in Figure 1.15. For GEMBE separations, short fused silica capillaries with 5 mm detection windows are used. One end of the capillary is placed into a 360 μm hole in the analyte reservoir with an electrode. The other end of the capillary is attached to a polypropylene syringe that contains ~1 mL of buffer. The syringe is grounded and connected to a ±69 kPa (10 psi) precision pressure controller, which used helium as the gas supply. The syringe plunger accommodates a high voltage supply and controls the pressure. All experiments are performed on a fluorescence microscope with 10 × objective (numerical aperature, NA = 0.3), Hg arc lamp, color CCD camera, (DXC-390, Sony, New York, NY) and appropriate fluorescence filter sets. Instrumental control and data acquisition uses Java 5.0 software. Raw data are transformed using Savitzky Golay smoothing [96].

1.6 Scope of Dissertation

This dissertation includes enhanced achiral, chiral, and protein separations using different chromatographic techniques, valuable to the pharmaceutical and biomedical industries. The versatility of PEM coatings are demonstrated for a wide range of analytes. Mixed mode separation is demonstrated for the first time with PEM coatings and molecular micelles. In addition, novel PEM coatings are applied for protein separation using OT-CEC and GEMBE.

Four cationic polymers are used to optimize chiral and protein separations with PEM

coatings in Chapters 2 and 3. Cationic polymers used in PEM coatings may affect the resolution, selectivity and retention time of the analytes being studied. In these chapters, the effect of changing four different cationic polymers, poly-L-lysine, poly-L-ornithine, poly-L-lysine-serine, and poly-L-glutamic acid-lysine, on the separation of two binaphthyl derivatives, one β-blocker, and four basic proteins (α-chymotrypsinogen A, lysozyme, ribonuclease A, and cytochrome *c*) was investigated. The anionic polymers used were poly-L-SULA, poly-L-SULV and sodium poly (undecylenic sulfate) (poly-SUS). The simultaneous effects of cationic polymer concentration, number of bilayers, temperature, applied voltage, and pH of the BGE on the separation of the chiral analytes, as well as the four basic proteins were analyzed using a Box Behnken experimental design. The influence of NaCl on the run-to-run reproducibility was investigated for PEM coatings containing each cationic polymer.

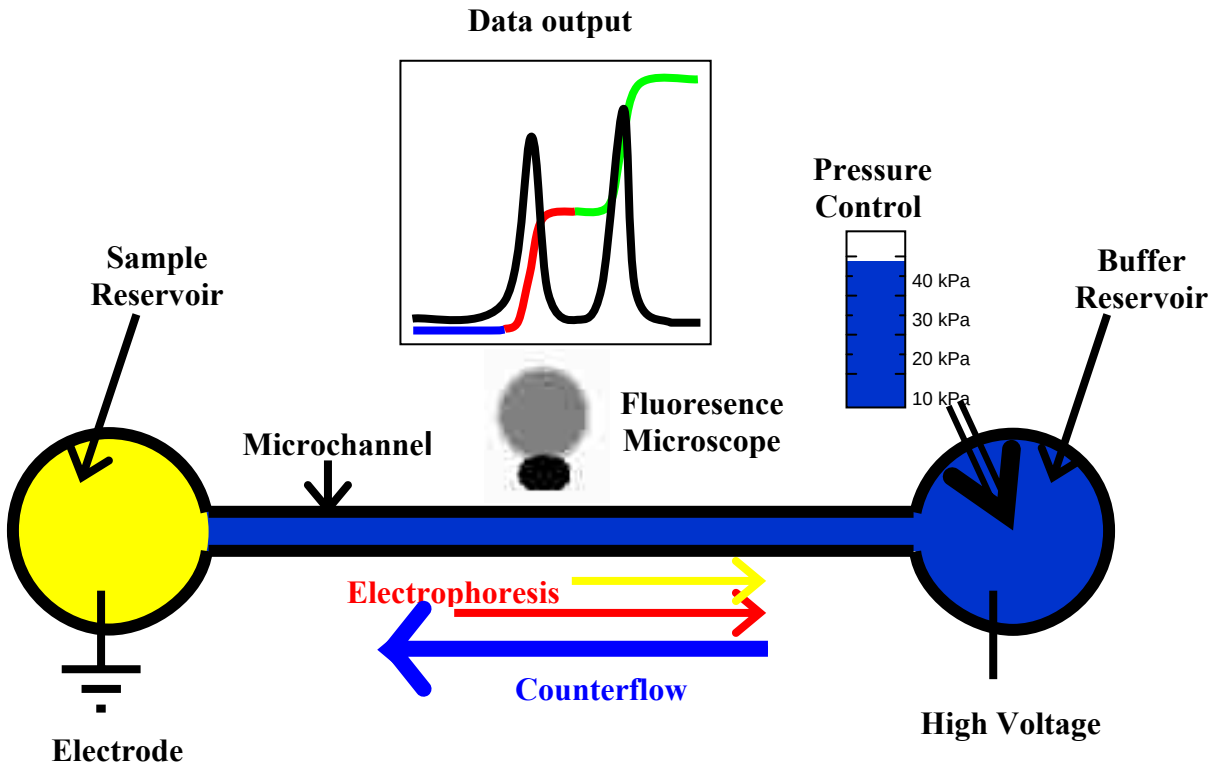


Figure 1.15 Illustration of GEMBE technique

Chapters 4 and 5 involve novel applications of PEM coatings for achiral, chiral and protein separations. Mixed mode separation using a combination of MEKC and PEM coatings is reported for the separation of achiral and chiral analytes in Chapter 4. Many analytes are difficult to separate by MEKC and PEM coatings alone. Therefore, the implementation of a mixed mode separation provides several advantages for overcoming the limitations of these well-established methods. In this study, achiral and chiral separations using MEKC and PEM coatings individually, were investigated using the molecular micelles (sodium poly (*N*-undecanoyl-L-glycinate) (poly-SUG) and poly-L-SULV. The molecular micelle concentration and bilayer number were varied to optimize these separations. The separation of achiral and chiral compounds from different compound classes demonstrates the versatility of this mixed mode approach.

In Chapter 5, PEM coatings were constructed using molecular micelles in OT-CEC and GEMBE for acidic protein separations. In OT-CEC, proteins were detected using both ultra violet (UV) and laser induced fluorescence (LIF) detection, while only LIF detection was used with the GEMBE technique. PEM coatings were constructed using the cationic polymer, poly-L-ornithine and the molecular micelles, poly-L-SULA and poly-L-SULV. Experimental variables, including bilayer number, type of molecular micelle, as well as pH of the BGE were studied for the separation of 6 acidic proteins (α-lactalbumin, β-lactoglobulin A, β-lactoglobulin B, albumin, myoglobin, and deoxyribonuclease I). The influences of pH of the background electrolyte, internal diameter, and the effective length of the capillary on the separation of three fluorescently labeled proteins were investigated using LIF detection. Protein separations using the new GEMBE technique was demonstrated for the first time. Different voltages were applied to a 3cm, 30μm capillary to investigate the influence on protein separations using PEM coatings and GEMBE.

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CHAPTER 2

SEPARATION OF CHIRAL ANALYTES USING POLYELECTROLYTE MULTILAYER COATINGS IN OPEN TUBULAR CAPILLARY ELECTROCHROMATOGRAPHY

2.1 Introduction

Chiral separations have captured the attention of the pharmaceutical, biomedical and environmental industries primarily because the interactions and behavior of the individual enantiomers have not been fully explored and there are thousands of chiral drugs in circulation [1]. One widely known example that illustrates the importance of the separation of chiral drugs is the thalidomide tragedy. In the 1960's thalidomide was given to pregnant patients in the racemate form to help lessen symptoms associated with morning sickness. However, it was later discovered that only the (R)-(+)-enantiomer was a sedative while, the (S)-(-) enantiomer caused horrible birth defects [2, 3, 7]. There are many other drugs that exhibit different pharmacokinetic behavior, and since then the United States Food and Drug Administration has recommended that the pharmacological and toxicological activity of each isomer be investigated and documented. Therefore, interest in methods to separate chiral analytes became more pressing.

In order to achieve chiral separation, enantiomers must be placed in a chiral environment. This is generally achieved through the use of a chiral selector or chiral discriminating agent able to differentially interact with the individual enantiomers. Molecular micelles are commonly used chiral selectors. Isiah M. Warner's research group at Louisiana State University has been one of the leaders in the use of molecular micelles for capillary electrophoresis enantioseparations due to their stability, rigidity and controllable sizes [4-8]. Molecular micelles are prepared by polymerizing surfactants at sufficiently high concentrations for micelles to form. The resulting polymer, thought to resemble a conventional micelle, is preferred over the unpolymerized micelles because molecular micelles have no critical micelle concentration. Therefore, molecular

micelles can be used at concentrations lower than the monomers' critical micelle concentration. In addition, molecular micelles contain covalent bonds which eliminate the dynamic equilibrium between micelles and surfactant monomers [1, 4, 5].

Capillary electrochromatography (CEC), a hybrid technique, combines the benefits, selectivity and efficiency, of electrophoresis and chromatography to separate charged and neutral analytes [9, 10, 11]. CEC is a versatile technique that has attracted the interest of separation scientists in recent years as an alternative to capillary zone electrophoresis. The separation of the analytes being investigated is based on electrophoretic mobility and their partitioning into the stationary phase. Neutral analytes are separated through interactions with the stationary phase which is adsorbed to the capillary wall, while charged analytes are separated due to their differences in charge and size as well as partitioning behavior [12].

In one type of CEC, open tubular capillary electrochromatography (OT-CEC), the stationary phase is adsorbed to the capillary wall and the mobile phase, which flows through the column, is driven by the electroosmotic flow (EOF) [13-15]. The chiral selector used as the stationary phase in chiral separations is an important part of achieving optimal separations. Coating the inner wall of a capillary can change the selectivity and control the EOF. Dynamic coatings are adsorbed to the capillary wall by electrostatic interactions and hydrogen bonding [6]. One widely used coating constructed by a physical adsorption process is polyelectrolyte multilayer (PEM) coating.

A PEM coating is formed by alternately exposing the hydrophilic inner wall of a silica capillary first to cationic and then anionic polymers. The combination of each is called a bilayer. The mechanism of a PEM coating formation is via ion exchange that results in stable coatings [16]. PEM coatings have been created as an alternative to micellar electrokinetic chromatography (MEKC) because the use of MEKC results in two main problems. The MEKC

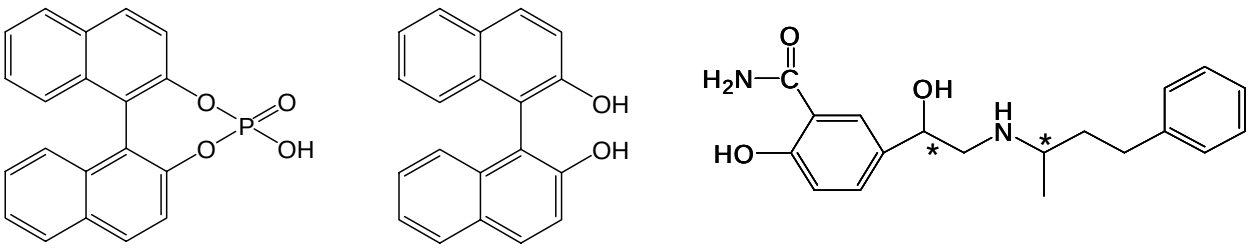
technique requires large amounts of chiral selector to achieve separation which is significantly reduced by using PEM coatings. Another main drawback of this technique is its inability to be coupled to a mass spectrometer (MS) because the presence of the chiral selector in the running buffer has the tendency to foul the ionization source [16]. PEM coatings are constructed using a simple rinse procedure and the columns can be coupled with MS detectors (CE/MS) [6, 16]. Usually, PEM coated capillaries are stable and robust and can be used to separate various chiral analytes [16-19].

Cationic polymers used in PEM coatings may affect the resolution, selectivity and retention time of the analytes being studied. The objective of the research demonstrated in this chapter is to investigate the effect of changing four different cationic polymers, poly-L-lysine, poly-L-ornithine, poly-L-lysine-serine, and poly-L-glutamic acid-lysine, on the separation of three chiral analytes. The choice of coating polymers can influence selectivity, resolution and migration time. These optically pure cationic polymers are commercially available and exhibit different structural properties and hydrophobicities. The most hydrophobic cationic polymer is poly-L-glutamic acid-lysine and the least hydrophobic cationic polymer is poly-L-ornithine. The anionic polymers used were poly (sodium N-undecanoyl-L-leucyl-alaninate) (poly-L-SULA), poly (sodium N-undecanoyl-L-leucyl-valinate) (poly-L-SULV). The molecular micelles poly-L-SULA and poly-L-SULV were used in the chiral studies since previous studies in our laboratory have shown that dipeptide surfactants resulted in better chiral separation than monopeptide surfactants when developing PEM coatings. Also, poly-L-SULA has been shown to have great selectivity for the binaphthyl derivatives. The effect of cationic polymer, applied voltage, temperature, concentration of anionic polymer and the number of bilayers on the separation and resolution of the chiral analytes is also investigated. The test analytes were 1,1´-bi-2-naphthyl-2,2`-dihydrogen phosphate (BNP), (±)-1,1`-bi-2-naphthol (BOH), and labetalol.

2.2. Materials and Methods

2.2.1 Chemicals

The cationic polymers poly-L-lysine hydrobromide, poly-L-ornithine hydrobromide, poly-L-glutamic acid-lysine hydrobromide and poly-L-lysine-serine hydrobromide were purchased from Sigma Chemical Company (St. Louis, MO), along with the enantiomerically pure chiral analytes (±)-1,1`-bi-2-naphthyl-2,2`-dihydrogen phosphate (BNP), (±)-1,1`-bi-2-naphthol (BOH), and labetalol. The molecular structures of the chiral analytes are illustrated in Figure 2.1. The chemicals used to synthesize surfactant monomers, N-hydroxysuccinimide, undecylenic acid, dicyclohexylcarbodiimide and sodium bicarbonate, were purchased from Fluka (Milwaukee, WI). The dipeptides (L,L)-leucyl-alaninate and (L,L)-leucyl-valinate were purchased from Bachem Bioscience, Inc. (King of Prussia, PA). Sodium hydroxide, ethyl acetate, methanol, and tetrahydrofuran were purchased from Sigma-Aldrich (Milwaukee, WI). The compounds used in the background electrolyte, tris(hydroxymethyl)aminomethane (Tris), phosphoric acid, sodium phosphate dibasic and sodium borate, were purchased from Fisher Scientific (Fair Lawn, NJ). All materials were used as received without any further purification.



(A) (B) (C)

Figure 2.1 Molecular structures of the chiral analytes investigated: (A) BNP; (B) BOH (C) Labetalol

2.2.2 Instrumentation

Separation of the chiral analytes was conducted on a Hewlett-Packard 3D CE instrument (model G1600AX) from Agilent (Palo Alto, CA), which uses the HP ChemStation software to

process and analyze the experimental data obtained. The analytes were detected at 220 nm using a UV diode array detector. The temperature was varied from 15 °C to 35 °C, the applied voltage ranged from 15 kV to 30 kV and the analytes were injected by pressure at 30 mbar for 3 s. Fused silica capillary columns purchased from Polymicro Technologies (Phoenix, AZ), with a total capillary length of 58 cm (50 cm effective length) and a 50 μm internal diameter were used for all separations.

2.2.3 Synthesis of Molecular Micelles

The chiral molecular micelles poly-L-SULA, and poly-L-SULV were synthesized according to the procedure outlined by Wang and Warner [20]. Briefly, undecylenic acid (UDC), dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) were combined with ethyl acetate in a round bottom flask. The mixture was left to stir for 16 hours. Then, the solution was filtered to remove the by-product, dicyclohexylurea and ethyl acetate was removed through rotary evaporation. Hot isopropanol was used to recrystalize the solution and placed in the freezer overnight. For purification, the slurry was washed with cold isopropanol and then dried for a two days using the lyophilizer. For synthesis of the surfactant monomer, the NHS ester is added to a solution containing sodium bicarbonate, deionized water, THF and the respective dipeptide amino acid. After removing THF with the rotary evaporator, dilute HCl was used to precipitate the surfactant monomer. The sodium salt of the monomer was formed by reacting the monomer with an equal amount of sodium bicarbonate. Irradiation of 100 mM solutions of the each surfactant monomer was achieved by using ^{60}Co γ-ray source for seven days.

2.2.4 Sample, Buffer, and Polymer Preparation

Stock solutions of the chiral analytes were prepared in a 50:50 methanol/ deionized water mixture. The analyte concentration was held constant at 0.2 mg/mL. The BGE used for chiral

separations consisted of 100 mM Tris and 10 mM sodium borate. A concentration of 0.1 M NaOH was used to adjust the pH of the buffer to 10.2. Before use, the buffers were sonicated for 15 minutes and filtered using a 0.45 μm polypropylene nylon filter. The cationic polymers used varied in concentration from 0.02% to 0.1% (w/v) and the anionic surfactant ranged from 0.25% to 0.75% (w/v).

2.2.5 PEM Coating Procedure

An untreated fused silica capillary was used for each PEM coating. All rinses were performed using the rinse function of the CE with applied pressure. First, the capillary was deprotonated by flushing with 1 M NaOH for 30 minutes followed by a 15 minute deionized water rinse. The first layer of the PEM coating was introduced to the capillary by flushing with the cationic polymer for 5 minutes followed by a 5 minute deionized water rinse. Then, the anionic polymer was flushed through the column for 5 minutes followed by a 5 minute deionized water rinse. The rinses with the cationic and anionic polymers were repeated until the desired number of bilayers was accomplished. The number of bilayers varied from 2 to 4 bilayers resulting in overall negative charge for the coating for the chiral analytes.

2.3 Results and Discussion

2.3.1 Comparison of the Separation of BNP in an Uncoated Capillary versus a PEM Coated Capillary

PEM coatings play an instrumental role in the separation of chiral analytes. Previous studies have shown that the binaphthyl derivative, BNP was successfully resolved using a PEM system created by 4 bilayers of 0.02% poly-L-lysine and 0.25% poly-L-SULA [18]. Using these parameters as a starting point, studies were performed to investigate the chiral selectivity of poly-L-ornithine, which is less hydrophobic than poly-L-lysine and the results are shown in Figure 2.2. BNP is an atropisomer since it has a chiral plane of symmetry instead of an asymmetric carbon. BNP was injected into an unmodified silica capillary and, as expected, the

enantiomers were not resolved because there was no chiral selector present. However, when BNP was injected into a capillary coated with 4 bilayers consisting of 0.02% poly-L-ornithine and 0.5% poly-L-SULA, baseline separation was achieved because the chiral selectivity of the system was enhanced. The migration time of BNP was longer in the PEM coated capillary because BNP, which is anionic under the experimental conditions, interacted with the hydrophobic core of the stationary phase. Similar results were obtained with other polymers (results are not shown). To optimize the separation achieved, the effects of voltage and temperature were investigated along with the effect of changing the cationic polymer and anionic polymer.

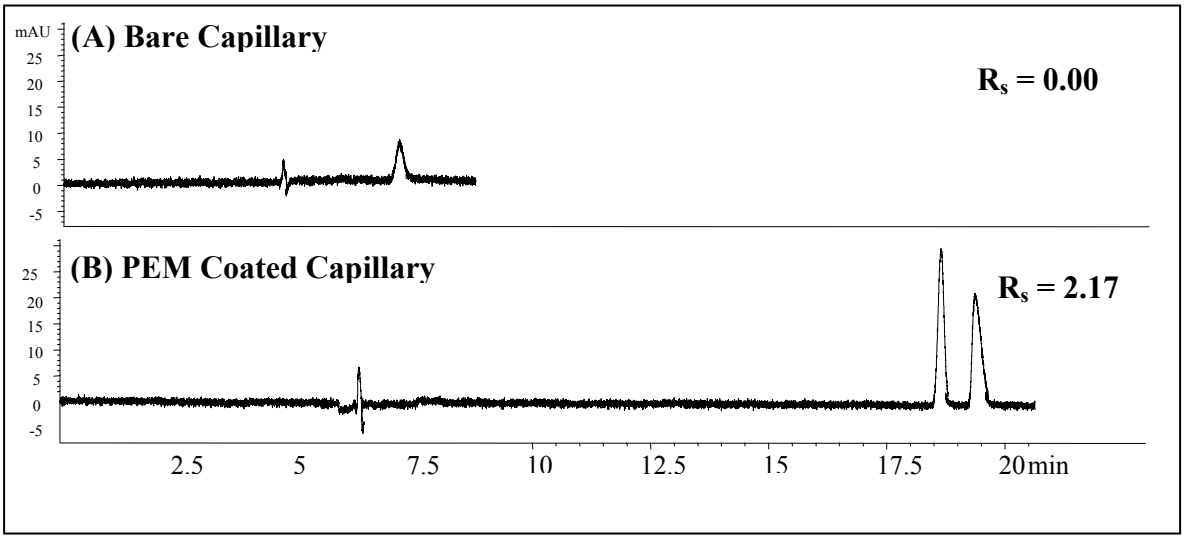


Figure 2.2 Comparison of the separation of BNP in an uncoated capillary versus a PEM coated capillary: Conditions: Cationic polymer: 0.02% (w/v) poly-L-ornithine; Anionic polymer: 0.5% (w/v) poly-L-SULA; Temperature: 15 °C; Voltage: 30 kV; Number of bilayers: 4; Capillary: 57 cm (50 cm effective length) × 50 μm i.d.; Buffer: 100 mM Tris/10 mM Borate; (pH 10.2); Analyte: 0.2 mg/mL BNP in MeOH:H_2O (50:50); Detection: 220 nm

2.3.2 Effect of Temperature on the Separation of Labetalol and BNP

In order to optimize separations, a PEM coating consisting of 4 bilayers was constructed to investigate the effect of temperature on the separation of the enantiomers of labetalol (Figure 2.3) and BNP (Figure 2.4) by varying the temperature from 15 °C to 35 °C. As temperature

increased, migration time and resolution decreased for each analyte. Because of a decrease in velocity, as temperature decreased, the analytes migration times slowed and each enantiomer interacted longer with the stationary phase resulting in increased resolution.

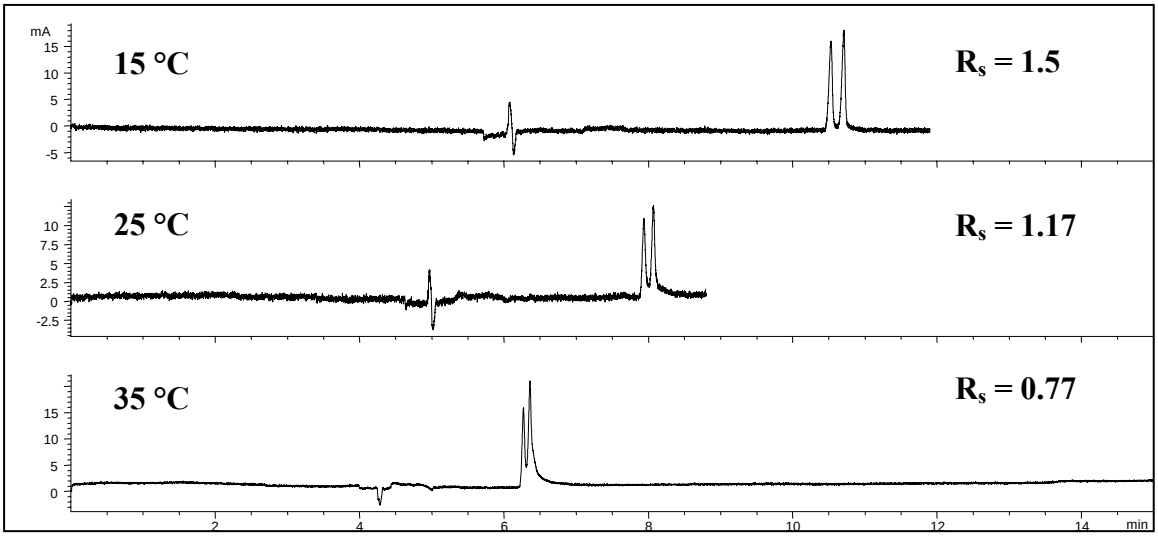


Figure 2.3 Effect of temperature on the separation of labetalol enantiomers: Conditions: Cationic polymer: 0.02% (w/v) poly-L-ornithine; Anionic polymer: 0.5% (w/v) poly-L-SULA; Temperature: 15 °C, 25 °C, 35 °C; Voltage: 30 kV; Number of bilayers: 4; Capillary*:* 57 cm (50 cm effective length) × 50 μm i.d.; Buffer: 100 mM Tris/10 mM Borate; (pH 10.2); Analyte: 0.2 mg/mL Labetalol in MeOH:H_2O (50:50); Detection: 220 nm

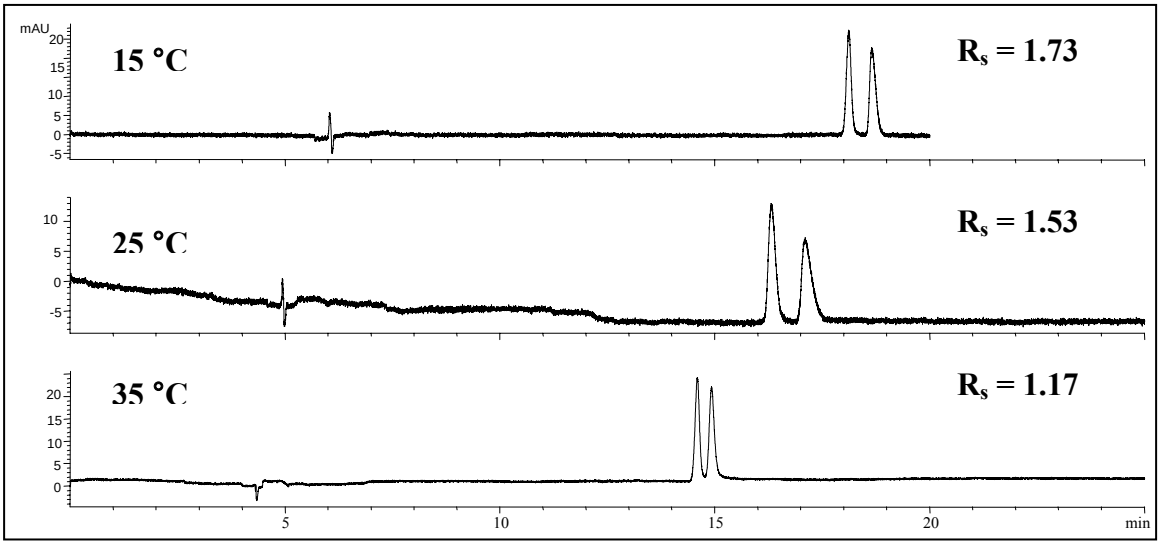


Figure 2.4 Effect of temperature on the separation of BNP: Conditions: same as in Figure 2.3

2.3.3 Effect of Voltage on the Separation of Labetalol and BNP

The effect of voltage on the separation of labetalol (Figure 2.5) and BNP (Figure 2.6) was investigated using a 4 bilayer PEM coated capillary to further optimize the separation of these analytes. The voltage was varied from 15 kV to 30 kV, using a 100 mM tris/10 mM borate, pH 10.2 running buffer. As expected, an increase in voltage to 30 kV resulted in a reduction in migration time for both labetalol and BNP. The resolution and peak efficiency were greater at higher voltages because the velocity of the analytes increased. At 15 kV, some peaked tailing was observed since the analyte interacted longer with the stationary phase causing some band broadening. This is corrected by increasing the temperature or voltage. As shown previously, 15 ˚C was the optimum temperature, therefore an increase in voltage made the migration time faster and decreased band broadening. The remaining separations used 30 kV as the optimum voltage.

2.3.4 Effect of Anionic Polymer Type on the Separation of BNP

An important consideration in this study is the choice of the anionic polymer used in a PEM coating. In this investigation, PEM coatings using 0.25% (w/v) poly- L-SULV or poly-L-SULA were studied. Poly-L-SULV is known as a versatile chiral selector and it has been used to separate a wide range of chiral compound classes. However, poly-L-SULA has been shown to be highly selective for the binaphthyl derivatives. In this study, a 4 bilayer coating consisting of 0.02% (w/v) poly-L-ornithine as the cationic polymer with either 0.25% (w/v) poly-L-SULV or 0.25% (w/v) poly-L-SULA as the anionic polymer for the separation of BNP. Representative electropherograms are shown in Figure 2.7. As seen in Figure 2.7, the migration time of BNP was faster using poly-L-SULV. However, the resolution of the enantiomers of BNP improved when poly-L-SULA was used as the anionic polymer, and only partial separation was obtained using poly-L-SULV. Therefore, poly-L-SULA was selected as the anionic polymer for all other PEM investigations.

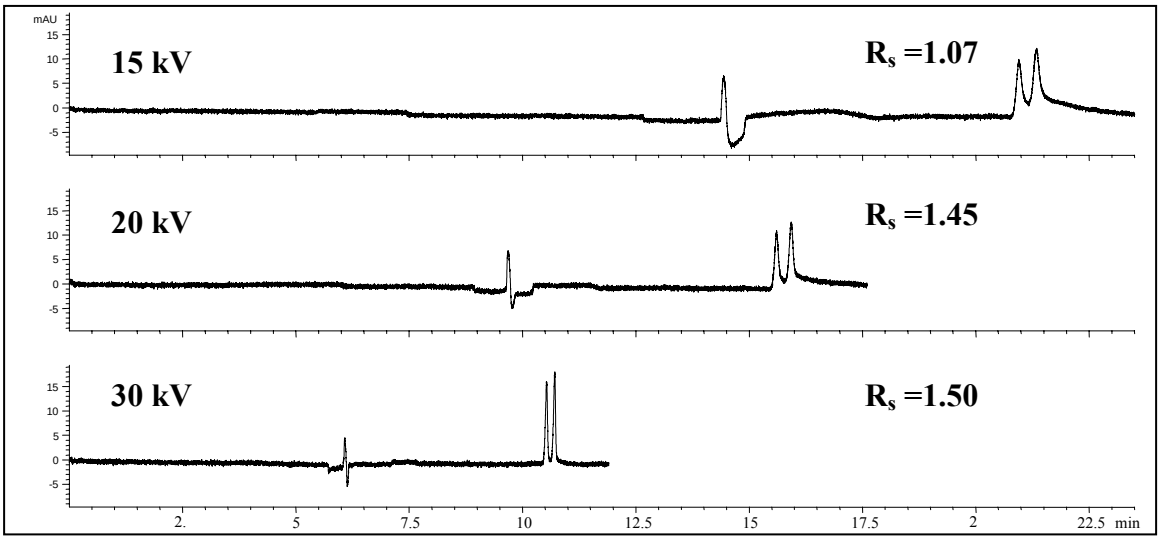


Figure 2.5 Effect of voltage on the separation of labetalol enantiomers: Conditions: Cationic polymer: 0.02% (w/v) poly-L-ornithine; Anionic polymer: 0.5% (w/v) poly-L-SULA; Temperature: 15 °C; Voltage: 15 kV, 20 kV, 30 kV; Number of bilayers: 4; Capillary: 57 cm (50 cm effective length) × 50 μm i.d.; Buffer: 100 mM Tris/10 mM Borate; (pH 10.2); Analyte: 0.2 mg/mL Labetalol in MeOH:H_2O (50:50); Detection: 220 nm

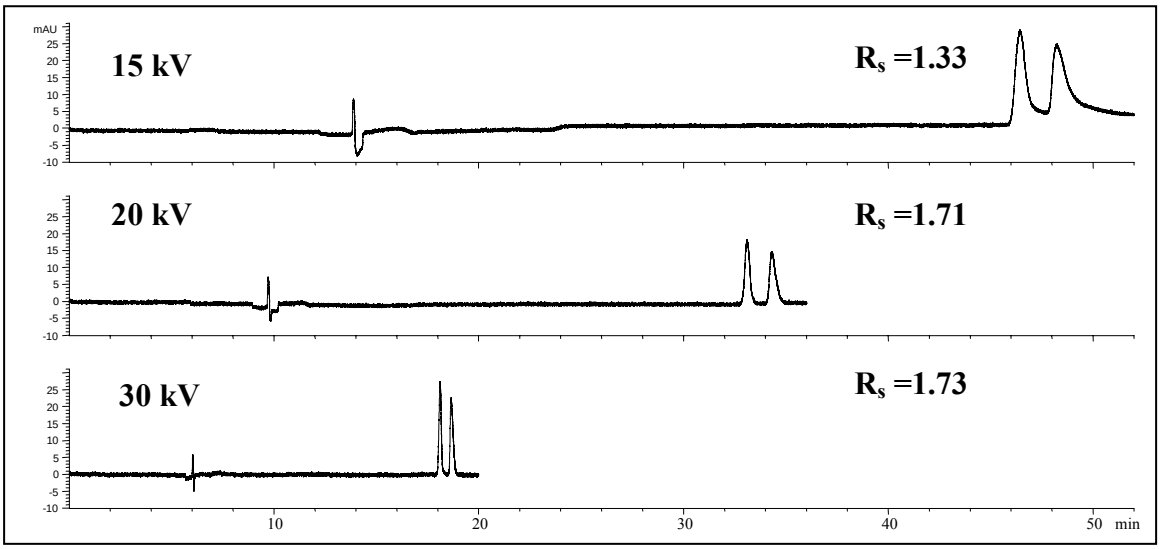


Figure 2.6 Effect of voltage on the separation of BNP: Conditions: Cationic polymer: 0.02% (w/v) poly-L-ornithine; Anionic polymer: 0.5% (w/v) poly-L-SULA; Temperature: 15 °C; Voltage: 15 kV, 20 kV, 30 kV; Number of bilayers: 4; Capillary: 57 cm (50 cm effective length) × 50 μm i.d.; Buffer: 100 mM Tris/10 mM Borate; (pH 10.2); Analyte: 0.2 mg/mL BNP in MeOH:H_2O (50:50); Detection: 220 nm

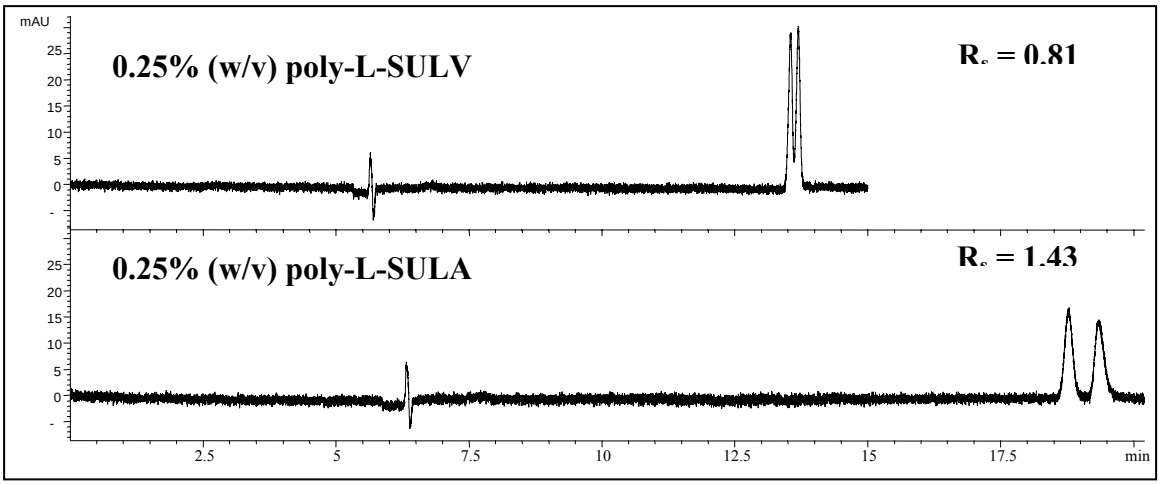


Figure 2.7 Comparison of the effect of poly-L-SULV and poly-L-SULA on the separation of BNP: Conditions: Cationic polymer: 0.02 % (w/v) poly-L-ornithine; Anionic polymer: 0.5% (w/v), poly-L-SULV/SULA; Voltage: 30 kV; Temperature: 15 ˚C; Number of bilayers: 4; Capillary: 57 cm (50 cm effective length) × 50 μm i.d.; Buffer: 100 mM Tris/10 mM Borate; (pH 10.2); Analyte: 0.2 mg/mL BNP in MeOH: H_2O (50:50); Detection: 220 nm

2.3.5 Effect of Anionic Polymer Concentration on the Separation of BNP

The effect of concentration of the anionic polymer, poly-L-SULA, was investigated to optimize separation efficiency. Previous studies have shown that an increased number of bilayers increased resolution due to increased analyte-PEM coating interactions. The results of the bilayer study are shown later, but 4 bilayers were expected to increase resolution for this particular study with BNP as the test analyte. Increasing the anionic polymer concentration is also expected to increase the PEM coating thickness, therefore, increasing analyte-PEM interactions. Figure 2.8 illustrates the electropherograms obtained when the concentration of poly-L-SULA was varied from 0.25% to 0.75% (w/v). The elution time of BNP was consistent for 0.25% and 0.5% (w/v) poly-L-SULA, and increased with 0.75% (w/v) poly-L-SULA. The highest resolution (R_S = 2.17) was observed when PEMs created using either 0.5% or 0.75% (w/v) poly-L-SULA. It is advantageous to minimize the amounts of reagents used, therefore, 0.5% poly-L-SULA was selected as the optimal concentration for the remaining studies.

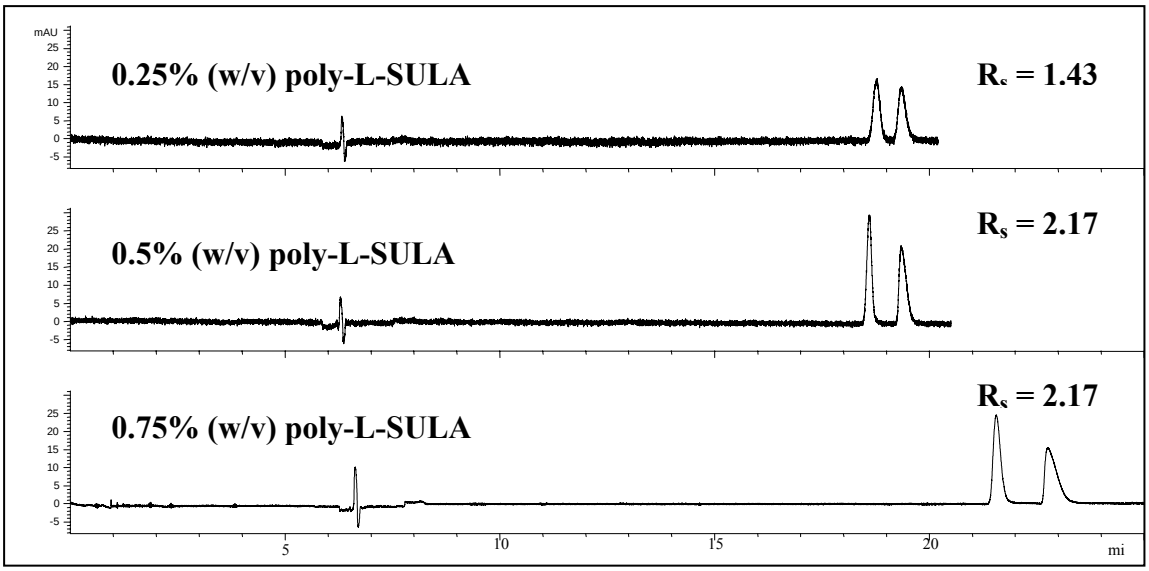


Figure 2.8 Effect of the concentration of poly-L-SULA on the separation of BNP enantiomers: Conditions: Cationic polymer: 0.02 % (w/v) poly-L-ornithine; Anionic polymer: 0.25%, 0.5%, 0.75% (w/v) poly-L-SULA; Voltage: 30 kV; Temperature: 15 ˚C; Number of bilayers: 4; Capillary: 57 cm (50 cm effective length) × 50 μm i.d.; Buffer: 100 mM Tris/10 mM Borate; (pH 10.2); Analyte: 0.2 mg/mL BNP in MeOH: H_2O (50:50); Detection: 220 nm

2.3.6 Effect of the Number of Bilayers on the Separation of Labetalol

The effect of the number of bilayers on the resolution and analysis time of labetalol was investigated. A bilayer in a PEM coating consists of one layer of a cationic polymer, in this case poly-L-ornithine, and one layer of an anionic polymer, poly-L-SULA. The coating used for these investigations consisted of 2, 3 and 4 bilayers. Previous investigations by Dubas and Schlenoff showed that an increase in the number of bilayers results in an enhanced film thickness [22]. This theory is supported by the results obtained in the electropherograms shown in Figure 2.9, where, as the number of bilayers increased from 2 to 4, the resolution and selectivity of labetalol increased. Also, the migration time increased as the bilayer number increased.

2.3.7 Effect of Variation of the Cationic Polymer on the Resolution of BNP

Another important factor in the investigation of PEM coatings is the cationic polymer used. Three cationic polymers were used in the separation of BNP: poly-L-lysine-serine, poly-L-

ornithine, and poly-L-lysine. While poly-L-lysine-serine, the most hydrophobic polymer, produced the shortest retention time, complete resolution of BNP enantiomers was achieved using poly-L-ornithine, the least hydrophobic polymer. Poly-L-glutamic acid-lysine was used as the cationic polymer to resolve BNP however, due to instability with the PEM coating, the separation was unsuccessful.

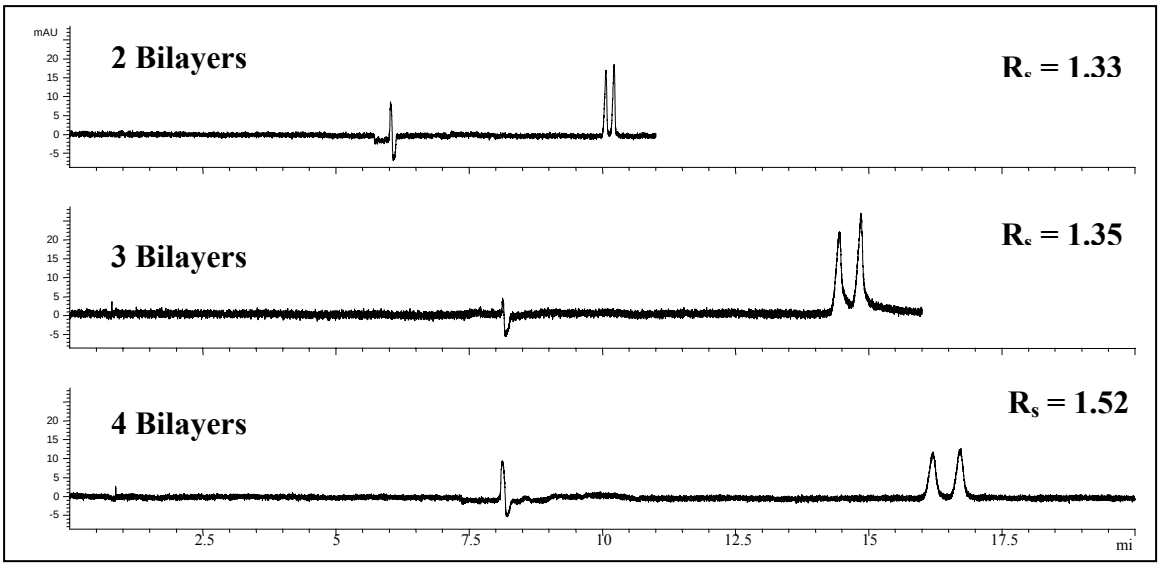


Figure 2.9 Effect of the number of bilayers on the separation of Labetalol enantiomers: Conditions: Cationic polymers: 0.02% (w/v), poly-L-glutamic acid-lysine; Anionic polymer: 0.5% (w/v) poly-L-SULA; Voltage: 30 kV; Temperature: 15 ˚C; Buffer: 100 mM Tris/10 mM Borate; (pH 10.2); Capillary: 57 cm (50 cm effective length) × 50 μm i.d.; Detection: 220 nm; Analyte: 0.2 mg/mL labetalol, MeOH: H_2O (50:50)

2.3.8 Effect of Variation of the Cationic Polymer on the Resolution of BOH

BOH is partially anionic under these experimental conditions and more hydrophobic than BNP. The cationic polymer in the PEM coating was also varied in order to investigate its influence on the separation of BOH. The electropherograms showing resolution and retention times are shown in Figure 2.11. A close inspection of Figure 2.11 shows optimal separation of BOH enantiomers was obtained when poly-L-ornithine was used to generate the PEM coating. Changes in migration time and resolution as the cationic polymer was varied are consistent with

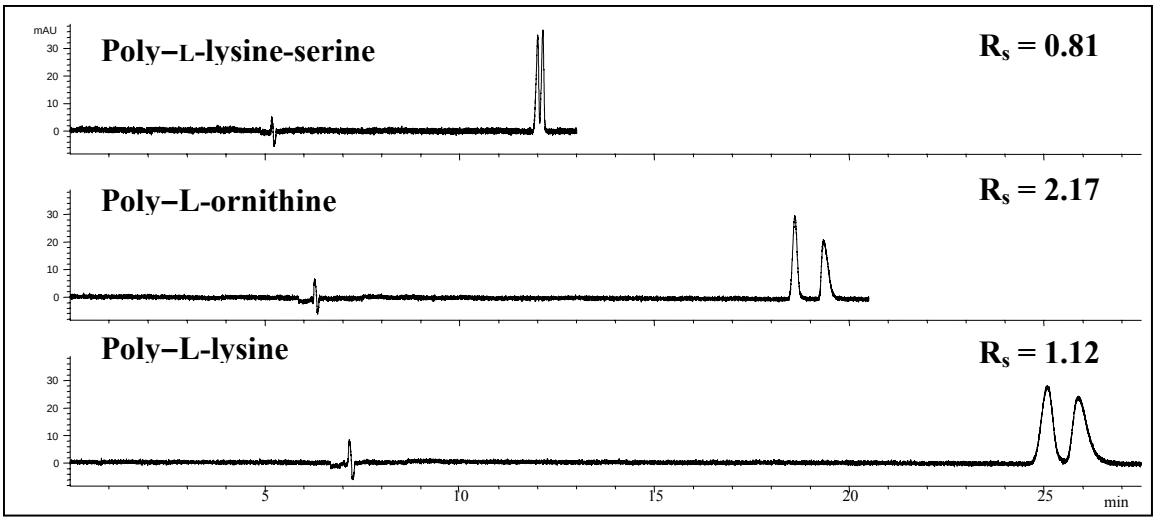


Figure 2.10 Effect of variation of the cationic polymer on the resolution of BNP enantiomers: Conditions: Cationic polymer: 0.02% (w/v); Anionic polymer: 0.5% (w/v), poly-L-SULA; Temperature: 15 ˚C; Voltage: 30 kV; Number of bilayers: 4; Capillary: 57 cm (50 cm effective length) × 50 μm i.d; Buffer: 100 mM Tris/10 mM Borate; (pH 10.2); Analyte: 0.2 mg/mL BNP in MeOH: H_2O (50:50); Detection: 220 nm

those observed in the separation of BNP. The reduction in peak efficiency of BOH relative to BNP may be the result of the higher hydrophobicity of BOH. Perhaps because of stronger interactions with BOH, the use of poly-L-glutamic acid-lysine, the most hydrophobic of the four cationic polymers, resulted in the slowest migration time. This supposition is supported by the observation of increased peak tailing when poly-L-glutamic acid-lysine is used to create the PEM coating.

2.3.9 Effect of Variation of the Cationic Polymer on the Resolution of Labetalol

Figure 2.12 are the electropherograms attained when the enantiomers of labetalol was separated to investigate the effect of varying the cationic polymer. Contrary to separations of binaphthyl derivatives, enantiomers of labetalol were baseline resolved when poly-L-glutamic acid-lysine was used as the cationic polymer in the PEM coating. However, it should be noted that the slight decrease in resolution achieved with the poly-L-ornithine coating was far outweighed by the reduction in analysis time and improvement in peak shape compared to poly-

L-glutamic acid-lysine coatings. Trends in labetalol migration time were similar to those observed for the binaphthyl derivatives as the cationic polymer was changed.

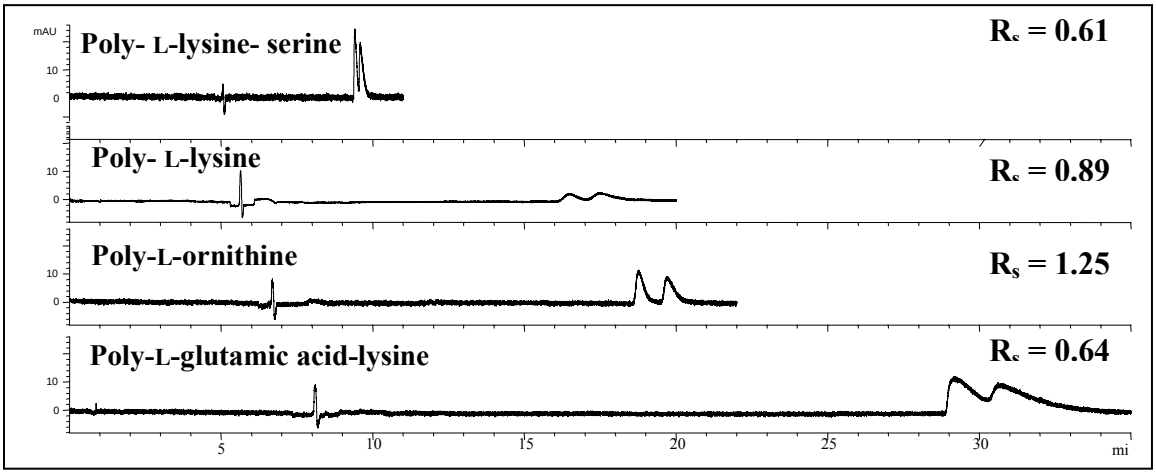


Figure 2.11 Effect of variation of the cationic polymer on the resolution of BOH enantiomers: Conditions: Cationic polymer: 0.02% (w/v); Anionic polymer: 0.5% (w/v), poly-L-SULA; Voltage: 30 kV; Temperature: 15 ˚C; Number of bilayers: 4; Capillary: 57 cm (50 cm effective length) × 50 μm i.d; Buffer: 100 mM Tris/10 mM Borate; (pH 10.2); Analyte: 0.2 mg/mL BOH in MeOH: H_2O (50:50); Detection: 220 nm

2.3.10 Investigation of the Reproducibility of a PEM Coated Capillary

The reproducibility of a PEM coating plays an important role in the determination of the column performance and stability. For all stability studies, the temperature and the voltage were maintained at 15 ˚C and 30 kV, respectively. Stability and reproducibility studies performed using a capillary coated with 4 bilayers of 0.02% (w/v) poly-L-ornithine and 0.5% (w/v) poly-L-SULA. The electropherograms in Figure 2.13 show the first, fourth, and sixth run on the same capillary. Table 1 demonstrates the excellent run-to-run reproducibility of the EOF. In order to demonstrate superior coating stability, the same PEM coated column was used over a period of five days (350 runs) to separate labetalol. Following each run the capillary was flushed with 100 mM Tris/10 mM borate buffer, pH 10.2, for 1 minute and the running buffer was replaced after 20 runs to maintain a stable current.

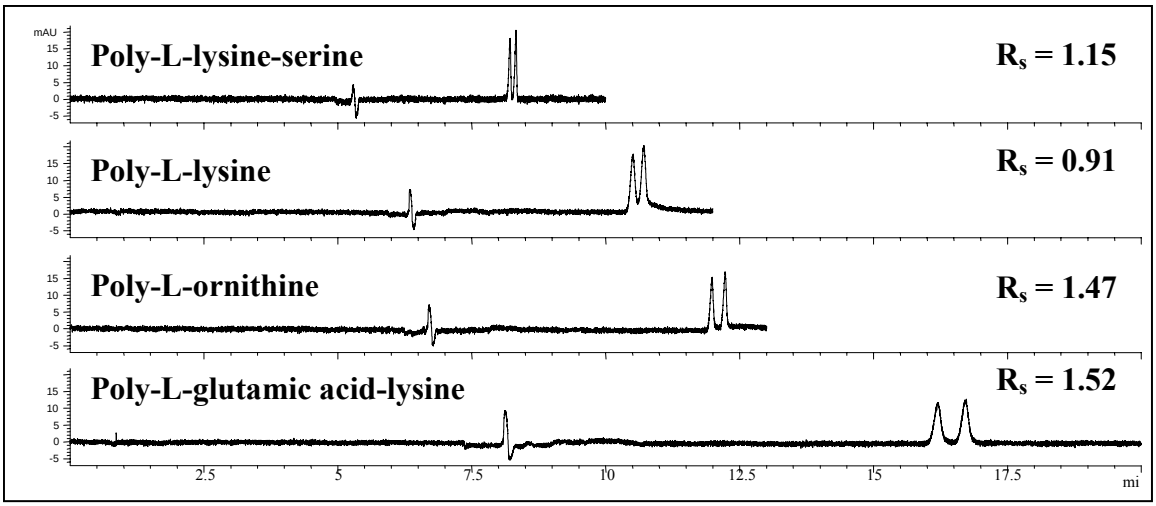


Figure 2.12 Effect of variation of the cationic polymer on the resolution of labetalol enantiomers: Conditions: Cationic polymer: 0.02% (w/v); Anionic polymer: 0.5% (w/v) poly-L-SULA; Temperature: 15 ˚C; Voltage: 30 kV; Number of bilayers: 4; Capillary: 57 cm (50 cm effective length) × 50 μm i.d; Buffer: 100 mM Tris/10 mM Borate; (pH 10.2); Analyte: 0.2 mg/mL, MeOH: H_2O (50:50); Detection: 220 nm

The column was able to endure over 350 runs with a %RSD of the EOF of 0.63%.

The determine column-to-column reproducibility, the separation of labetalol was performed on five different columns using the same type of PEM coating. Five consecutive runs were used to obtain the %RSD for each of the five columns investigated. Table 2 shows the %RSD values of the EOF for the five columns investigated to be 0.91%.

2.4 Concluding Remarks

In this study, novel PEM coatings were constructed using four different cationic polymers: poly-L-ornithine, poly-L-lysine, poly-L-lysine-serine and poly-L-glutamic acid-lysine. 0.5% poly-L-SULA was chosen as the optimal concentration and molecular micelles to be used as the anionic polymer in the PEM coatings studied. As previously stated, it was shown that the resolution of labetalol increased with increasing number of bilayers. Poly-L-ornithine gave the best resolution for both BNP and BOH when compared to the other cationic polymers while poly-L-glutamic acid-lysine showed the best resolution of labetalol. The PEM coated column

endured over 350 runs and the %RSD of the run-to-run reproducibility of the EOF was 0.63% with 4 bilayers. The %RSD of the column-to-column reproducibility of the EOF was 0.91% with 4 bilayers. In the future, the effect of the concentration of the cationic polymers will be investigated and also the separation of more chiral analytes will be explored

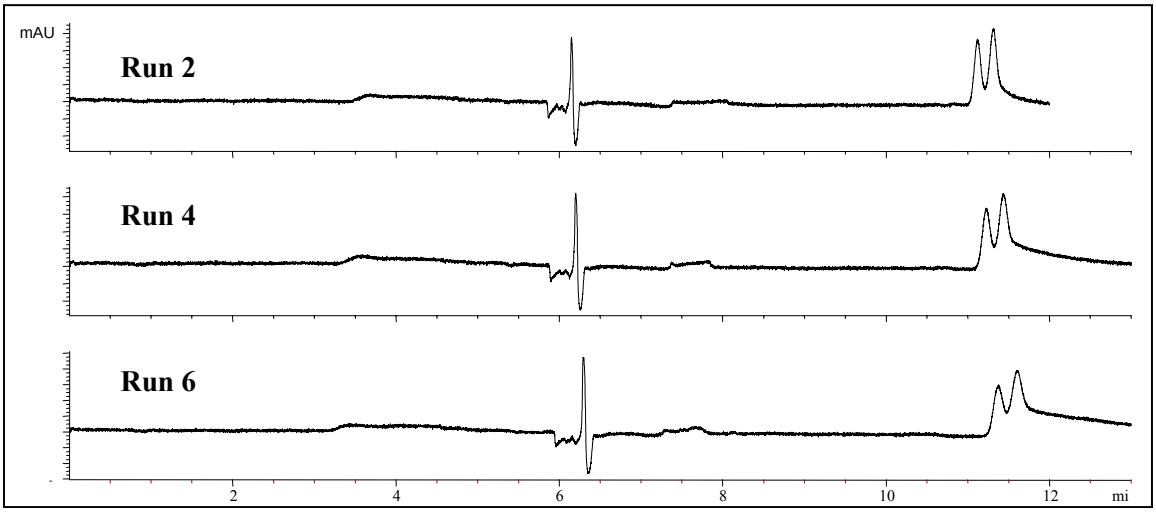


Figure 2.13 Investigation of the run-to-run reproducibility of a PEM coated capillary: Conditions: Total number of runs: 350; Cationic polymer: 0.02% (w/v) poly-L-ornithine; Anionic polymer: 0.5% (w/v) poly-L-SULA; Number of bilayers: 4; Temperature: 15 °C; Voltage: 30 kV; Capillary: 57 cm (50 cm effective length) × 50 μm i. d.; Buffer: 100 mM Tris/10 mM Borate (pH 10.2); Analyte: 0.2 mg/ml Labetalol MeOH: H_2O (50:50); Detection: 220 nm

Table 2.1 Run-to-Run Reproducibility**:** Conditions: Same as in Figure 2.13

Number of Columns	1
Column Endurance (total number of runs)	> 350
Consecutive Runs	5
%RSD of EOF	0.63

Table 2.2 Column-to-Column Reproducibility**:** Conditions: Same as Figure 2.13

Number of Columns	5
Consecutive Runs	5
%RSD of EOF	0.91

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CHAPTER 3

BASIC PROTEIN SEPARATIONS USING PEM COATINGS WITH MOLECULAR MICELLES IN OPEN TUBULAR CAPILLARY ELECTROCHROMATOGRAPHY*

3.1 Introduction

It is well established that the separation of relatively large basic molecules, such as proteins and peptides, are problematic when using capillary zone electrophoresis (CZE) [1-4]. Proteins are generally difficult to separate in capillary electrophoresis (CE) because of structural similarities, chemical properties, and adsorption to the negatively charged walls of the capillary under conditions where the proteins are cationic. Thus, protein adsorption due to electrostatic interactions and/or hydrogen bonding, may cause several problems such as peak tailing, unstable baselines, poor run-to-run reproducibility, low peak efficiencies, and low sample recovery [5, 6].

Capillary electrochromatography (CEC) has been employed to overcome the above cited problems through prevention of sample adsorption as well as providing other beneficial interactions [5, 6, 23, 30]. CEC is a versatile combination of CE and high performance liquid chromatography (HPLC) that has attracted the interest of separation scientists in recent years. This is likely because the CEC method successfully combines the selectivity of HPLC and the efficiency of CE, allowing the separation of charged, as well as neutral analytes [7, 8]. In general, separation of charged analytes using CEC is based on combined differences in electrophoretic mobility and partitioning into the stationary phase. In contrast, neutral analytes are separated solely through interactions with the stationary phase coating on the capillary wall [8]. For protein separations using CEC, the stationary phase has three main functions. It is used to coat the silanol groups at the capillary wall, hence, minimizing protein adsorption on the negatively charged walls. Secondly, the stationary phase can control or modify the EOF.

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Finally, the stationary phase provides a medium for protein interactions.

Stationary phases for open tubular capillary electrochromatography (OT-CEC), one form of CEC, have been prepared using several different approaches. These include covalent bonding and/or crosslinking [10], formation of porous silica layers [11], chemical bonding after etching [12], sol gel [13] and adsorption [9]. In the case of adsorption, the stationary phase can either be dynamically or physically adsorbed to the capillary wall in order to effectively shield the negatively charged silanol groups with a layer of the coating material [9, 14]. In a dynamically coated capillary, the adsorption of the coating material to the capillary wall is relatively weak, and thus the coating material is placed in the running buffer to ensure the stability of the coating. In contrast, if the adsorption of the coating material is strong, i.e. it is strongly physically adsorbed to the wall, then the addition of the coating material to the running buffer is not necessary [9, 14]. One of the most stable coatings, obtained by a physical adsorption process, is a polyelectrolyte multilayer (PEM) coating, particularly when a molecular micelle is used as the anionic phase [7, 15, 17].

PEM coatings are constructed by use of several electrostatic interactions involving ion exchange, often resulting in extremely stable coatings [15-20]. A PEM coating is formed by alternately rinsing the anionic inner wall of a silica capillary, first with cationic polymers followed by anionic polymers. The combination of one cationic layer and one anionic layer is referred to as a bilayer [15, 19, 21, 22]. PEM coatings offer several advantages over other stationary phases because a wide range of coating polymers of different structural and chemical properties can be used [23]. These polymers include those that are commercially available or polymers that are synthesized in research laboratories, e.g. our molecular micelles [24-33]. Polymers used in PEM coatings may affect the resolution, selectivity and retention time of the analytes because the interactions between the PEM coating and analyte may vary. Furthermore,

several studies have also proven that PEM coatings are robust and stable over a wide pH range [14, 16, 18, 20].

Protein separations are achieved by suppressing protein adsorption to the capillary wall due to electrostatic repulsion of like charges between proteins and polymers as well as protein interactions with the stationary phase. The interactions between proteins and PEM coatings have become an area of growing interest, especially in bio-analytical chemistry. Proteins have been successfully separated using PEM coated columns and the mechanisms of separation are based on differences in protein-PEM interactions [34]. The overall charge of the last layer of PEM coatings and the net charge of proteins play an important role in protein adsorption and interaction with PEM coatings. Salloum and Schlenoff [34] have investigated protein adsorption onto PEM coatings as well as the influence of surface charge, ionic strength, and thickness of the PEM coating on protein-PEM interactions. Results from that study showed that the last layer of PEM coatings determines how proteins adsorb and interact with the coating. Furthermore, the net surface charge of PEM coatings can be tailored so as to allow or retard protein adsorption. Protein adsorption on oppositely-charged surfaces is due to electrostatic interactions, whereas adsorption on like-charged surfaces can be attributed to non-electrostatic interactions [34]. In addition, Salloum and Schlenoff [34] have also demonstrated that proteins can be adsorbed and interact within the multilayers of PEM coatings if proteins and PEM coatings are of opposite charge. However, when proteins are of the same charge as PEM coatings, interactions occur at the surface layer regardless of PEM coating thickness [34].

Several studies involving the separations of cationic proteins using a positively charged wall coating have been previously performed [5, 6, 23, 34, 35]. Wang and Dubin [23] have investigated the influence of an immobilized adsorbed coating using poly(dimethyldiallyammonium chloride) (PDADMAC) as the coating polymer for the separation

of cationic proteins. Results indicated that high molecular weight polymers at high ionic strengths provided optimal coating conditions. In addition, the authors reported that the coating polymer formed loops and tails which were important for the reversal of the EOF and provided a stationary phase for protein interactions as well as increased the stability and efficiency of the coated column [23]. Graul and Schlenoff [5] have used PDADMAC and poly (styrene sulfonate) (PSS) as polyelectrolytes in PEM coatings for the separation of four basic proteins. Also, the authors investigated the influence of the number of bilayers on protein resolution and reported that the use of 6.5 bilayers improved protein resolution [5].

The optimization of separation parameters to achieve high resolution separations in a reasonable analysis time continues to be an active area of research [36-38]. Examples of such parameters include the choice and concentration of the cationic and anionic polymer, as well as the applied voltage, temperature, background electrolyte pH, and the number of bilayers in the PEM coating. The conventional approach to optimizing a PEM coating can be a tedious process since only one parameter is usually varied at a time. In recent studies, experimental design and multivariate analysis have been widely used to simultaneously optimize separation parameters, leading to higher resolution in relatively short analysis times [26, 39-44]. We have previously reported the use of experimental design and multivariate analysis for the optimization of separation parameters to predict the migration time, resolution, and resolution per unit time of several chiral and achiral analytes using micellar electrokinetic chromatography [26]. We also previously used experimental design to optimize the separation conditions of two different stationary phases for the separation of four basic peptides in OT-CEC [39]. Yang et al. [44] have used experimental design, specifically central composite design, in CEC to simultaneously optimize the separation parameters of 11 nucleosides and nucleobases in Cordyceps sinensis, a traditional Chinese medicine.

In the study reported in this chapter, a Box Behnken experimental design is used to optimize separation parameters using four different chiral cationic polymers and the anionic molecular micelle, poly-L-SULA for PEM coatings in the OT-CEC separation of four basic proteins: α-chymotrypsinogen, lysozyme, ribonuclease A and cytochrome c. The cationic polymers investigated were poly-L-glutamic acid-lysine, poly-L-lysine-serine, poly-L-lysine, and poly-L-ornithine. The anionic polymers were the chiral molecular micelles sodium poly(*N*-undecanoyl-L-leucyl-alaninate) (poly-L-SULA), sodium poly(*N*-undecanoyl-L-leucyl-valinate) (poly-L-SULV) as well as the achiral molecular micelle sodium poly(undecylenic sulfate) (poly-SUS). The effects of the type and concentration of polymers, applied voltage, temperature, background electrolyte pH, as well as the number of bilayers on the resolution of the four basic proteins were investigated. In addition, the influence of added NaCl to the PEM coatings and the effect of PEM coating thickness on the reproducibility of protein separations were investigated.

3.2 Materials and Methods

3.2.1 Materials

The cationic polymers, poly-L-glutamic acid-lysine hydrobromide, poly-L-lysine-serine hydrobromide, poly-L-lysine hydrobromide, and poly-L-ornithine hydrobromide, as well as the proteins, α-chymotrypsinogen A (type II from bovine pancreas), lysozyme (from chicken egg white), ribonuclease A (from bovine pancreas), and cytochrome c (from bovine pancreas) were purchased from Sigma Chemical Company (St. Louis, MO). The chemicals used to synthesize surfactant monomers *N*-hydroxysuccinimide, undecylenic acid, chlorosulfonic acid, dicyclohexylcarbodiimide, and sodium bicarbonate were purchased from Fluka (Milwaukee, WI). The dipeptides (L,L)-leucyl-alaninate and (L,L)-leucyl-valinate were purchased from Bachem Bioscience, Inc. (King of Prussia, PA). Sodium hydroxide, ethyl acetate, and tetrahydrofuran were purchased from Sigma-Aldrich (Milwaukee, WI). The background

electrolyte, sodium phosphate dibasic, methanol, and acetone were purchased from Fisher Scientific (Fair Lawn, NJ). All materials were used as received.

3.2.2 Instrumentation

The protein separations were conducted using a Beckman P/ACE MDQ capillary electrophoresis system, equipped with a photodiode array detector (Fullerton, CA). A fused silica capillary, with an internal diameter of 50μm was purchased from Polymicro Technologies (Phoenix, AZ). The total length of the capillaries used in this study was 37cm (30cm effective length). The temperatures used in this study were varied from 15°C to 35°C using a liquid coolant. The applied voltage ranged from 15kV to 30kV. The analytes were detected at 214 nm and the samples were injected using 5kV for 5s.

3.2.3 Syntheses of Molecular Micelles

The chiral molecular micelles poly-L-SULA and poly-L-SULV used in the PEM coating were synthesized according to a procedure previously described by Wang and Warner [27] and in chapter 2. The achiral molecular micelle, sodium poly(undecylenic sulfate), (poly-SUS) was synthesized according to the procedure previously reported by Shamsi et al [45]. Briefly, in a round bottom flask, chlorosulfonic acid, undecenyl alcohol and pyridine were combined and refluxed for 3 hours. Then, a solution of sodium hydroxide and sodium carbonate in deionized water was added to the flask and stirred for 16 hours. To isolate the SUS monomer, n-butanol and pyridine were evaporated using the rotary evaporator and dessicator. Hot isopropanol was then added to the resulting solution followed by overnight refrigeration. The following day, purification was completed with a cold isopropanol rinse and the sample was then dried on the lyophilizer. Polymerization of the monomers at a concentration of 100mM was achieved using a ^{60}Co γ-ray irradiation source. Representations of the chemical structures of the molecular micelles as well as the chiral cationic polymers used in this study are presented in Figure 3.1.

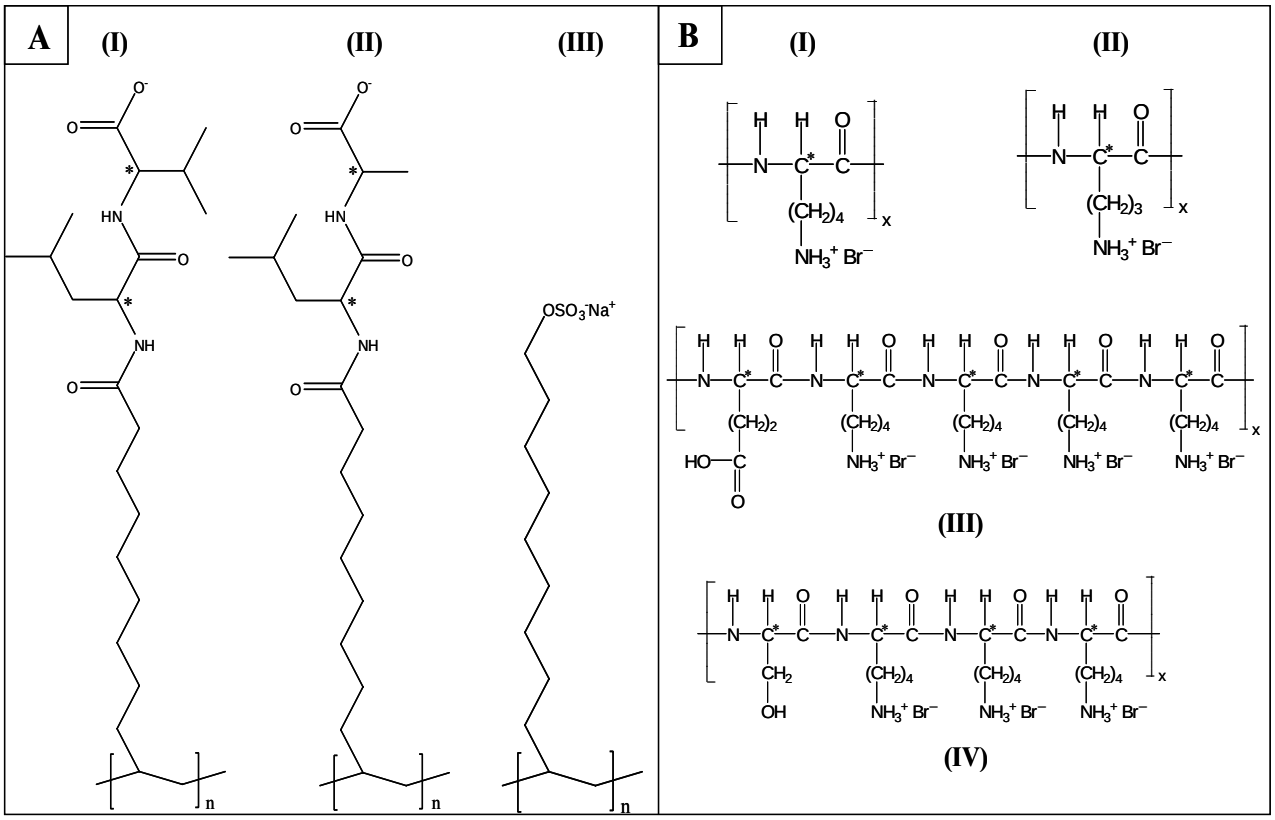

Figure 3.1 Representative molecular structures of polymers used in PEM coatings: A: Anionic polymer: (I) Poly-L-SULV; (II) Poly-L-SULA; (III) Poly-SUS (* indicates the chiral center) B: Cationic Polymers: (I) Poly-L-lysine hydrobromide; (II) Poly-L-ornithine hydrobromide; (III) Poly-L-glutamic acid-lysine hydrobromide; (IV) Poly-L-lysine-serine hydrobromide

3.2.4 Sample and Buffer Preparation

The pH of the background electrolyte (20mM phosphate buffer) was adjusted using 1M HCl. The buffer was filtered using a 0.45µm polypropylene nylon filter and sonicated for 15 minutes before use. Protein stock solutions were prepared in 20mM phosphate buffer at pH 3, 4, and 5. All protein analyte concentration were set at 0.5mg/mL. The cationic polymers, poly-L-glutamic acid-lysine hydrobromide, poly-L-lysine-serine hydrobromide, poly-L-lysine hydrobromide, and poly-L-ornithine hydrobromide (Figure 3.1) varied in concentration from 0.01% to 0.03% (w/v) in deionized water. The concentrations of the anionic molecular micelles,

poly-L-SULA, poly-L-SULV, and poly SUS were held constant at 0.5% (w/v) in deionized water for studies.

3.2.5 Polyelectrolyte Multilayer Coating Procedure

A fused silica capillary was deprotonated by flushing the capillary with 1M NaOH for 30 minutes followed by a 15 minute deionized water rinse. The first layer of the PEM coating was obtained by rinsing the capillary with the cationic polymer for 10 minutes, followed by a 5 minute deionized water rinse. Then, the anionic polymer was rinsed through the column for 5 minutes, followed by a 5 minute deionized water rinse. Unless otherwise noted, alternate 5 minute rinses with the cationic and anionic polymers were repeated to obtain the desired number of bilayers. The number of bilayers were varied from 1.5 to 3.5, and the outermost layer of the PEM coating was maintained to achieve a positive charge. All rinses were performed using the rinse function of the CE instrument, with applied pressure of 20psi. All experiments run with PEM coated capillaries were completed in reverse polarity.

3.2.6 Experimental Design Procedure and Data Analysis

In general, the usual goal of a separation is to achieve baseline resolution ($R_{s1} = 1.5$) with minimum analysis time. Traditionally, routine optimization of separation parameters in chromatography involves a variation of one parameter at a time with numerous trials, which may only result in local optimum conditions. In contrast, the use of experimental design enables the determination of global optimum separation conditions, since all the parameters are simultaneously optimized. Separation parameters such as the cationic polymer, cationic polymer concentration, number of bilayers, temperature, applied voltage, and background electrolyte pH may significantly influence the resolution and migration time of analytes in OT-CEC. In this study, a Box Behnken experimental design was used to simultaneously optimize these parameters (design variables) for protein separations. Box Benken experimental design was

selected as the design technique, since it requires relatively few experiments for optimization. This approach was used to study the design variables at three levels (low, medium, and high), allowing investigation of the primary and interactive effects on protein resolution (response). The design variables and the levels used for this optimization are shown in Table 3.1.

Table 3.1 Design variables used in box behnken design

Parameter	*Levels*		
	Low	**Medium**	**High**
Temperature (°C)	15	25	35
Voltage (kV)	15	20	30
Number of bilayers	1.5	2.5	3.5
Cationic Polymer concentration (%w/v)	0.01	0.02	0.03
Background electrolyte	3	4	5

A total of 43 experiments were conducted for each polymer under a variety of experimental conditions. The experimental design and data analysis were performed using The Unscrambler (CAMO, Corvallis, OR, version 9.1) chemometric software. Reproducibility studies were performed for each cationic polymer and the experiments were completed in triplicate under optimum separation conditions.

3.3 Results and Discussion

3.3.1 Separation of Four Basic Proteins Using a PEM Coated Capillary

The primary aims of this study were to minimize the adsorption of proteins onto the capillary wall during the separation process by using a PEM coated capillary, and secondly to influence protein separations by providing a stationary phase for protein interactions. In figure 3.2, the separation of proteins in an uncoated capillary performed in reverse polarity resulted in poor reproducibility, poor peak efficiency, and peak tailing. As the protein injection number increased, the quality of the protein separations decreased. Figure 3.3 illustrates the advantage of

using PEM coated columns in the separation of basic proteins (α-chymotrypsinogen A, ribonuclease A, lysozyme, and cytochrome c) as compared with an uncoated capillary column.

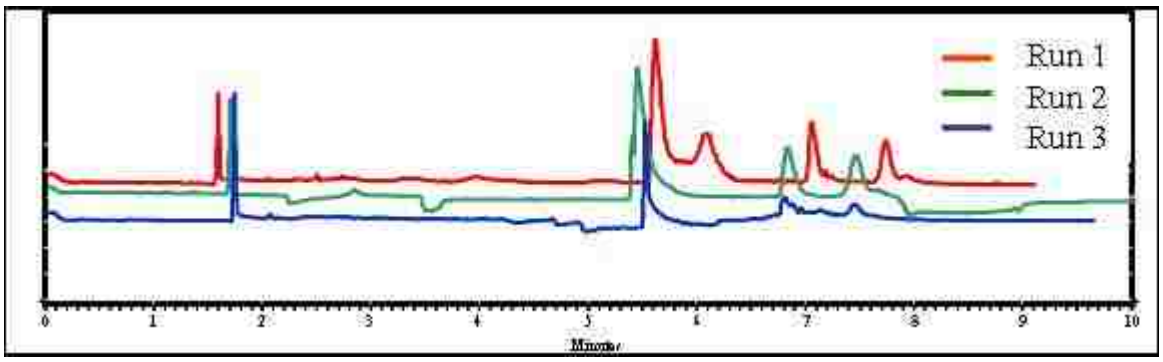

Figure 3.2 Protein separations in an uncoated capillary: Conditions: Bare Capillary *(reverse polarity)*; Analytes: 1. α-chymotrypsinogen A; 2. ribonuclease A; 3. lysozyme; 4. cytochrome c; Temperature: 15 ºC; Voltage: 15 kV. Buffer: 20 mM phosphate, pH 4

In contrast to an uncoated column (Figure 3.3A), better resolution, higher peak efficiency, and greater reproducibility were obtained when PEM coated capillaries were used for protein separations. Figure 3.3B shows the results obtained when one layer of 0.03% (w/v) poly-L-ornithine was used to coat the capillary wall. This 0.5 bilayer coating provided a reversal of EOF since the overall charge on the capillary wall was positive. Separation of α-chymotrypsinogen A and ribonuclease A (peaks 1 and 2) improved as compared to the uncoated capillary. However, lysozyme and cytochrome c (peaks 3 and 4) were only partially separated. There was in fact an increase in the retention time of the protein mixture corresponding to decreased electroosmotic mobility. Good run to run reproducibility was attained using 0.5 bilayer coated column. The addition of the molecular micelle, poly-L-SULA in Figure 3.3C produced increased resolution of peaks 3 and 4 (lysozyme and cytochrome c) as compared to 0.5 bilayers (no molecular micelle) in Figure 3B. Figure 3.3C and 3.3D illustrates protein separations using 1.5 and 2.5 bilayers respectively, obtained with 0.03% (w/v) poly-L-ornithine as the cationic polymer and 0.5% (w/v) poly-L-SULA as the anionic polymer. Increasing

resolution between peaks 3 and 4 as the number of bilayers increased (Figure 3.3B-3.3D) results from increased interaction of peak 4 (cytochrome c) with the stationary phase.

The separation of these four proteins in OT-CEC was further optimized using a Box Behnken design with four different PEM coatings in which the cationic polymer was varied and the anionic polymer, poly-L-SULA, was held constant. The resolution results for various separation conditions with the four cationic polymers are shown in Figure 3.4 (Tables of resolution values of each cationic polymer are reported in Tables 3.2 - 3.5).

Resolution (R_s) values were calculated for each experiment using the equation:

$$R_s = [2(t_2 - t_1)/(w_1 + w_2)] \quad (1)$$

where, t_1 and t_2 are the elution times of the first and second peak, and w_1 and w_2 are the baseline widths of each peak. Three resolution values were calculated for each experimental run. The resolution between peaks 1 and 2 was termed R_{s1}, peaks 2 and 3, R_{s2}, and peaks 3 and 4, R_{s3} respectively. All investigated cationic polymers successfully resolved all four proteins. However, the use of poly-L-glutamic acid-lysine (Figure 3.4A) generally resulted in higher resolution for the four proteins. Poly-L-lysine-serine (Figure 3.4B) and poly-L-ornithine (Figure 3.4D) have similar protein resolution capabilities, while the lowest protein resolution was obtained when poly-L-lysine was used as the cationic polymer for the PEM coating (Figure 3.4C).

Response surface plots for the influence of temperature and voltage on protein resolution using poly-L-glutamic acid-lysine as the cationic polymer and poly-L-SULA as the anionic polymer in the PEM coating were generated and the results are shown in Figure 3.5. In general, increasing the temperature resulted in faster migration times due to an increase in the electroosmotic mobility.

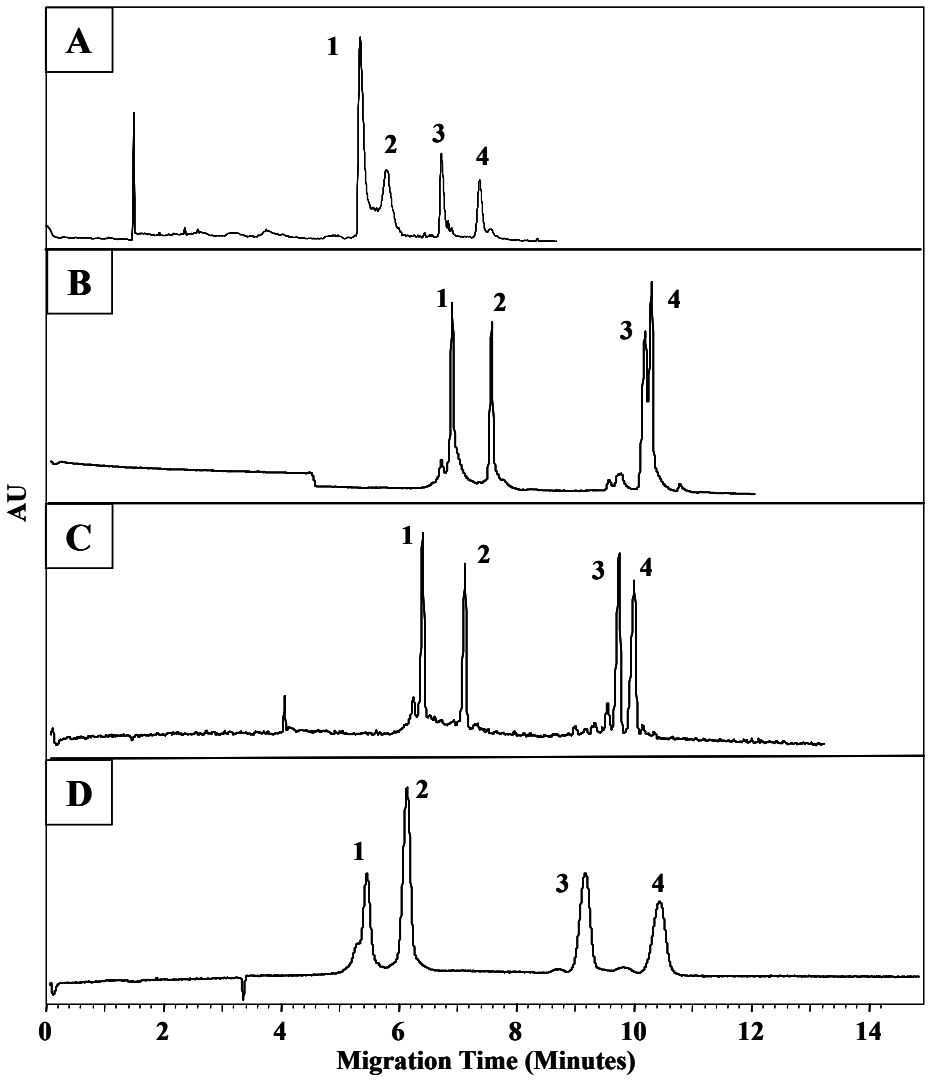

Figure 3.3 Separation of basic proteins using an uncoated capillary and PEM coated capillaries: Conditions*:* Coating: (A): uncoated capillary (normal polarity) (B): 0.5 bilayers (reverse polarity); (C): 1.5 bilayers (reverse polarity); (D) 2.5 bilayers (reverse polarity); Cationic Polymer: 0.03% (w/v) poly-L- ornithine; Anionic Polymer: 0.5% (w/v) poly-L-SULA; Analytes: 1.α-chymotrypsinogen A; 2. ribonuclease A; 3. lysozyme; 4 .cytochrome c; Buffer: 20 mM phosphate, pH 4; Analyte concentration: 0.5 mg/ml; Capillary length: 37 cm total (30 cm effective length); Capillary i.d.: 50 μm; Temperature: 15 ºC; Voltage: 15 kV; Injection: 5 kV for 5 s; Detection: 214 nm

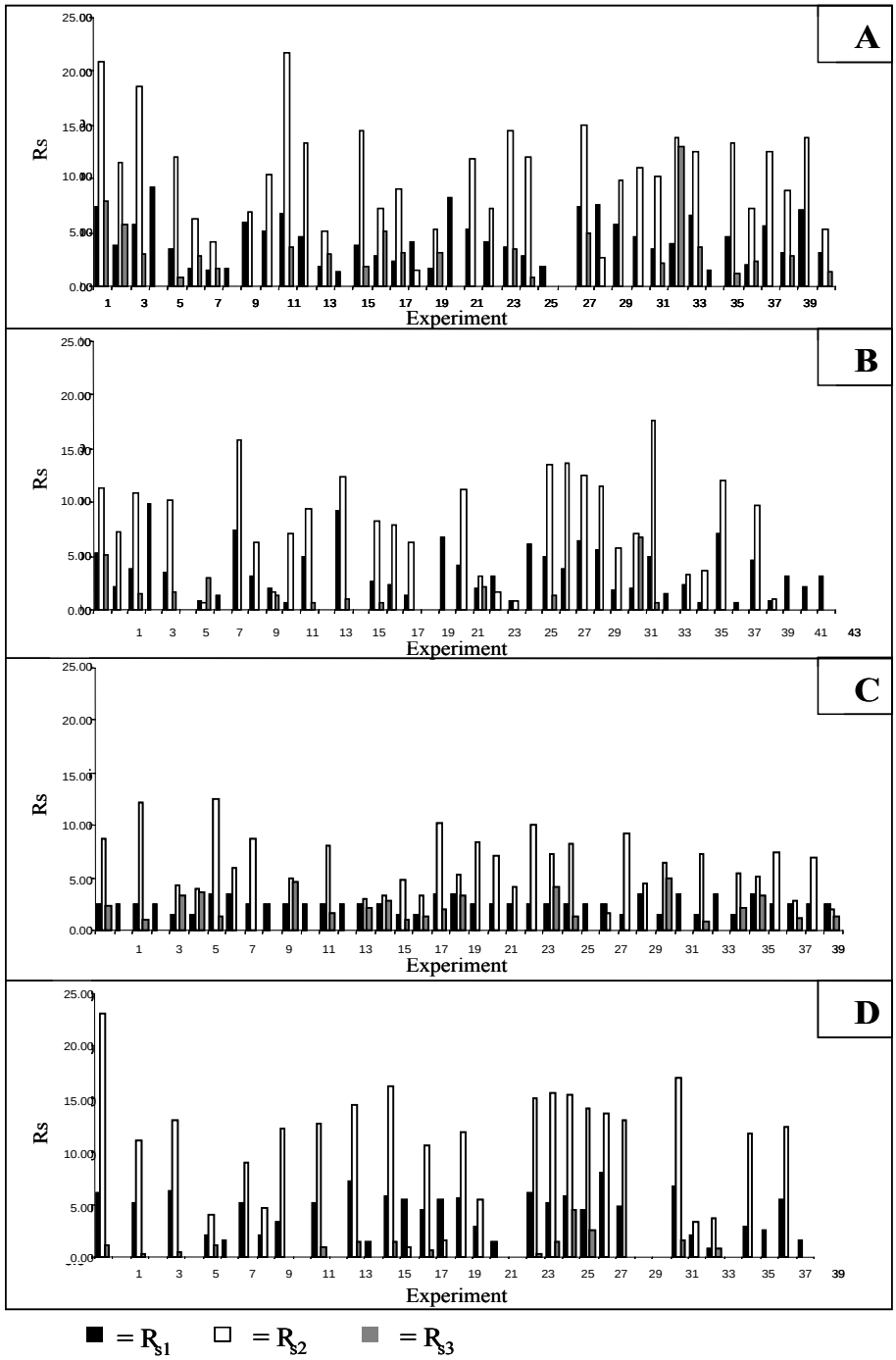

Figure 3.4 Resolution values obtained for various cationic polymer used in PEM coatings: Conditions: Cationic Polymer: (A) poly-L-glutamic acid-lysine; (B) poly-L-lysine-serine; (C) poly-L-lysine; (D) poly-L-ornithine; Anionic Polymer: 0.5% (w/v) poly-L-SULA; Analytes: 1. α- chymotrypsinogen A; 2. ribonuclease A; 3. lysozyme; 4. cytochrome c. All other conditions same as in Figure 3.3. [R_{s1} indicates the resolution between peaks 1 and 2; R_{s2} indicates the resolution between peaks 2 and 3, and R_{s3} indicates the resolution between peaks 3 and 4]

Table 3.2 Experimental parameters and resolution values for poly-L-glutamic acid-lysine as the cationic polymer in the PEM coating

No.	Temp (˚C)	Volt (kV)	Bilayers	[Polymer] (%w/v)	pH	EOF	MT1	MT2	MT3	MT4	Rs1	Rs2	Rs3
1	15	15	2.5	0.02	4	5.45	9.93	11.42	17.21	19.75	7.42	20.92	7.83
2	35	15	2.5	0.02	4	4.10	7.43	8.55	13.26	16.45	3.75	11.58	5.68
3	15	30	2.5	0.02	4	2.59	4.50	5.13	7.99	8.52	5.69	18.54	2.90
4	35	30	2.5	0.02	4	1.87	3.71	5.65	0.00	0.00	9.29	0.00	0.00
5	15	20	1.5	0.02	4	4.53	8.22	9.51	16.62	17.18	3.53	12.04	0.86
6	35	20	1.5	0.02	4	3.28	6.41	7.26	10.36	12.00	1.71	6.33	2.83
7	15	20	3.5	0.02	4	4.57	8.16	9.18	13.79	16.39	1.52	4.09	1.71
8	35	20	3.5	0.02	4	3.35	7.14	8.29	0.00	0.00	1.65	0.00	0.00
9	15	20	2.5	0.02	3	3.68	7.86	9.08	12.37	0.00	5.95	6.96	0.00
10	35	20	2.5	0.02	3	2.49	5.85	6.67	9.34	0.00	5.07	10.33	0.00
11	15	20	2.5	0.02	5	4.37	7.00	7.89	12.20	12.86	6.78	21.68	3.62
12	35	20	2.5	0.02	5	3.38	4.96	5.55	8.28	0.00	4.58	13.36	0.00
13	15	20	2.5	0.01	4	4.34	7.67	8.66	12.83	15.43	1.81	5.06	3.04
14	35	20	2.5	0.01	4	3.38	6.94	7.89	0.00	0.00	1.31	0.00	0.00
15	15	20	2.5	0.03	4	4.34	7.72	8.84	13.99	14.78	3.86	14.41	1.89
16	35	20	2.5	0.03	4	3.15	5.91	6.90	11.03	14.37	2.73	7.27	5.03
17	25	15	1.5	0.02	4	4.37	7.93	9.21	15.14	18.30	2.33	9.03	3.09
18	25	30	1.5	0.02	4	1.97	3.71	5.88	6.74	0.00	4.11	1.44	0.00
19	25	15	3.5	0.02	4	5.29	10.59	12.07	18.73	24.09	1.56	5.20	3.14
20	25	30	3.5	0.02	4	2.41	5.13	9.69	0.00	0.00	8.24	0.00	0.00
21	25	15	2.5	0.02	3	4.04	9.11	10.49	14.51	0.00	5.21	11.78	0.00
22	25	30	2.5	0.02	3	1.83	4.04	4.66	6.38	0.00	4.07	7.17	0.00
23	25	15	2.5	0.02	5	4.89	7.52	8.42	13.58	15.46	3.62	14.55	3.43
24	25	30	2.5	0.02	5	2.13	3.32	3.77	6.17	6.40	2.81	12.07	0.88
25	25	15	2.5	0.01	4	5.12	9.86	11.25	0.00	0.00	1.84	0.00	0.00
26	25	30	2.5	0.01	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
27	25	15	2.5	0.03	4	4.57	8.09	9.28	14.18	16.02	7.32	14.95	5.00
28	25	30	2.5	0.03	4	2.22	4.44	7.02	8.25	0.00	7.55	2.55	0.00
29	25	20	1.5	0.02	3	3.02	6.77	7.82	10.19	0.00	5.76	9.91	0.00
30	25	20	3.5	0.02	3	2.95	6.64	7.63	10.49	0.00	4.62	10.96	0.00
31	25	20	1.5	0.02	5	3.87	6.10	7.03	12.21	13.56	3.41	10.12	2.06
32	25	20	3.5	0.02	5	4.14	6.21	6.94	10.86	17.90	3.93	13.79	12.92
33	25	20	1.5	0.01	4	3.44	5.96	6.86	11.10	12.04	6.59	12.43	3.55
34	25	20	3.5	0.01	4	4.07	8.32	9.54	0.00	0.00	1.40	0.00	0.00
35	25	20	1.5	0.03	4	2.96	4.75	5.28	7.13	7.30	4.65	13.35	1.16
36	25	20	3.5	0.03	4	3.64	6.48	7.33	11.02	12.77	2.00	7.21	2.33
37	25	20	2.5	0.01	3	3.02	6.64	7.66	10.49	0.00	5.52	12.47	0.00
38	25	20	2.5	0.01	5	4.00	6.44	7.43	13.26	15.73	3.07	8.88	2.75
39	25	20	2.5	0.03	3	3.11	6.84	7.88	10.87	0.00	7.03	13.83	0.00
40	25	20	2.5	0.03	5	3.93	6.18	7.03	12.67	15.08	3.17	5.30	1.30
41	25	20	2.5	0.02	4	3.33	6.13	6.87	10.11	11.32	5.56	11.87	3.59
42	25	20	2.5	0.02	4	3.30	5.82	6.67	10.13	11.48	3.99	9.32	3.94
43	25	20	2.5	0.02	4	3.41	5.85	6.70	10.13	11.68	3.84	11.16	4.13

Table 3.3 Experimental parameters and resolution values for poly-L-lysine-serine as the cationic polymer in the PEM coating

No.	Temp (˚C)	Volt (kV)	Bilayers	[Polymer] (%w/v)	pH	EOF	MT1	MT2	MT3	MT4	Rs1	Rs2	Rs3
1	15	15	2.5	0.02	4	4.59	7.58	8.49	11.34	12.49	5.21	11.34	5.11
2	35	15	2.5	0.02	4	5.62	12.24	14.68	27.58	0.00	2.21	7.21	0.00
3	15	30	2.5	0.02	4	2.05	3.09	3.40	4.44	4.60	3.83	10.87	1.42
4	35	30	2.5	0.02	4	3.03	3.86	6.55	0.00	0.00	9.84	0.00	0.00
5	15	20	1.5	0.02	4	3.08	4.41	4.75	5.97	6.15	3.52	10.27	1.58
6	35	20	1.5	0.02	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7	15	20	3.5	0.02	4	5.15	8.62	8.96	9.43	10.46	0.83	0.72	3.02
8	35	20	3.5	0.02	4	3.70	12.64	15.27	0.00	0.00	1.36	0.00	0.00
9	15	20	2.5	0.02	3	3.97	9.44	11.05	16.42	0.00	7.43	15.74	0.00
10	35	20	2.5	0.02	3	2.15	5.08	5.69	7.17	0.00	3.15	6.21	0.00
11	15	20	2.5	0.02	5	4.74	7.06	7.89	10.42	13.23	1.98	1.69	1.27
12	35	20	2.5	0.02	5	4.11	5.71	6.04	13.60	0.00	0.59	7.04	0.00
13	15	20	2.5	0.01	4	3.37	5.11	5.61	7.81	7.98	4.97	9.33	0.61
14	35	20	2.5	0.01	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15	15	20	2.5	0.03	4	3.22	4.36	5.27	6.90	7.01	9.19	12.41	0.93
16	35	20	2.5	0.03	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
17	25	15	1.5	0.02	4	3.89	6.18	6.84	9.91	10.17	2.70	8.16	0.62
18	25	30	1.5	0.02	4	1.83	2.77	3.08	4.43	0.00	2.26	7.82	0.00
19	25	15	3.5	0.02	4	4.76	8.19	9.51	17.51	0.00	1.38	6.26	0.00
20	25	30	3.5	0.02	4	2.23	4.65	0.00	0.00	0.00	0.00	0.00	0.00
21	25	15	2.5	0.02	3	4.73	14.48	18.47	0.00	0.00	6.69	0.00	0.00
22	25	30	2.5	0.02	3	1.96	4.93	5.94	9.54	0.00	4.10	11.26	0.00
23	25	15	2.5	0.02	5	5.88	9.08	10.20	14.46	18.65	1.97	3.20	2.07
24	25	30	2.5	0.02	5	2.43	3.64	5.36	8.29	0.00	3.06	1.60	0.00
25	25	15	2.5	0.01	4	8.13	11.97	13.47	15.20	0.00	0.89	0.78	0.00
26	25	30	2.5	0.01	4	1.86	3.40	4.87	0.00	0.00	6.00	0.00	0.00
27	25	15	2.5	0.03	4	3.81	6.15	6.83	9.36	9.64	4.89	13.52	1.38
28	25	30	2.5	0.03	4	1.83	2.79	3.08	4.15	0.00	3.80	13.70	0.00
29	25	20	1.5	0.02	3	2.98	6.40	7.52	10.59	0.00	6.39	12.52	0.00
30	25	20	3.5	0.02	3	2.93	6.50	7.48	10.36	0.00	5.55	11.46	0.00
31	25	20	1.5	0.02	5	2.14	6.33	7.56	11.24	0.00	1.82	5.77	0.00
32	25	20	3.5	0.02	5	2.89	3.83	4.10	5.15	6.21	1.98	7.13	6.79
33	25	20	1.5	0.01	4	2.78	4.18	4.60	6.10	6.19	4.88	17.61	0.68
34	25	20	3.5	0.01	4	3.04	5.66	6.60	0.00	0.00	1.56	0.00	0.00
35	25	20	1.5	0.03	4	3.06	5.04	5.55	7.41	0.00	2.34	3.32	0.00
36	25	20	3.5	0.03	4	3.35	6.00	6.51	10.15	0.00	0.60	3.66	0.00
37	25	20	2.5	0.01	3	3.15	7.12	8.24	11.91	0.00	7.04	11.96	0.00
38	25	20	2.5	0.01	5	6.33	11.10	13.10	0.00	0.00	0.65	0.00	0.00
39	25	20	2.5	0.03	3	3.21	7.29	8.51	12.23	0.00	4.56	9.70	0.00
40	25	20	2.5	0.03	5	5.25	9.07	10.48	12.79	0.00	0.77	0.93	0.00
41	25	20	2.5	0.02	4	3.58	9.31	12.17	0.00	0.00	3.20	0.00	0.00
42	25	20	2.5	0.02	4	3.84	8.72	10.53	0.00	0.00	2.21	0.00	0.00
43	25	20	2.5	0.02	4	3.65	8.75	10.49	0.00	0.00	3.20	0.00	0.00

Table 3.4 Experimental parameters and resolution values for poly-L-lysine as the cationic polymer in the PEM coating

No.	Temp (˚C)	Volt (kV)	Bilayers	[Polymer] (%w/v)	pH	EOF	MT1	MT2	MT3	MT4	Rs1	Rs2	Rs3
1	15	15	2.5	0.02	4	4.80	7.28	7.98	10.59	11.15	4.27	8.76	2.23
2	35	15	2.5	0.02	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	15	30	2.5	0.02	4	2.25	3.45	3.83	5.22	5.33	3.93	12.25	0.94
4	35	30	2.5	0.02	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	15	20	1.5	0.02	4	4.01	4.82	5.03	6.24	7.41	0.84	4.29	3.21
6	35	20	1.5	0.02	4	1.93	3.50	3.64	4.50	5.32	0.64	3.97	3.65
7	15	20	3.5	0.02	4	3.61	5.72	6.38	8.79	9.01	5.59	12.42	1.33
8	35	20	3.5	0.02	4	3.22	5.22	5.83	8.07	0.00	1.95	5.97	0.00
9	15	20	2.5	0.02	3	3.43	6.73	7.54	9.64	0.00	4.30	8.79	0.00
10	35	20	2.5	0.02	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
11	15	20	2.5	0.02	5	4.32	5.64	6.01	7.91	10.16	1.94	4.85	4.54
12	35	20	2.5	0.02	5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
13	15	20	2.5	0.01	4	3.13	4.42	4.73	5.82	6.06	2.49	8.02	1.65
14	35	20	2.5	0.01	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15	15	20	2.5	0.03	4	3.37	4.01	4.21	5.02	5.76	0.75	2.98	2.18
16	35	20	2.5	0.03	4	2.32	2.77	2.92	3.48	4.08	0.94	3.37	2.84
17	25	15	1.5	0.02	4	4.20	6.01	6.55	8.99	9.51	1.99	4.76	1.01
18	25	30	1.5	0.02	4	1.95	2.84	3.07	3.99	4.25	1.07	3.22	1.31
19	25	15	3.5	0.02	4	4.27	6.77	7.53	10.66	11.42	2.95	10.20	1.93
20	25	30	3.5	0.02	4	2.12	2.62	2.80	3.50	4.29	1.23	5.20	3.24
21	25	15	2.5	0.02	3	3.81	7.82	8.79	11.38	0.00	3.81	8.40	0.00
22	25	30	2.5	0.02	3	1.74	3.48	3.93	5.06	0.00	3.97	7.07	0.00
23	25	15	2.5	0.02	5	4.76	6.60	7.22	10.96	0.00	1.23	4.18	0.00
24	25	30	2.5	0.02	5	1.74	2.59	2.87	4.14	0.00	2.87	10.00	0.00
25	25	15	2.5	0.01	4	4.89	6.86	7.47	10.25	14.28	1.60	7.28	4.16
26	25	30	2.5	0.01	4	1.68	2.43	2.63	3.36	3.49	2.63	8.19	1.27
27	25	15	2.5	0.03	4	4.37	6.25	8.41	0.00	0.00	1.58	0.00	0.00
28	25	30	2.5	0.03	4	2.23	3.63	4.93	6.34	0.00	1.49	1.58	0.00
29	25	20	1.5	0.02	3	2.77	5.59	6.27	8.05	0.00	4.12	9.19	0.00
30	25	20	3.5	0.02	3	2.69	4.84	5.31	6.43	0.00	2.39	4.47	0.00
31	25	20	1.5	0.02	5	3.72	4.50	4.72	5.90	7.35	1.59	6.49	4.90
32	25	20	3.5	0.02	5	3.18	5.50	6.56	0.00	0.00	1.90	0.00	0.00
33	25	20	1.5	0.01	4	2.23	3.06	3.27	3.95	4.03	2.66	7.32	0.87
34	25	20	3.5	0.01	4	3.19	5.13	5.68	0.00	0.00	1.11	0.00	0.00
35	25	20	1.5	0.03	4	3.33	5.74	6.42	9.87	11.29	1.12	5.39	2.09
36	25	20	3.5	0.03	4	3.42	6.08	6.94	11.02	14.71	1.21	5.13	3.25
37	25	20	2.5	0.01	3	2.59	4.90	5.46	6.79	0.00	3.97	7.47	0.00
38	25	20	2.5	0.01	5	3.37	4.06	4.25	5.41	6.60	0.69	2.73	1.17
39	25	20	2.5	0.03	3	2.49	4.70	5.18	6.44	0.00	3.17	6.92	0.00
40	25	20	2.5	0.03	5	2.98	3.72	3.89	4.83	5.92	0.50	2.05	1.24
41	25	20	2.5	0.02	4	3.13	3.93	4.20	5.38	7.03	1.43	5.29	3.78
42	25	20	2.5	0.02	4	3.30	4.58	4.79	6.05	8.55	0.54	4.26	3.17
43	25	20	2.5	0.02	4	3.19	3.20	3.29	4.24	5.45	0.53	2.60	1.86

Table 3.5 Experimental parameters and resolution values for poly-L-ornithine as the cationic polymer in the PEM coating

No.	Temp (˚C)	Volt (kV)	Bilayers	[Polymer] (%w/v)	pH	EOF	MT1	MT2	MT3	MT4	Rs1	Rs2	Rs3
1	15	15	2.5	0.02	4	4.84	7.45	8.20	11.09	11.30	6.14	23.12	1.07
2	35	15	2.5	0.02	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	15	30	2.5	0.02	4	2.23	3.41	3.77	5.13	5.18	5.17	11.01	0.37
4	35	30	2.5	0.02	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	15	20	1.5	0.02	4	3.57	5.56	6.16	8.45	8.54	6.18	12.92	0.49
6	35	20	1.5	0.02	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7	15	20	3.5	0.02	4	3.89	6.23	6.86	9.33	9.99	2.03	4.05	1.10
8	35	20	3.5	0.02	4	3.27	7.30	8.97	0.00	0.00	1.61	0.00	0.00
9	15	20	2.5	0.02	3	3.36	6.31	7.08	9.00	0.00	5.12	9.02	0.00
10	35	20	2.5	0.02	3	2.75	5.86	6.60	8.78	0.00	2.02	4.66	0.00
11	15	20	2.5	0.02	5	5.09	7.91	8.93	14.07	0.00	3.37	12.21	0.00
12	35	20	2.5	0.02	5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
13	15	20	2.5	0.01	4	3.53	5.44	5.99	8.08	8.24	5.19	12.69	0.94
14	35	20	2.5	0.01	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15	15	20	2.5	0.03	4	3.62	5.80	6.46	8.92	9.18	7.28	14.40	1.47
16	35	20	2.5	0.03	4	2.94	5.37	6.10	0.00	0.00	1.45	0.00	0.00
17	25	15	1.5	0.02	4	3.99	6.34	7.05	9.67	9.93	5.84	16.17	1.47
18	25	30	1.5	0.02	4	1.99	3.73	5.02	5.26	0.00	5.45	0.94	0.00
19	25	15	3.5	0.02	4	4.36	7.32	8.28	11.93	12.16	4.53	10.60	0.57
20	25	30	3.5	0.02	4	2.11	4.09	5.59	6.11	0.00	5.49	1.66	0.00
21	25	15	2.5	0.02	3	3.04	7.81	8.88	11.57	0.00	5.60	11.83	0.00
22	25	30	2.5	0.02	3	1.22	3.70	4.18	5.46	0.00	2.86	5.42	0.00
23	25	15	2.5	0.02	5	6.22	10.02	11.37	0.00	0.00	1.49	0.00	0.00
24	25	30	2.5	0.02	5	4.25	0.00	0.00	16.80	0.00	0.00	0.00	0.00
25	25	15	2.5	0.01	4	4.00	6.47	7.22	10.14	10.22	6.06	15.08	0.39
26	25	30	2.5	0.01	4	1.55	3.03	3.40	4.74	4.89	5.13	15.53	1.41
27	25	15	2.5	0.03	4	4.07	6.73	7.53	10.15	11.01	5.75	15.42	4.42
28	25	30	2.5	0.03	4	1.84	3.04	3.39	4.62	4.88	4.46	14.11	2.50
29	25	20	1.5	0.02	3	2.81	5.50	6.23	8.09	0.00	7.97	13.55	0.00
30	25	20	3.5	0.02	3	2.43	5.35	6.00	7.72	0.00	4.83	13.04	0.00
31	25	20	1.5	0.02	5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	25	20	3.5	0.02	5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
33	25	20	1.5	0.01	4	2.95	4.70	5.36	7.56	7.82	6.70	17.02	1.62
34	25	20	3.5	0.01	4	3.27	6.16	7.20	9.65	0.00	2.01	3.30	0.00
35	25	20	1.5	0.03	4	3.26	5.31	5.77	8.05	8.40	0.88	3.73	0.76
36	25	20	3.5	0.03	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
37	25	20	2.5	0.01	3	2.50	5.35	5.85	7.43	0.00	2.89	11.64	0.00
38	25	20	2.5	0.01	5	4.73	8.10	9.57	0.00	0.00	2.50	0.00	0.00
39	25	20	2.5	0.03	3	4.02	5.40	6.04	7.73	0.00	5.38	12.36	0.00
40	25	20	2.5	0.03	5	4.60	8.07	9.52	0.00	0.00	1.59	0.00	0.00
41	25	20	2.5	0.02	4	3.42	5.92	6.65	9.22	10.03	1.38	4.71	1.41
42	25	20	2.5	0.02	4	3.45	6.07	6.83	9.81	10.90	1.18	5.04	1.72
43	25	20	2.5	0.02	4	3.59	6.73	7.52	11.09	13.01	1.12	4.41	2.17

3.3.2 Influence of Temperature, Voltage and pH on Protein Resolution

Response surface plots for the influence of temperature and voltage on protein resolution using poly-L-glutamic acid-lysine as the cationic polymer and poly-L-SULA as the anionic polymer in the PEM coating were generated and the results are shown in Figure 5. In general, increasing the temperature resulted in faster migration times due to an increase in the electroosmotic mobility. Since the electroosmotic mobility increased, the interactions between the proteins and the stationary phase decreased, thereby, resulting in decreased resolution of proteins. However, an increase in voltage reduced migration times for all four proteins and decreased the resolution of R_{s2} and R_{s3}. Conversely, separation at higher voltages resulted in higher resolution (R_{s1}) of peaks 1 (α-chmyotrypsinogen) and 2 (ribonuclease A). Analysis of the response plot verifies that the separation at lower temperatures and lower voltages should result in higher resolution of the four proteins.

The influence of pH on protein separation was also investigated. The pH of the background electrolyte, 20mM phosphate, was varied at three pH values (3, 4, 5). In general, a cationic outer layer at an acidic pH value is most effective for the electrophoretic separation of basic proteins due to reduced adsorption to the capillary wall. The isoelectric point (pI) of the studied proteins ranged from 8 to 11. Therefore, acidic pH values at which these proteins are cationic were chosen for investigation. The highest resolution for all four proteins was achieved at pH 4 for all cationic polymers, while pH 3 allowed resolution of only three of the four proteins. Cytochrome c (peak 4) could not be separated from lysozyme at pH 3. Separation of the four proteins at pH 5 was achieved only under specific conditions. Hence, pH 4 was determined to be the optimum pH for these separations. While this optimum will likely be different for different protein mixtures, the results from this model system indicate that the lowest pH is not always the optimum. We speculate these results are due to the complex

interaction of both electrophoretic and chromatographic separation mechanisms involved in CEC.

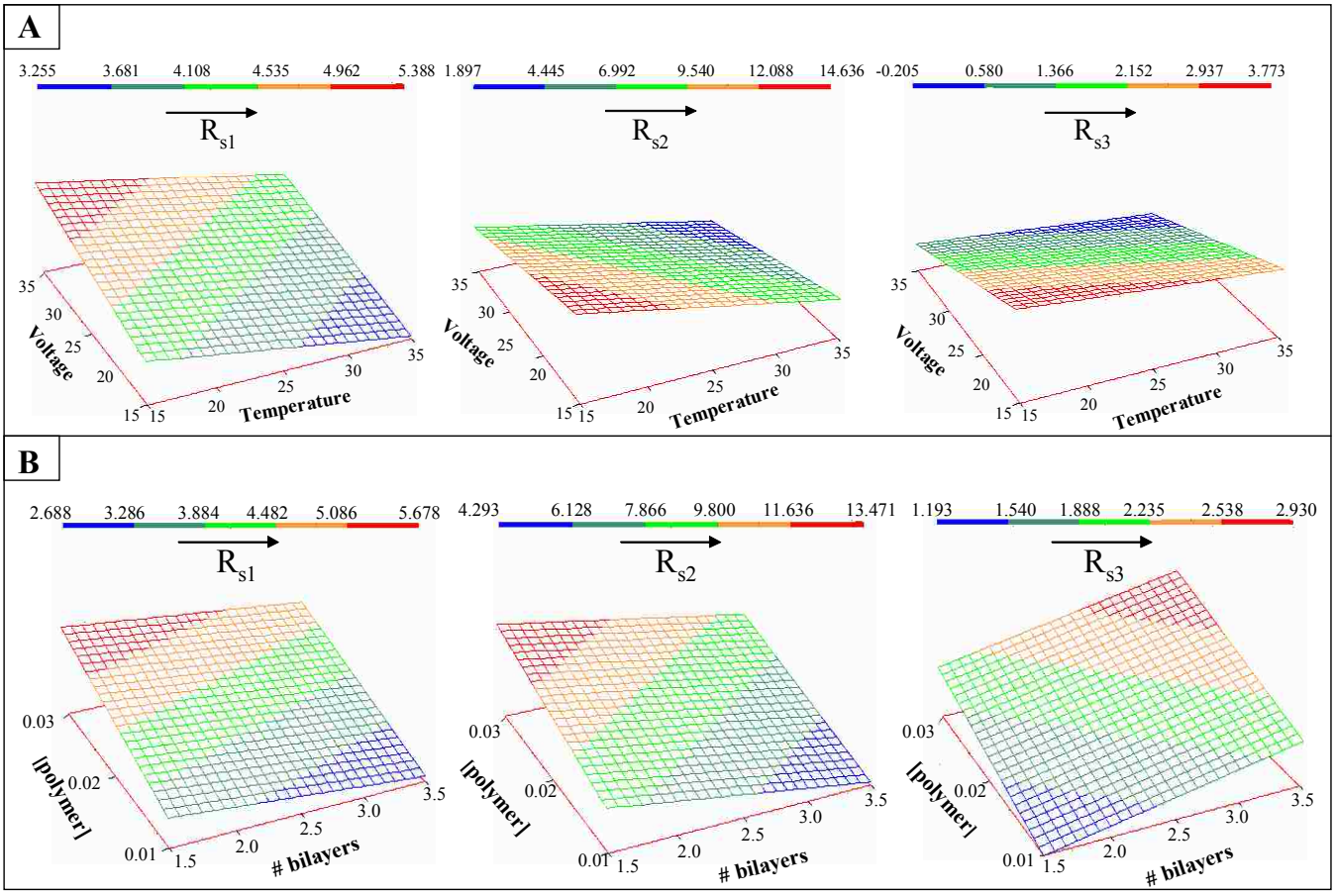

Figure 3.5 Response surface plots of design variables: A: Influence of temperature and voltage on protein resolution: Conditions: A: Coating: 1.5, 2.5, 3.5 bilayers of 0.01% (w/v), 0.02% (w/v), 0.03% (w/v) poly-L-glutamic acid-lysine; Anionic Polymer: 0.5% (w/v) poly-L-SULA; Analytes: 1. α-chymotrypsinogen A; 2. ribonuclease A; 3. lysozyme; 4. cytochrome c; Temperature: 15 ºC, 25 ºC, 35 ºC; Voltage: 15 kV, 20 kV, 30 kV. All other conditions same as in Figure 3.3. B: Effect of the Number of Bilayers and Cationic Polymer Concentration on Protein Resolution: Conditions: Same as in A

3.3.3 Effect of the Number of Bilayers and Cationic Polymer Concentration on Protein Resolution

The response plot for the influence of the number of bilayers and cationic polymer concentrations on protein resolution, using poly-L-glutamic acid-lysine as the cationic polymer and poly-L-SULA as the anionic polymer in the stationary phase were generated (Figure 3.5B).

A bilayer in a PEM coating consists of one layer of a cationic polymer and one layer of the anionic polymer. The PEM coatings used in this study consisted of 1.5, 2.5, and 3.5 bilayers with the outer layer always cationic and run in reverse polarity. Three methods of increasing the thickness of PEM coatings are 1) by increasing the rinse time of the coating polymers, 2) by increasing the number of bilayers used in the PEM coating, and 3) by increasing the concentration of the coating polymers. In this study, the influence of the thickness of the PEM coating was investigated by increasing the number of bilayers. When using the cationic polymer, poly-L-glutamic acid-lysine, R_{s1} and R_{s2} were noted to decrease as the number of bilayers increased. However, an increase in the number of bilayers produces an increase in R_{s3}. Overall, the use of 2.5 bilayers resulted in higher protein resolution. Increasing the number of bilayers, increases protein resolution to a point (in this case 2.5 bilayers) and it is dependent on the proteins under investigation; however, having a PEM coating that is too thick may adversely affect protein separations through peak broadening.

The influence of the thickness of the PEM coating, by variation of the cationic polymer concentration used in PEM coatings on protein separations, was also investigated. An increase in the cationic polymer concentration (poly-L-glutamic acid-lysine) resulted in an increase in the resolution of the four proteins (R_{s1}, R_{s2} and R_{s3}). It is suspected that as the concentration of poly-L-glutamic acid-lysine increased to an optimum, the PEM coating thickness increased, thereby enhancing the protein interactions with the stationary phase.

3.3.4 Effect of Cationic Polymer Type on Protein Resolution

An important factor in the investigation of PEM coatings is the choice of cationic polymer used in the stationary phase. Separations performed using PEM coated capillaries may be affected by the properties of the cationic polymer, such as the molecular structure, molecular weight, ionic strength, and the hydrocarbon content of the polymers. To investigate the

influence of cationic polymers in PEM coatings on these protein separations, poly-L-glutamic acid-lysine, poly-L-lysine-serine, poly-L-lysine, and poly-L-ornithine (Figure 3.1B) were used as cationic polymers in the stationary phase of the PEM coating. These polymers vary widely in molecular structure, molecular weight, and hydrocarbon content. Based on the molecular structures, poly-L-ornithine has the least hydrocarbon content, followed by poly-L-lysine, poly-L-lysine-serine, and poly-L-glutamic acid-lysine, with the most hydrocarbon content.

Figure 3.6 shows electropherograms for the highest resolution separation conditions obtained for each cationic polymer. Overall, all cationic polymers achieved successful separations. However, the use of poly-L-glutmatic acid-lysine, the highest hydrocarbon content polymer (Figure 3.6A), resulted in the highest resolution and highest peak efficiencies ($N \approx 60{,}000$) of the four proteins investigated. Resolution values of R_{s1} (7.42), R_{s2} (20.92), and R_{s3} (7.83) are consistently higher than those obtained using other cationic polymers in the PEM coating. These results may be attributed to stronger protein interactions between the highest hydrocarbon content and highest molecular weight cationic polymer and the proteins. Figure 3.6B and 3.6C illustrate the protein separation obtained using poly-L-ornithine ($R_{s1} = 5.75$, $R_{s2} = 15.42$, and $R_{s3} = 4.42$) and poly-L-lysine-serine ($R_{s1} = 5.21$, $R_{s2} = 11.34$, and $R_{s3} = 5.11$), respectively, as the cationic polymers. These two polymers resulted in lower resolution values, as compared to the resolution obtained using poly-L-glutamic acid-lysine. However, baseline resolution was obtained in a shorter migration time but with lower peak efficiency ($N \approx 50, 000$). Figure 3.6D, is a presentation of the results of protein separations using poly-L-lysine as the cationic polymer, where the lowest resolution values ($R_{s1}= 4.27$, $R_{s2} = 8.76$ and $R_{s3} = 2.23$) and the lowest peak efficiencies ($N \approx 30{,}000$) as compared to the other three cationic polymers were obtained. The overall best separation conditions achieved using different cationic polymers is shown in Table 3.6, where two sets of optimum conditions are obtained for each cationic

polymer. Optimum conditions are defined as (a) the highest protein resolution achieved and (b) baseline resolution in the shortest migration time.

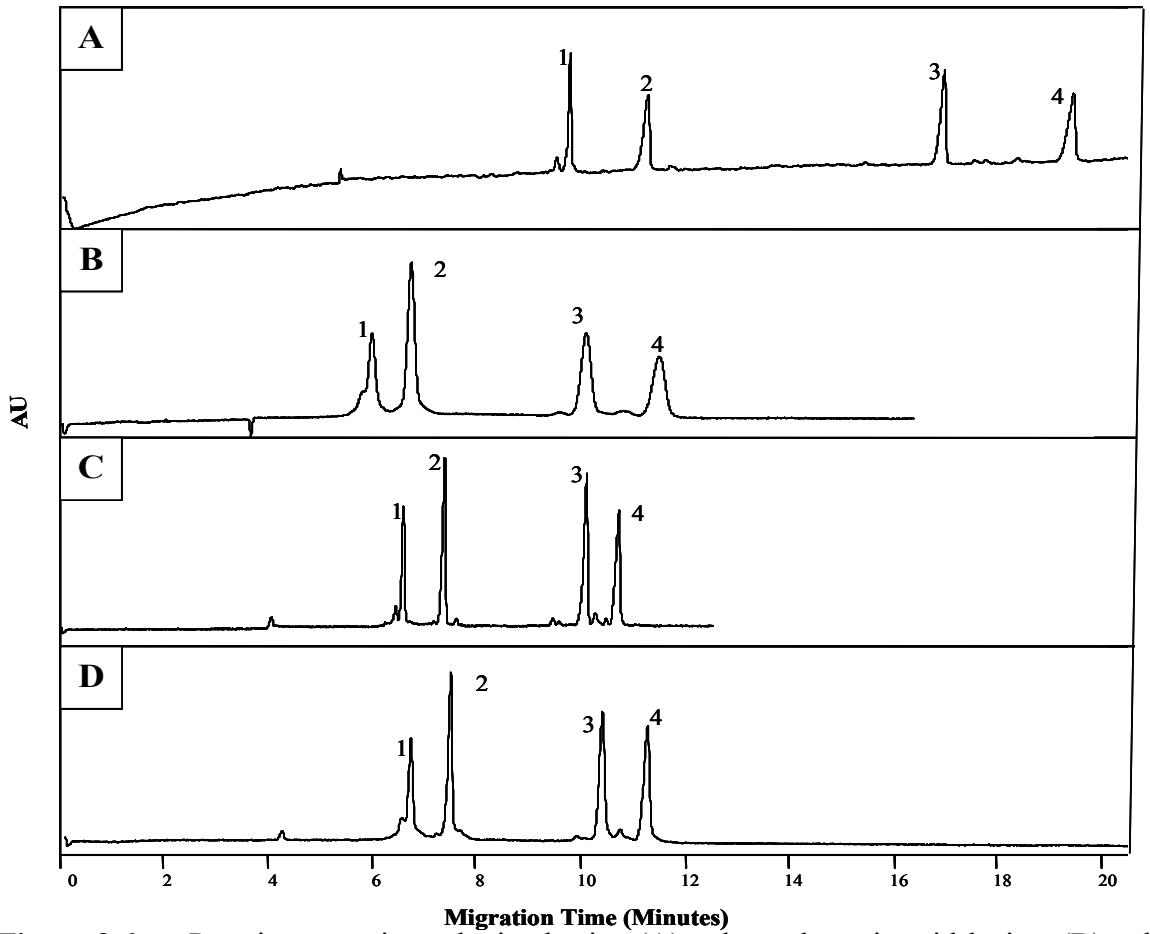

Figure 3.6 Protein separations obtained using (A) poly-L-glutamic acid-lysine, (B) poly-L-ornithine, (C) poly-L-lysine-serine and (D) poly-L-lysine as the cationic polymer in PEM coating: Conditions: Coating: (A) 2.5 bilayers of 0.02% (w/v) poly-L-glutamic acid-lysine; (B) 2.5 bilayers of 0.03% (w/v) poly-L-ornithine, (C) 2.5 bilayers of 0.02% (w/v) poly-L-lysine-serine, (D) 2.5 bilayers of 0.02% (w/v) poly-L-lysine; Analytes: 1.α-chymotrypsinogen A; 2. ribonuclease A; 3.lysozyme; 4. cytochrome c; All other conditions same as in Figure 3.3 and are all completed in the reverse polarity mode on the CE instrument.

In general, the highest resolutions were obtained at 15 ˚C, 15 kV, 20 mM phosphate, pH 4, 2.5 bilayers and 0.02% (w/v) for each cationic polymer. Clearly, the results of this study demonstrated that interactions occur between the stationary phase of the PEM coating and the proteins. This is in agreement with previous studies where interactions of proteins and a stationary phase using PDADMAC in a PEM coated capillary were reported by Wang and Dubin

[23]. In addition, similar conclusions were drawn for the interactions of chiral molecules with PEM coatings of chiral stationary phases [7, 15, 37].

Table 3.6 Optimum conditions for each cationic polymer used in the PEM coating (* indicates migration time of the last peak)

	Run time* (min)	Temp (˚C)	Volt (kV)	# Bilayers	[Polymer] (%w/v)	pH	Rs1	Rs2	Rs3
Poly-L-glutamic acid-lysine	(a) 19.20	15	15	2.5	0.02	4	7.42	20.92	7.83
	(b) 8.50	15	30	2.5	0.02	4	5.69	18.54	2.90
Poly-L-ornithine	(a) 11.01	25	15	2.5	0.03	4	5.75	15.42	4.42
	(b) 4.88	25	30	2.5	0.03	4	4.46	14.11	2.50
Poly-L-lysine-serine	(a) 12.49	15	15	2.5	0.02	4	5.21	11.34	5.11
	(b) 6.15	15	20	1.5	0.02	4	3.52	10.27	1.58
Poly-L-lysine	(a) 11.15	15	15	2.5	0.02	4	4.27	8.76	2.23
	(b) 6.06	15	20	2.5	0.01	4	2.49	8.02	1.65

3.3.5 Reproducibility of a PEM Coated Capillary

The reproducibility of a PEM coating plays an important role in the determination of column performance and separation reproducibility. Optimum conditions, where the highest resolution was obtained (Table 3.6a), were used to investigate the EOF reproducibility for each cationic polymer. The concentration of the anionic polymer, poly-L-SULA, was held constant at 0.5% (w/v). The protein separations were performed under optimum conditions for each polymer in triplicate and the percent relative standard deviation (%RSD) was calculated. All cationic polymers demonstrated good run-to-run reproducibility with a %RSD of the EOF of less than 3%.

Previous studies in our laboratory have shown that the presence of NaCl in the cationic polymer may significantly improve the reproducibility of successive experimental runs [15, 33]. Therefore, reproducibility studies were also performed with 0.1M NaCl present in the cationic

polymer under previously established optimum conditions (Table 3.6). As expected, the %RSD of the EOF improved to less than 1% for all four cationic polymers.

3.3.6 Influence of the Thickness of the Cationic Polymer Layer in PEM Coating on Protein Separations

The influence of the thickness of the cationic layer on protein separations was examined by varying the rinse time of the cationic polymer for the PEM coating from 2 to 45 minutes. Other separation parameters were held constant and the protein separations were performed under optimum conditions (Table 3.6a) using poly-L-ornithine as the cationic polymer in the PEM coating. Figure 3.7A provides an illustration of the protein separation using a PEM coated capillary obtained by rinsing the capillary with the cationic polymer for 2 minutes. Only peaks 1 and 2 were fully resolved, while peaks 3 and 4 co-eluted. Figures 3.7B and 3.7C illustrate the resolution obtained when the column was rinsed for 5 and 15 minutes, respectively. Resolution values for 5 and 15 minute rinse times (R_{s1} = 6.05, 6.44; R_{s2} = 16.16, 17.22; R_{s3} = 2.71, 3.11) were approximately the same for all proteins. In addition, high peak efficiencies were obtained, and all four proteins were completely resolved within 13 minutes. However, an increase in resolution and decrease in peak efficiency were observed when the cationic polymer was rinsed for 30 minutes (Figure 3.7D). Furthermore, increasing the rinse time to 30 minutes resulted in increased migration times, with the last protein eluting at 18 minutes.

Figure 3.7E shows the result of the protein separation obtained when the last cationic layer of the PEM coating was rinsed with poly-L-ornithine for 45 minutes. Evidently, no separation was achieved under these conditions and all protein peaks co-eluted with longer migration times of approximately 30 minutes. It is clear from the result of this study that the rinse time of the cationic polymer layer has a significant influence on the thickness of the PEM coating, thus influencing the protein separation. At relatively shorter rinse times, the amount of cationic polymer in the PEM coating is small, resulting in thinner PEM coatings. As a result,

weak protein-PEM coating interactions at the surface are observed. The highest protein resolution and peak efficiencies with the shortest migration time were obtained using PEM coatings produced by rinsing the cationic polymer between 5-15 minutes. At relatively longer rinse times (45 minutes), the coating is very thick, thereby, impeding flow in the capillary column and resulting in no protein separation. These results further confirmed that the observed separations are a result of a CEC process since protein separation is determined by interactions with the stationary phase, i.e. the PEM coating.

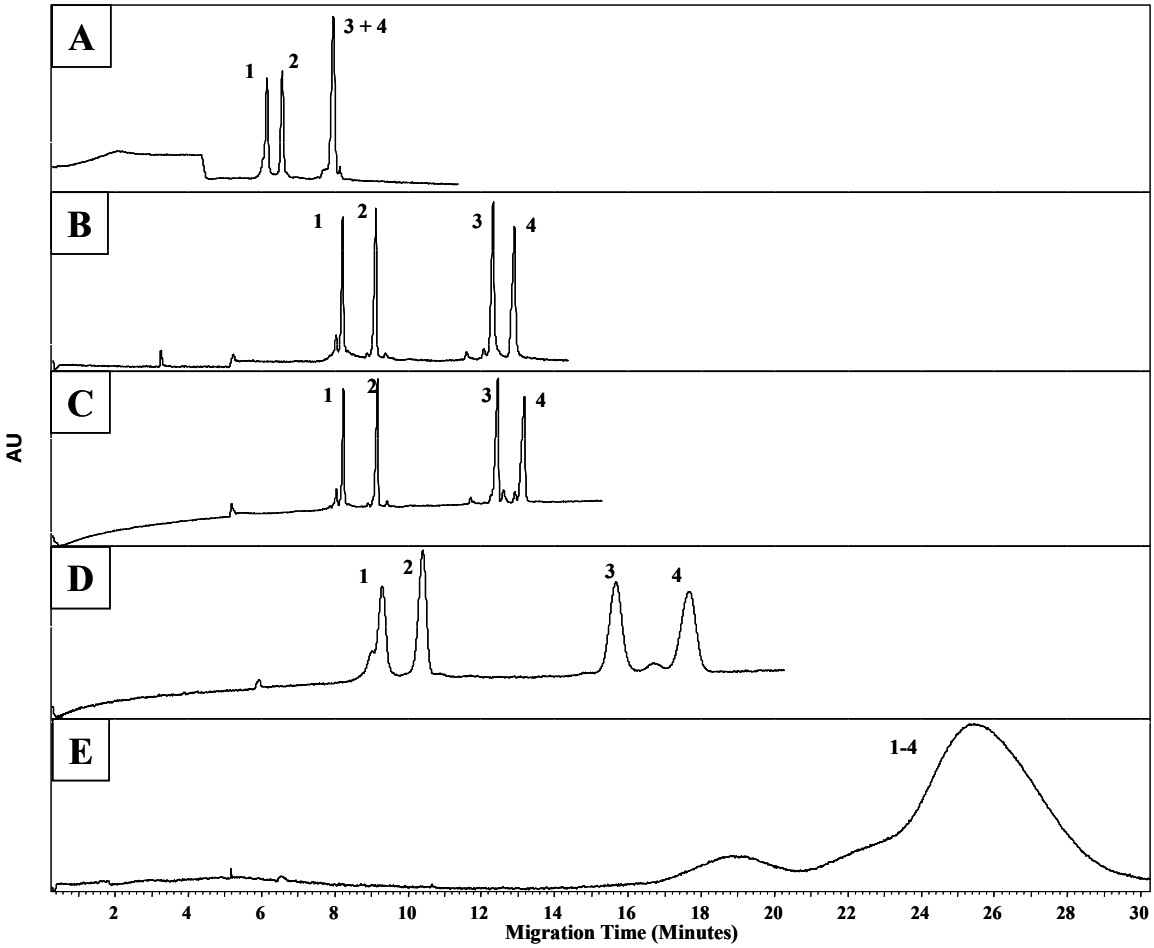

Figure 3.7 Influence of cationic layer thickness on protein separations: Conditions: Coating: 2.5 bilayers; Cationic Polymer: 0.03% (w/v) poly-L-ornithine with 0.1 M NaCl; Anionic Polymer: 0.5% (w/v) poly-L-SULA. (A) 2 minutes, (B) 5 minutes, (C) 15 minutes, (D) 30 minutes, (E) 45 minutes. Analytes: 1. α-chymotrypsinogen A; 2. ribonuclease A; 3. lysozyme; 4. cytochrome c; All other conditions same as in Figure 3.3 and are all completed in the reverse polarity mode on the CE instrument.

3.3.7 Effect of Anionic Polymer Type on Protein Resolution

The choice of anionic polymer is another important parameter to consider when using PEM coatings as the stationary phase in OT-CEC. In this study, three different molecular micelles were used as anionic polymers to investigate their influence on protein separations. Poly-SUS is an achiral molecular micelle and poly-L-SULV and poly-L-SULA are dipeptide chiral molecular micelles that possess two chiral centers. Figure 3.8 shows the results obtained when columns were coated with 2.5 bilayers of 0.03% (w/v) poly-L-ornithine and 0.5% (w/v) of poly-SUS, poly-L-SULV, and poly-L-SULA, respectively. In Figure 3.8A, the achiral poly-SUS was used as the anionic polymer and only partial separation was achieved for peaks three (lysozyme) and four (cytochrome c). Figure 3.8B and 3.8C shows the use of the chiral molecular micelles, poly-L-SULA and poly-L-SULV, respectively. Baseline resolution was attained using these two chiral molecular micelles; however, poly-L-SULV achieved higher peak efficiencies of the protein peaks and resulted in a reversal of the elution order of peaks three and four (Figure 3.8C). Use of poly-L-SULV (Figure 3.8C) resulted in higher Rs_1 values but lower Rs_2 and Rs_3, as compared to poly-L-SULA (Figure 3.8B). In contrast to poly-SUS and poly-L-SULA, cytochrome c eluted before lysozyme when poly-L-SULV was used. These results also confirm protein interactions with the PEM coatings, i.e. CEC, since higher protein resolution was achieved when the chiral molecular micelles were used and also the elution order of cytochrome c and lysozyme were different when using a different chiral molecular micelle. This suggests the possibility of chiral interactions between the proteins and the molecular micelles.

3.3.8 Influence of Anionic Polymer Order in PEM Coatings on Protein Separations

The results of the influence of the anionic type on protein separation revealed that superior protein resolution was achieved using chiral molecular micelles and also that the elution order changed.

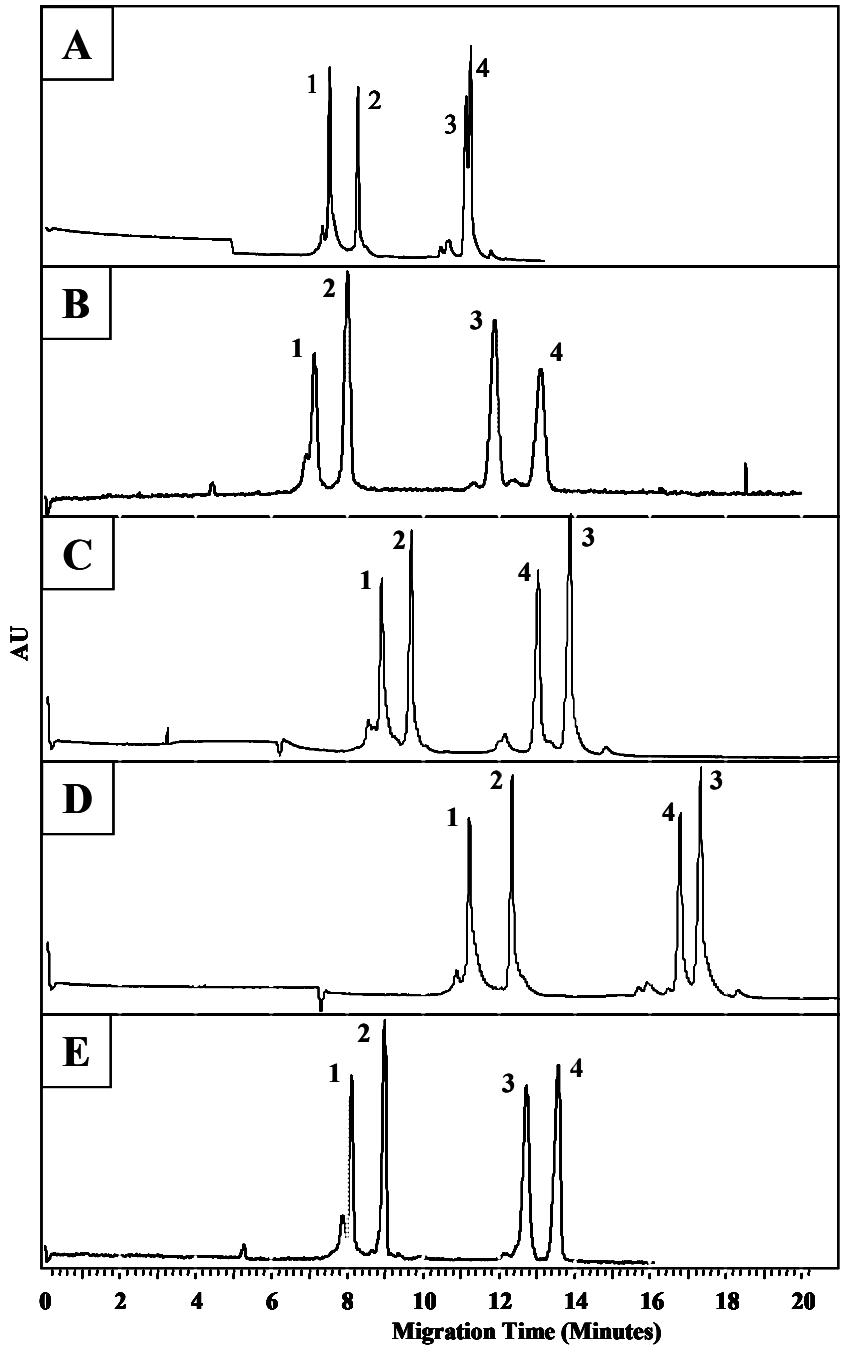

Figure 3.8 Influence of anionic polymer on protein separations: (A) Poly-SUS, (B) Poly-L-SULA, (C) Poly-L-SULV, (D) First bilayer: anionic polymer, Poly-L-SULA; Second bilayer: anionic polymer, Poly-L-SULV; (E) First bilayer: anionic polymer, Poly-L-SULV; Second bilayer: anionic polymer, Poly-L-SULA: Conditions: 2.5 bilayers; Cationic Polymer: 0.03% (w/v) poly-L-ornithine; Anionic Polymer: 0.5% (w/v) Analytes: 1. α-chymotrypsinogen A; 2. ribonuclease A; 3. lysozyme; 4. cytochrome c; All other conditions same as in Figure 3.3 and are all completed in the reverse polarity mode on the CE instrument.

Therefore, further studies were performed using these anionic polymers. Protein separations were performed using 2.5 bilayers of 0.03% (w/v) poly-L-ornithine as the cationic polymer. Figures 3.8B and 3.8C show baseline resolution of all four proteins when poly-L-SULA and poly-L-SULV chiral anionic molecular micelles were used as PEM coating materials. However, as stated earlier, the use of poly-L-SULV as an anionic polymer resulted in higher protein peak efficiencies and a change in the elution order of cytochrome c and lysozyme.

To further investigate the influence of anionic polymers on protein separation, PEM coatings were constructed using poly-L-SULA as the anionic polymer in the first bilayer, and poly-L-SULV in the second bilayer. The results of the protein separation using this PEM coating are shown in Figure 3.8D. The electropherogram shows that the protein elution order is in agreement with the results obtained with the use of only poly-L-SULV (Figure 3.8C), resulting in cytochrome c eluting before lysozyme. In contrast, Figure 3.8E shows the results obtained when poly-L-SULV was used in the first bilayer of the PEM coating, and poly-L-SULA used as the anionic polymer in the second bilayer which resulted in lysozyme being eluted before cytochrome c. This elution order agrees with the results obtained when only poly-L-SULA was used in the PEM coating (Figure 3.8B). Overall, it seems that when poly-L-SULA is the anionic polymer in the last bilayer of the PEM coating, cytochrome c elutes last and conversely, when poly-L-SULV is in the last bilayer, lysozyme elutes last. In addition, reversal of the elution order of the last two proteins when the anionic polymer is changed shows that the layers of the PEM coatings are interpenetrating since like charged PEM coatings and proteins only interact at the surface. It should not be overlooked that when the anionic polymer was changed in the last bilayer, the last layer of the PEM coating was in fact cationic (poly-L-ornithine). Therefore, these results suggest that the underlying layers of the PEM coating are instrumental in achieving the selectivity of the coatings. Furthermore, these results suggest that the type, as well as the

position of the anionic polymer in the PEM coating has a significant affect on protein-PEM interactions, resulting in differences in protein elution.

3.4 Concluding Remarks

In this study, novel PEM coatings were constructed using four different cationic polymers: poly-L-glutamic acid-lysine, poly-L-lysine-serine, poly-L-ornithine, and poly-L-lysine and three anionic molecular micelles, poly-L-SULA, poly-L-SULV, and poly-SUS to separate four basic proteins (α-chymotrypsinogen, lysozyme, ribonuclease A and cytochrome c). Several factors such as operating temperature, applied voltage, type and concentration of cationic polymer, and background electrolyte pH were optimized using a Box Behnken experimental design for protein separations. Generally, all four cationic polymers successfully resolved all four proteins with different resolutions and migration times. However, the use of the most hydrophobic polymer, poly-L-glutamic acid-lysine, in the PEM coating resulted in the highest resolution and poly-L-lysine the lowest. The presence of the molecular micelle in the PEM coating enhanced the protein-PEM interaction and resulted in better protein separation than use of a simple cationic polymer alone. Chiral molecular micelles (poly-L-SULA and poly-L-SULV) resulted in higher protein resolution than an achiral molecular micelle (poly-SUS) as well as in different elution orders of the proteins investigated. Clearly, the choice of both the cationic and anionic polymers used in a PEM coating has significant influence on protein separations since these polymers affect column selectivity. This is due to different protein-PEM interactions when different stationary phases are used. Definitive confirmation that these separations involve wall interactions, and thus a CEC process, is provided. Overall, the experimental findings reported in this manuscript provide valuable new knowledge to the biotechnological arena that will aid in the optimization of different coatings for separation of additional protein mixtures.

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CHAPTER 4

ACHIRAL AND CHIRAL SEPARATIONS USING MICELLAR ELECTROKINETIC CHROMATOGRAPHY, POLYELECTROLYTE MULTILAYER COATINGS, AND MIXED MODE SEPARATION TECHNIQUES WITH MOLECULAR MICELLES

4.1 Introduction

Chiral separations are important in the pharmaceutical, biomedical and environmental industries, primarily because the interactions and behavior of individual enantiomers have not been fully explored. The importance of these studies is further magnified by the mass circulation of thousands of chiral drugs [1]. It is well established that enantiomers of the same drug may have very different clinical effects in the human body since the body metabolizes these isomers through different pathways. Many drugs exhibit dissimilar pharmacokinetic behavior [1]. As a result, the United States Food and Drug Administration requires that the pharmacological and toxicological activity of each isomer be investigated and documented. Therefore, the development of methods to separate chiral analytes continues to garner much attention.

In order to achieve chiral separations, enantiomeric interactions must occur in a chiral environment. This is generally achieved through the use of a chiral selector or chiral discriminating agent able to differentially interact with each individual enantiomer. Several chiral selectors have been used in capillary electrophoresis (CE) separations; these include bile acids [2], crown ethers [3, 4], polysaccharides [5], proteins [6, 7], cyclodextrins [8, 9] and molecular micelles [11, 12]. Our group is among the pioneers in the use of molecular micelles for CE enantioseparations. Molecular micelles are prepared by polymerizing surfactants at sufficiently high concentrations for formation of micelles. These resulting polymers are thought to resemble a conventional micelle and are preferred over unpolymerized micelles because molecular micelles have virtually no critical micelle concentration and, thus, can be used at concentrations below the CMC. In addition, the covalent bonds formed during the

polymerization process eliminate the dynamic equilibrium between monomer and micelle. Experimental parameters such as pH and concentration of added organic solvent, known to disrupt the formation of conventional micelles, have been shown not to seriously damage molecular micellar interactions [1, 10, 11]. The addition of molecular micelles to a separation system may result in the resolution of a wide variety of compounds. For example, Rizvi *et al.* investigated the separation of the β-blockers, labetalol, and nadalol by using variations of the chiral selector, polyalkenoxy amino acid polymers [1]. Also, Shamsi *et al.* used CE and resolved several different compound classes by using the versatile chiral selector, poly (sodium N-undecanoyl-L-leucylvalinate) (poly-SULV) [11].

The technique of CE has emerged as one of the leading separation approaches because high separation efficiencies are achieved with relatively low consumption of analyte and chiral selector. Thus, CE has the added advantages of relatively simple method development and short analysis times [14, 15]. Micellar electrokinetic chromatography (MEKC) and capillary electrochromatography (CEC) are hybrids of CE which combine the benefits of electrophoresis and chromatography to separate both charged and neutral analytes [13, 14, 15, 22]. In MEKC, a pseudostationary phase is created by the introduction of a surfactant at a concentration above the CMC, to the mobile phase. Separation of both charged and neutral analytes are based on hydrophobic and ionic interactions of the analytes with micelles in the pseudostationary phase [17]. MEKC has been used to separate both achiral and chiral analytes [18, 19, 20].

The methodology of CEC combines both the selectivity of HPLC and the efficiency of CE [12, 23]. Separation is based on differences in electrophoretic mobilities and partitioning of the analytes into the stationary phase. Neutral analytes are separated through interactions with the stationary phase which is adsorbed to the capillary wall, while charged analytes are separated due to differences in charge and size as well as partitioning behavior [23]. CEC has shown great

potential in the separation of both achiral and chiral analytes. The stationary phase can be prepared by several methods including adsorption, where the stationary phase can either be dynamically or physically adsorbed to the capillary wall to shield the negatively charged silanol groups with a layer of the coating material [24, 25]. In one mode of CEC, open tubular capillary electrochromatography (OT-CEC), the stationary phase is adsorbed to the capillary wall and the mobile phase, which flows through the column, is driven by the electroosmotic flow (EOF). Several chiral selectors have been used as stationary phases in OT-CEC. For example, Liu *et al.* used avidin, a basic protein, as the adsorbed stationary phase to separate a total of sixteen different enantiomers [4]. Another method of creating the stationary phase is dynamic coatings. Dynamic coatings are adsorbed to the capillary wall by electrostatic interactions and hydrogen bonding [12]. One widely used coating constructed by a physical adsorption process is a polyelectrolyte multilayer (PEM) coating.

A PEM coating is formed by alternately exposing the hydrophilic inner wall of a silica capillary first to cationic and then anionic polymers. The combination of each is called a bilayer and the mechanism of a PEM coating formation is via ion exchange that results in stable coatings [33]. PEM coatings are constructed using chiral cationic and anionic polymers and have been used to separate a number of chiral analytes. Rmaile and Schlenoff used the polymers poly-L-lysine and poly-L-glutamic acid, among others, to resolve chiral probes such as ascorbic acid and a chiral viologen [34]. In our laboratory, Kamande *et al.* used poly-L-lysine hydrobromide and poly (sodium N-undecanoyl-L-leucyl-alaninate) (poly-L-SULA) to separate three binaphthyl derivatives and two β-blockers [35]. We note that it is not necessary for both polymers to be chiral in order to separate chiral analytes. For example, Kapnissi *et al.* optimized several experimental parameters using PEMs generated with achiral poly (diallyldimethylammonium chloride) (PDADMAC) and chiral poly (sodium N-undecanoyl-L- leucyl-valinate) (poly-L-

SULV) to resolve chiral analytes. In that study the authors created up to a 12 bilayer capillary using ionic liquids as additives [36].

In this chapter, the influence of separation mode (MEKC, PEM coatings and mixed mode separation technique) on the resolution of achiral and chiral analytes is investigated. The effects of applied voltage, bilayer number, polymer concentration and polymer type on analyte separation were investigated. The molecular micelles poly(*N*-undecanoyl-L-glycinate) (poly-SUG) and (poly-L-SULV) were used for the achiral and chiral separations respectively.

4.2 Materials and Methods

4. 2.1 Materials

The achiral cationic polymer, poly(diallyldimethylammonium chloride) (PDADMAC) was purchased from Sigma Chemical Company (St. Louis, MO). The chemicals used to synthesize both achiral and chiral surfactant monomers, *N*-hydroxysuccinimide, undecylenic acid, dicyclohexylcarbodiimide, and sodium bicarbonate were purchased from Fluka (Milwaukee, WI). The peptides leucine-valine and glycine were purchased from Bachem Bioscience, Inc. (King of Prussia, PA). Sodium hydroxide, ethyl acetate, and tetrahydrofuran were purchased from Sigma-Aldrich (Milwaukee, WI). The buffer used in these experiments, were composed of sodium phosphate dibasic, monobasic sodium phosphate,sodium borate tris[hydroxymethyl]aminomethane, methanol, and acetone were purchased from Fisher Scientific (Fair Lawn, NJ). The achiral alkyl aryl ketones, acetophenone, propiophenone, butyrophenone, valerophenone, hexanophenone, heptanophenone, octanophenone, decanophenone, as well as the chiral analytes, temazepam, benzoin, aminoglutethimide, coumachlor, and benzoin methyl ether were also purchased from Sigma (St. Louis, MO). All materials were used as received without any further purifications. The molecular structures of the analytes investigated are shown in Figure 4.1.

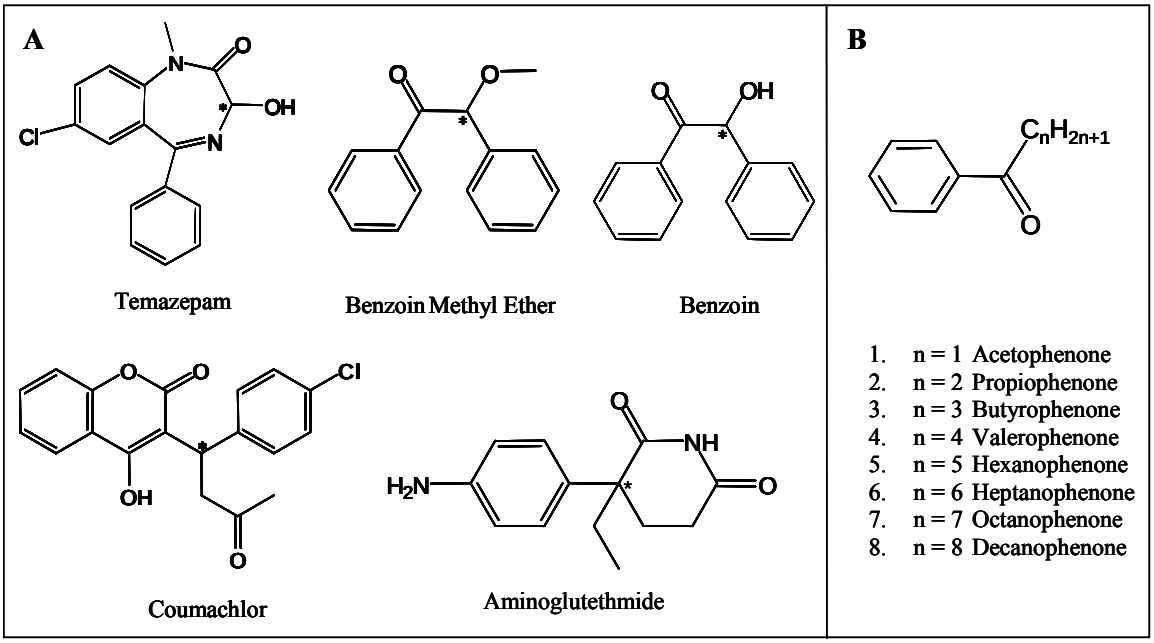

Figure 4.1 Structures of analytes; A. Chiral analytes; B: Achiral aryl ketones

4.2.2 Instrumentation

A roll of fused silica capillary, with an internal diameter of 50μm was purchased from Polymicro Technologies (Phoenix, AZ). A piece of this material with a total length of 60 cm (50 cm effective length) was used for the experiments described in this manuscript. All separations were conducted using a Beckman P/ACE MDQ capillary electrophoresis system, equipped with a photodiode array detector (Fullerton, CA). Liquid coolant was used to maintain the temperature at 15°C. The applied voltage ranged from 15kV to 30kV. The achiral and chiral analytes were detected at 220nm and 254 nm. All analytes were injected using 0.5psi for 5s.

4.2.3 Synthesis of Molecular Micelles

The achiral molecular micelle, poly-SUG as well as the chiral dipeptide molecular micelle poly-L-SULV were synthesized according to a procedure previously described by Wang and Warner [37]. Solutions containing 100 mM of the monomers were polymerized by use of a

^{60}Co γ-ray irradiation source. The molecular structures of the molecular micelles and the cationic polymer used in this study are illustrated in Figure 4.2.

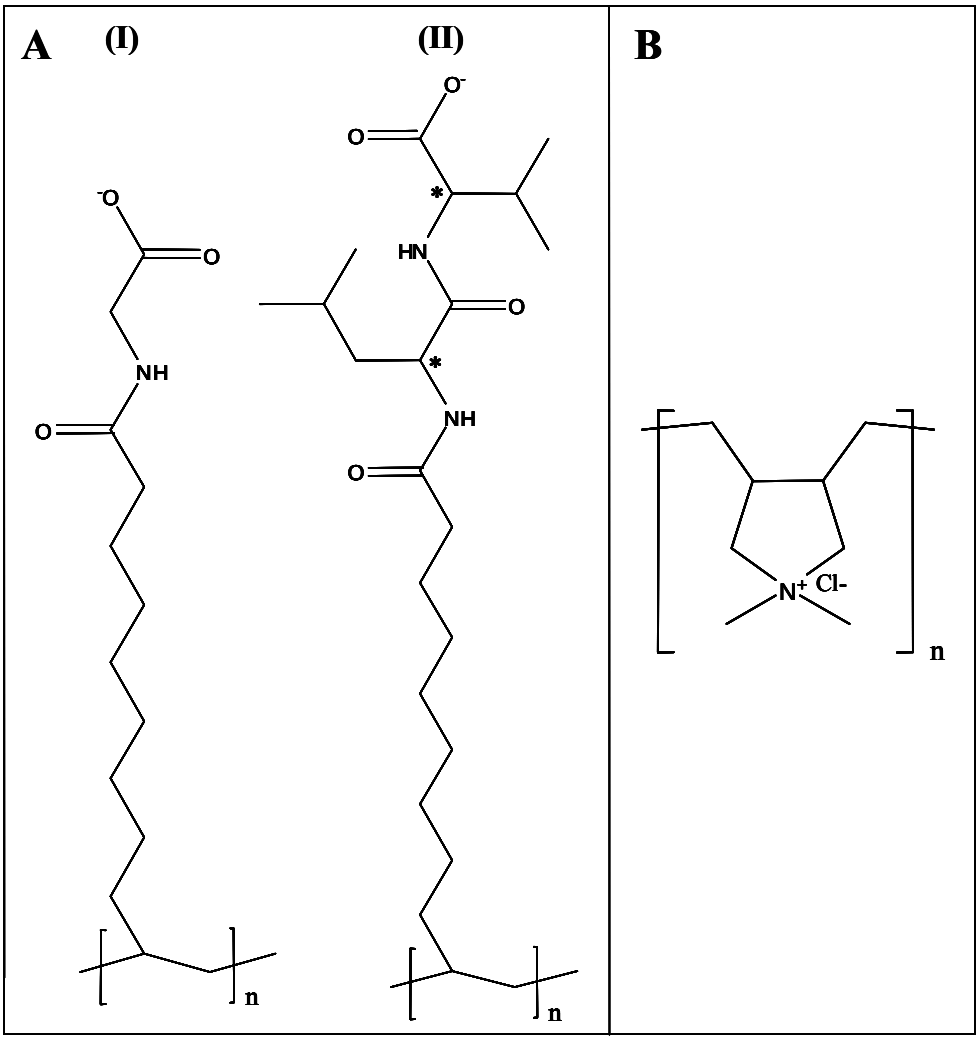

Figure 4.2 A. Structural representation of (I) Poly-SUG and (II) Poly-L-SULV
B. Structural representation of PDADMAC.

4.2.4 Sample and Buffer Preparation

In all experiments, 1M NaOH and 1M HCl were used to adjust the pH of the background electrolyte. Prior to use, all buffers were filtered using a 0.45 μm polypropylene nylon filter and sonicated for 15 minutes. Solutions of 8 alkyl aryl ketones (0.1 mg/mL) were dissolved in MeOH for the achiral investigations and 0.2 mg/mL solutions were prepared in 50:50

methanol/water for use in the chiral studies. The achiral cationic polymer, PDADMAC was used at 0.5% (w/v) in deionized water. Achiral and chiral anionic molecular micelles (poly-SUG and poly-L-SULV) were varied from concentrations of 0.25% (w/v) to 1.50% (w/v) for all separation studies.

4.2.5 Micellar Electrokinetic Chromatography Procedure

An untreated silica capillary is rinsed with 1M NaOH for 30 minutes to deprotonate the capillary wall followed by a 15 minute rinse with deionized water. The background electrolyte is then flushed through the capillary for 5 minutes prior to analyte injection. The capillary is rinsed with 0.1M NaOH for 2 minutes, deionized H_2O for 2 minutes and the background electrolyte for 5 minutes between each run.

4.2.6 Polyelectrolyte Multilayer Coating Procedure

As in the MEKC procedure, a fused silica capillary is deprotonated with 1M NaOH for 30 minutes followed by a 15 minute deionized water rinse. A 20 minute rinse with the cationic polymer followed by a 5 minute rinse with deionized water initiates the first layer of the PEM coating. To complete the bilayer, the anionic polymer is rinsed through the column for 5 minutes, followed by a 5 minute deionized water rinse. All other bilayers were created with alternate 5 minute rinses of the cationic and anionic polymers. The number of bilayers was varied from 2 to 4. All rinses were performed using the rinse function of the CE instrument, with applied pressure of 20psi.

4.2.7 Mixed Mode Separation Technique

The mixed mode separation method combines both the MEKC and PEM coatings procedures. First, the PEM coatings are constructed with the desired coating polymer and polymer concentration as well as the number of bilayers needed for the study (Procedure outlined in Section 4.2.6). Then, the respective molecular micelles are added to the mobile phase of the

coated capillary at the desired concentrations and various studies are completed.

4.3 Results and Discussion

4.3.1 Effect of Concentration of Poly-L-SUG on the Separation of 8 Achiral Alkyl Aryl Ketones Using MEKC

To investigate the influence of molecular micelle concentration on the separation of 8 achiral alkyl aryl ketones using MEKC, five different mobile phases were prepared. The molecular micelle concentration was varied from 0.25% (w/v) to 1.00% (w/v) poly-SUG in 100 mM Tris at pH 10 (results shown in Figure 4.3). At 0.25% (w/v), only the first four aryl ketones, acetophenone, propiophenone, butyrophenone, and valerophenone were resolved with moderate peak shape and efficiency. As the molecular micelle concentration increased to 1.00% (w/v), the resolution of the aryl ketones increased with a slight increase in migration time. The elution order of these analytes is indicated in the figure with the least hydrophobic, acetophenone, eluting first and the most hydrophobic, decanophenone, eluting last. All peaks were identified by spiking the concentration of one analyte at a time. At 1.00% (w/v) poly-SUG, the resolution of the first five analytes increased. However, there was only partial separation of the last three aryl ketones. The shapes and efficiencies of the latter peaks were due to the more hydrophobic aryl ketones being retained in the micellar phase longer than the aqueous phase. Baseline resolution of all 8 aryl ketones was not achieved using these parameters in MEKC.

4.3.2 Effect of Concentration and Bilayer Number of Poly-L-SUG on the Separation of 8 Achiral Alkyl Aryl Ketones Using PEM Coatings

The influence of the anionic molecular micelle concentration, poly-SUG, on the resolution of the 8 aryl ketones was investigated with PEM coatings. Different PEM coatings were constructed using 0.25% (w/v), 0.5% (w/v), and 1.00% (w/v) poly-SUG as the anionic layer. The cationic layer was held constant using 0.5% (w/v) PDADMAC. All concentrations of poly-SUG resulted in only partial separation of the aryl ketones (Figure 4.4). The optimum

anionic polymer concentration was chosen to be 0.5% (w/v) poly-SUG since the highest resolution with a relatively short migration time was achieved (Figure 4.4B).

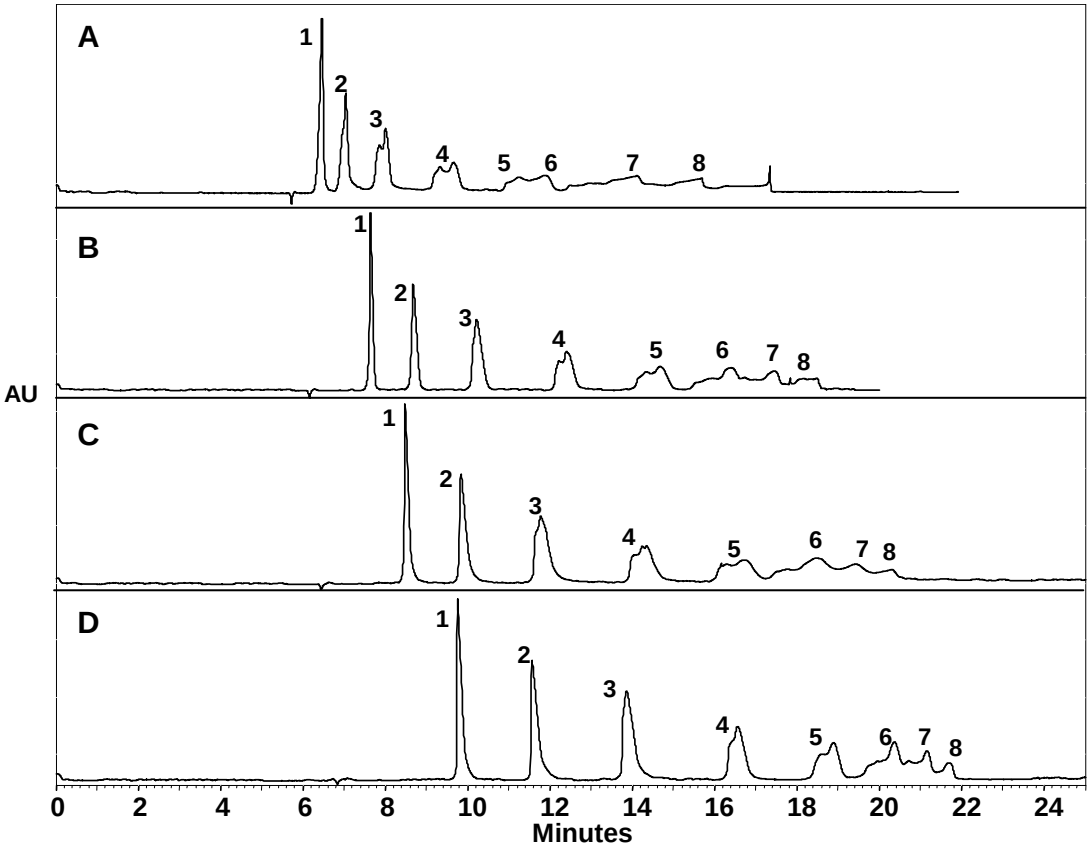

Figure 4.3 Influence of poly-SUG concentration on the separation of 8 aryl ketones using MEKC: Conditions: A: 0.25% (w/v) p-SUG; B: 0.5% (w/v) p-SUG; C: 0.75% (w/v) p-SUG; D: 1.00% (w/v) p-SUG Buffer: 100mM Tris, pH 10; Analyte concentration: 0.1mg/ml, Capillary Length: 57cm total (50 cm effective length); Capillary I.D.: 50 µm; Temperature: 15 ºC; Voltage: 15kV, Injection: 5psi for 5s; Detection: 220nm; Analytes: 1. Acetophenone, 2. Propiophenone, 3. Butyrophenone, 4. Valerophenone, 5. Hexanophenone, 6. Heptanophenone, 7. Octanophenone, 8. Decanophenone

Three different PEM coatings were achieved using 0.5% (w/v) PDADMAC and 0.5% (w/v) poly-SUG consisting of 2, 3 and 4 bilayers (Figure 4.5). The influence of bilayer number on the separation of 8 aryl ketones was investigated. The column coated with 2 bilayers resulted in partial separation of the aryl ketones within 10 minutes. Even though the resolutions increased as the number of bilayers increased, there was still only partial separation ($R_s < 1.5$) of

the ketones using the 3 bilayer system with an elution time of 14 minutes and 20 minutes for the 4 bilayer coating. It has been well established in PEM coatings that an increase in the number of bilayers results in increased interactions between the analyte and the coatings which in turn results in higher resolutions. As a result, it was concluded that baseline resolution of all 8 aryl ketones would not be possible using PEM coatings alone.

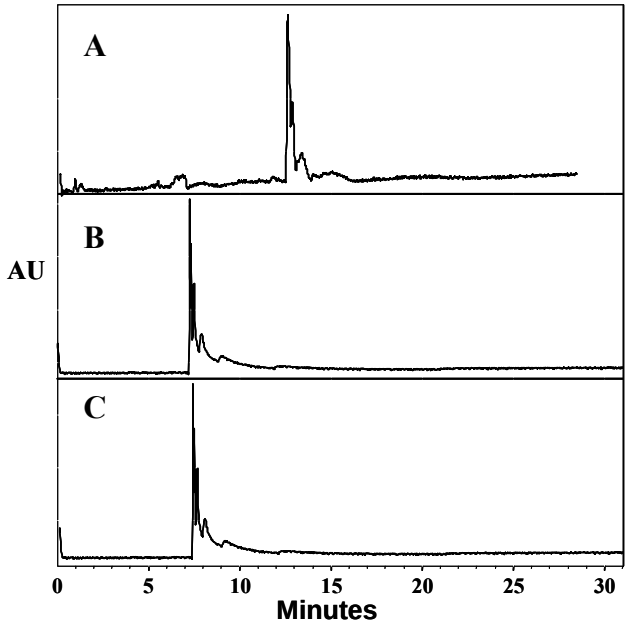

Figure 4.4 Effect of concentration of poly-L-SUG on the separation of 8 achiral alkyl aryl ketones using PEM coatings. Conditions: PEM Coating: 2 Bilayers; 0.5%w/v PDADMAC; A.0.25%; B. 0.5% C. 1.00% (w/v) poly-SUG ; Buffer: 100 mM Tris, pH 10; Analyte concentration: 0.1 mg/ml; Capillary Length: 57 cm total (50 cm effective length); Capillary I.D.: 50 µm; Temperature: 15 ºC; Voltage: 15 kV; Injection: 5 psi for 5 s; Detection: 254 nm ; Analytes: 1. Acetophenone, 2. Propiophenone, 3. Butyrophenone, 4. Valerophenone, 5. Hexanophenone, 6. Heptanophenone, 7. Octanophenone, 8.Decanophenone

4.3.3 Effect of Concentration of Poly-L-SUG and Voltage on the Separation of 8 Achiral Alkyl Aryl Ketones Using Mixed Mode Separation Technique

The use of MEKC and PEM coatings alone resulted in only partial separation of the 8 alkyl aryl ketones. Therefore, a different approach, i.e. mixed mode separation, was employed in order to achieve baseline resolution. In mixed mode separation, the analytes are able to partition

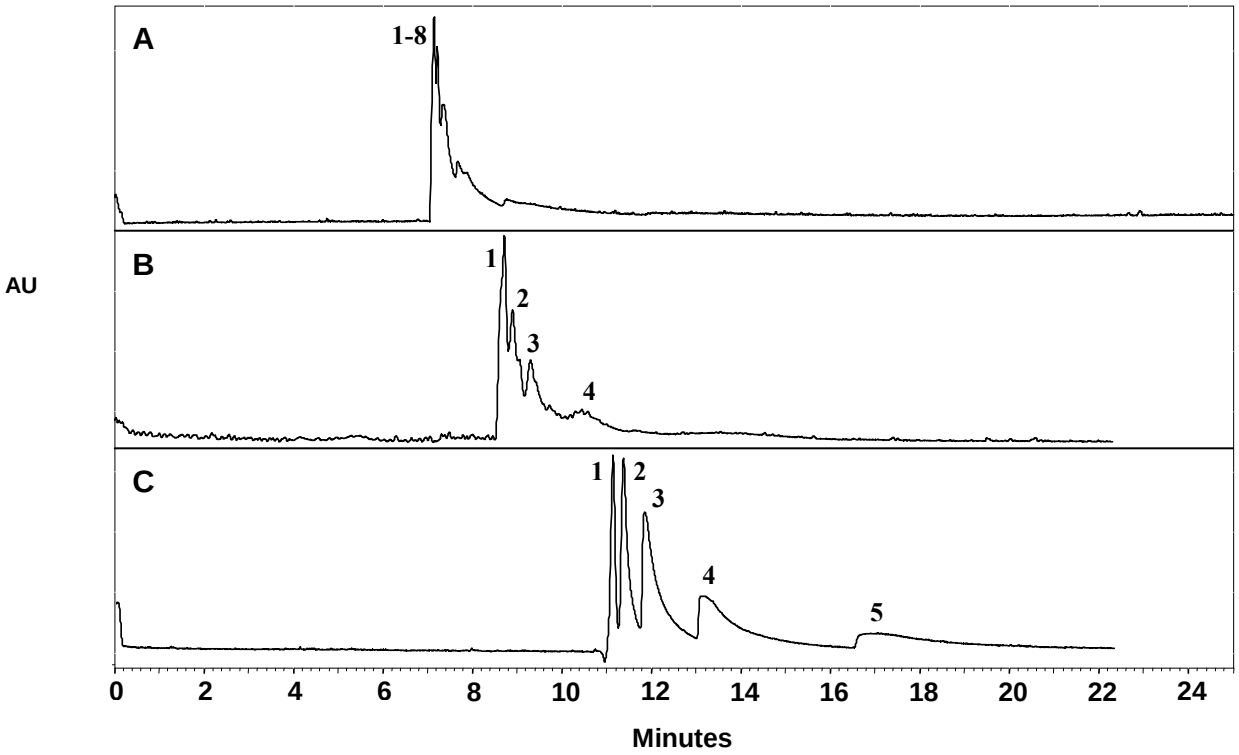

Figure 4.5 Influence of bilayer number on the separation of 8 aryl ketones using PEM coatings.Conditions: A: 2 bilayers; B: 3 bilayers; C: 4 bilayers; Coating: 0.5% (w/v) PDADMAC and 0.5% (w/v) p-SUG; Buffer: 100 mM Tris, pH 10; Analyte concentration: 0.1 mg/ml, Capillary Length: 57 cm total (50 cm effective length); Capillary I.D.: 50 μm; Temperature: 15 ºC; Voltage: 15 kV, Injection: 5 psi for 5 s; Detection: 220 nm; Analytes: 1. Acetophenone, 2. Propiophenone, 3. Butyrophenone, 4. Valerophenone, 5. Hexanophenone, 6. Heptanophenone, 7. Octanophenone, 8. Decanophenone

into the stationary phase as well as the mobile phase. Increased interactions between analytes and molecular micelles results in higher resolution. In Figure 4.6, each capillary was coated with 2 bilayers of 0.5% PDADMAC and 0.5% p-SUG (PEM coating), also, different concentrations of p-SUG were placed in the mobile phase. In Figure 4.6A, 0.25% p-SUG in the mobile phase resulted in the separation in seven (7) of the eight (8) aryl ketones. As the concentration of the molecular micelle increased (figure 4.6B-4.6D), the resolution of the aryl ketones increased and all 8 ketones were baseline resolved. Also, higher concentrations of poly-SUG provided longer migration times and higher peak efficiencies. The electrophoretic mobility of anionic molecular

micelles is opposite to that of the EOF and the hydrophobic aryl ketones interact strongly with poly-SUG. Therefore, the analytes are retained in the column longer, hence, longer migration times. The optimum concentration of p-SUG was 0.75% (w/v) since the highest resolution in a reasonable migration time as well as higher peak efficiencies were obtained (Figure 4.6C).

The effect of voltage on the separation of 8 aryl ketones was also investigated. The voltage was varied from 15kV to 30 kV (Figure 4.7). As the voltage increased, the viscosity of the electrolyte increased and the analytes moved at a faster rate through the column. At these separation conditions shorter migration times (18 minutes) and higher peak efficiencies were obtained when 30 kV was used (Figure 4.7C).

4.3.4 Effect of the Separation Mode on the Resolution of the Chiral Benzodiazepine, Temazepam

Due to the success of the mixed mode approach to the separation of achiral analytes, experiments were conducted to investigate its effect on chiral separation. Each of the 3 separation techniques were also applied to chiral analytes of different compound classes. The results obtained using each separation mode for temazepam is shown in Figure 4.8. In Figure 4.8A, PEM coatings were constructed with 2 bilayers of 0.5% (w/v) PDADMAC and 0.75% (w/v) poly-L-SULV. Only partial separation was achieved for temazepam (Rs = 0.39) in a migration time of 5 minutes. Next, the separation of temazepam was investigated using four concentrations (0.25 - 1.00% (w/v)) of poly-L-SULV. The technique used for this study was MEKC. In Figure 4.8B, the highest resolution (Rs = 0.75) obtained when 1.00% (w/v) poly-L-SULV was used is illustrated. Examination of the electropherogram reveals an increase in resolution compared to PEM coatings as well as increases in peak efficiencies and migration times. However, baseline resolution could not be achieved by using either method alone. Therefore, the mixed mode separation approach was employed to fully resolve this compound.

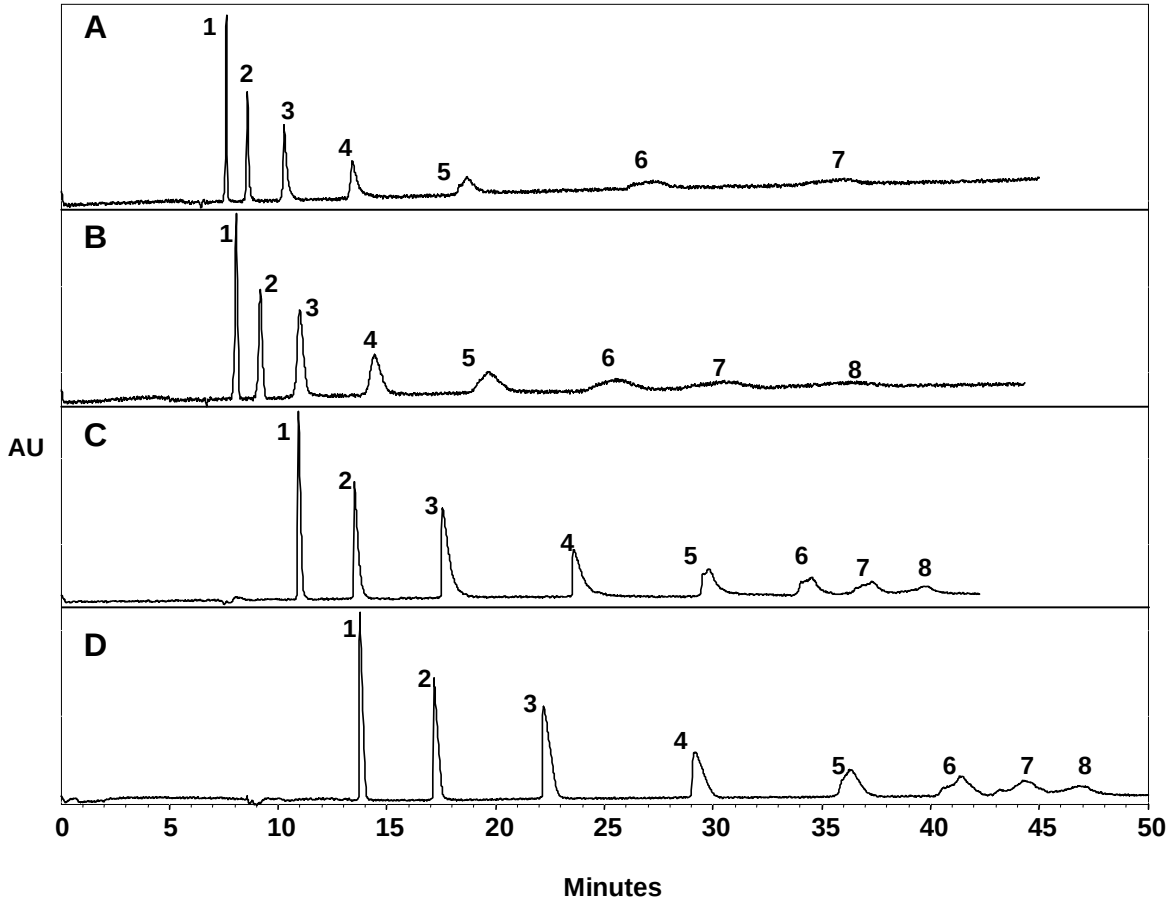

Figure 4.6 Influence of poly-SUG concentration on the separation of 8 aryl ketones using mixed mode separation technique. Conditions: All PEM coatings were constructed using 2 bilayers of 0.5% PDADMAC and 0.5% p-SUG (A-D); A: MEKC: 0.25% (w/v) p-SUG; B: MEKC: 0.5% (w/v) p-SUG; C: MEKC: 0.75% (w/v) p-SUG; D: MEKC: 1.00% (w/v) p-SUG; Buffer: 100 mM Tris, pH 10; Analyte concentration: 0.1 mg/ml, Capillary Length: 57 cm total (50 cm effective length); Capillary I.D.: 50 µm; Temperature: 15 ºC; Voltage: 15 kV, Injection: 5 psi for 5 s; Detection: 220 nm; Analytes: 1. Acetophenone, 2. Propiophenone, 3. Butyrophenone, 4. Valerophenone, 5. Hexanophenone, 6. Heptanophenone, 7. Octanophenone, 8. Decanophenone

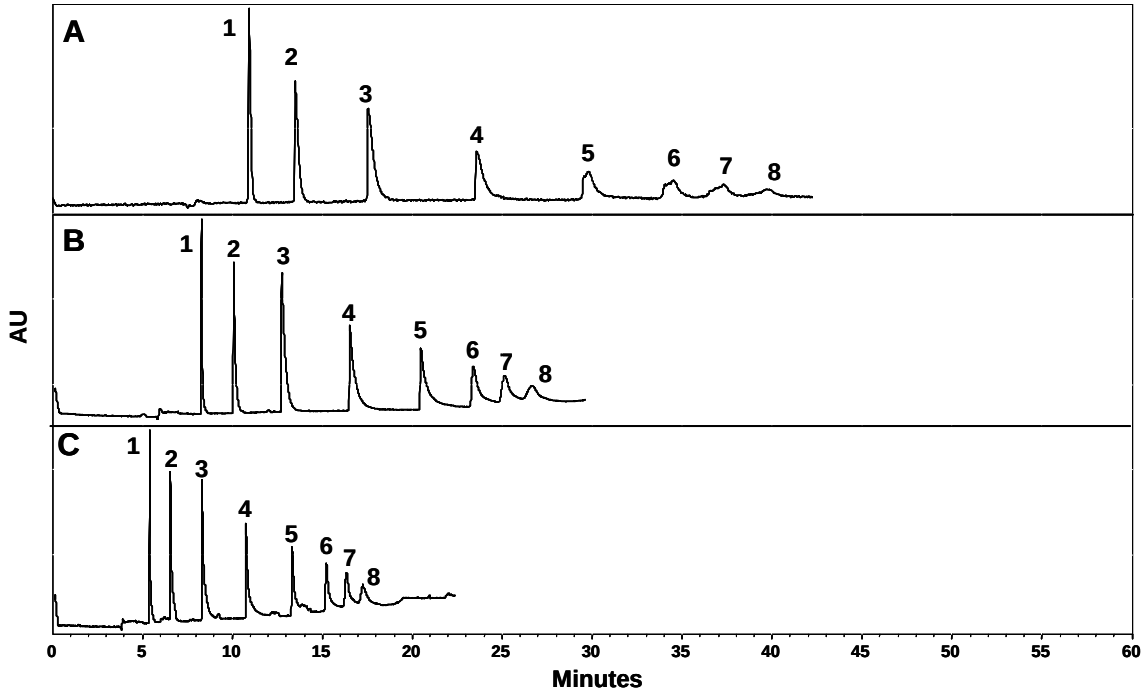

Figure 4.7 Influence of applied voltage on the separation of 8 aryl ketones using mixed mode separation technique. Conditions: All PEM coatings were constructed using 2 bilayers of 0.5% PDADMAC and 0.5% p-SUG; MEKC: 1.00% (w/v) poly-SUG; Buffer: 100 mM Tris, pH 10; Analyte concentration: 0.1 mg/ml, Capillary Length: 57 cm total (50 cm effective length); Capillary I.D.: 50 µm; Temperature: 15 ºC; Voltage: A. 15 kV, B. 20 kV, C. 30 kV Injection: 5 psi for 5 s; Detection: 220 nm; Analytes: 1. Acetophenone, 2. Propiophenone, 3. Butyrophenone, 4. Valerophenone, 5. Hexanophenone, 6. Heptanophenone, 7. Octanophenone, 8. Decanophenone

In Figure 4.8C, the combination of 2 bilayers (PEM coating) on the capillary wall as well as varying concentrations (0.25 – 1.00% (w/v)) of poly-L-SULV in the mobile phase (MEKC) is illustrated. As seen in figure 4.8C-I, the resolution of temazepam increased slightly to 0.76 when 0.25% (w/v) was used. When the concentration of poly-L-SULV increased from 0.5% (w/v) to 1.00% (w/v) (Figure 4.8C-II-IV), the resolution of temazepam increased to 1.76. The increase in resolution is to due to the increased partitioning and interactions (hydrogen bonding, dipole-dipole, and dispersive) between temazepam and the chiral molecular micelle, poly-L-SULV which is located both in the stationary and mobile phases.

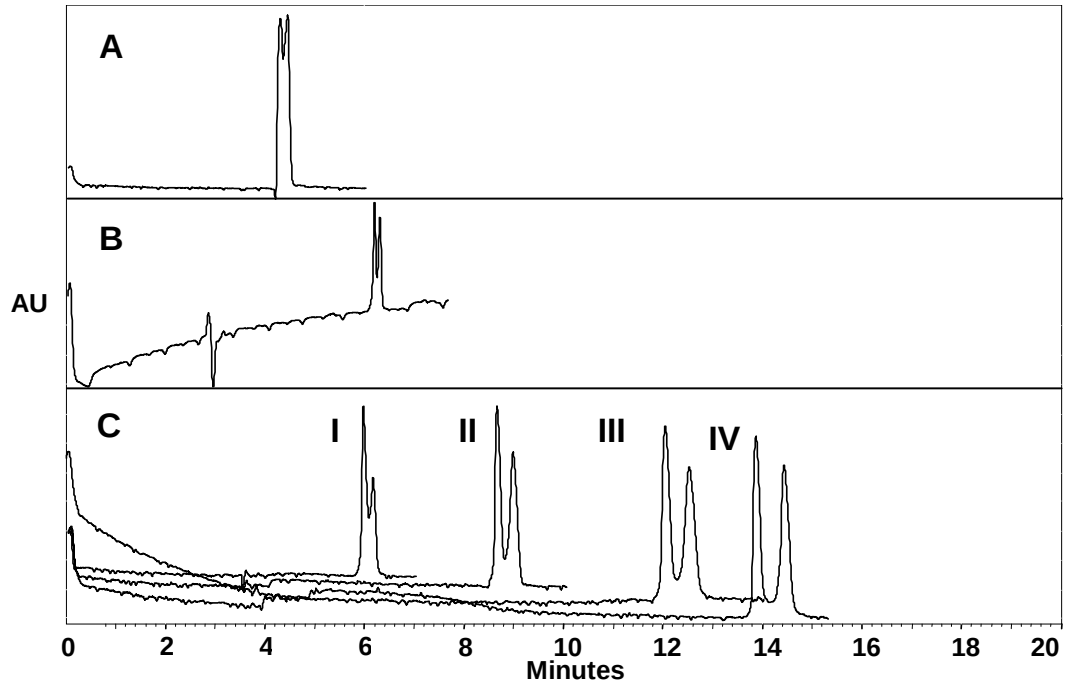

Figure 4.8 Influence of separation mode on the resolution of temazepam. Conditions: A. PEM coatings: 2 bilayers of 0.5% (w/v) PDADMAC and 0.75% (w/v) p-SULV; B. MEKC: 1.00% (w/v) p-SULV; C: Mixed mode: PEM Coating: 2 bilayers of 0.5% (w/v) PDADMAC and 0.75% (w/v) p-SULV and MEKC: I. 0.25% (w/v); II. 0.5% (w/v); III. 0.75% (w/v); IV. 1.00% (w/v) poly-L-SULV; Buffer: 50 mM phosphate, pH 9.2; Analyte concentration: 0.2 mg/ml, Capillary Length: 57 cm total (50 cm effective length); Capillary I.D.: 50 μm; Temperature: 15 ºC; Voltage: 30 kV, Injection: 5 psi for 5 s; Detection: 254 nm

4.3.5 Influence of the Polymer Concentration on the Resolution of Aminoglutethimide Using Mixed Mode Separation Technique

Examination of previous results suggest superiority of the mixed mode separation technique as compared to MEKC or PEM coatings alone in terms of analyte resolution. In this study, the influence of polymer concentration in the mobile phase using the mixed mode technique was investigated. The PEM coatings were first constructed using 2 bilayers of 0.5% (w/v) PDADMAC and 0.75% (w/v) poly-L-SULV. Then, the concentration of poly-L-SULV in the mobile phase was varied from 0.25-1.5% (w/v). The results obtained are shown in Figure 4.9. When 0.25% (w/v) poly-L-SULV was used, partial separation (Rs = 0.29) of aminoglutethimide was achieved within 10 minutes (Figure 4.9A). Figure 4.9B shows an

increase in resolution (Rs = 0.75) when using 0.5% (w/v) poly-L-SULV in a similar elution time. As expected, when the concentration of poly-L-SULV in the mobile phase increased from 1.00-1.50% (w/v) (Figure 4.9C-D), the resolution (Rs = 1.40 – 1.68) as well as the migration time (13 – 16 minutes) increased. The optimum condition for separation (baseline resolution) was achieved when 1.5% (w/v) poly-L-SULV was used. The presence of the molecular micelles both in the stationary and mobile phases plays a seminal role in increasing the polymer-analyte interactions, hence, increased resolution.

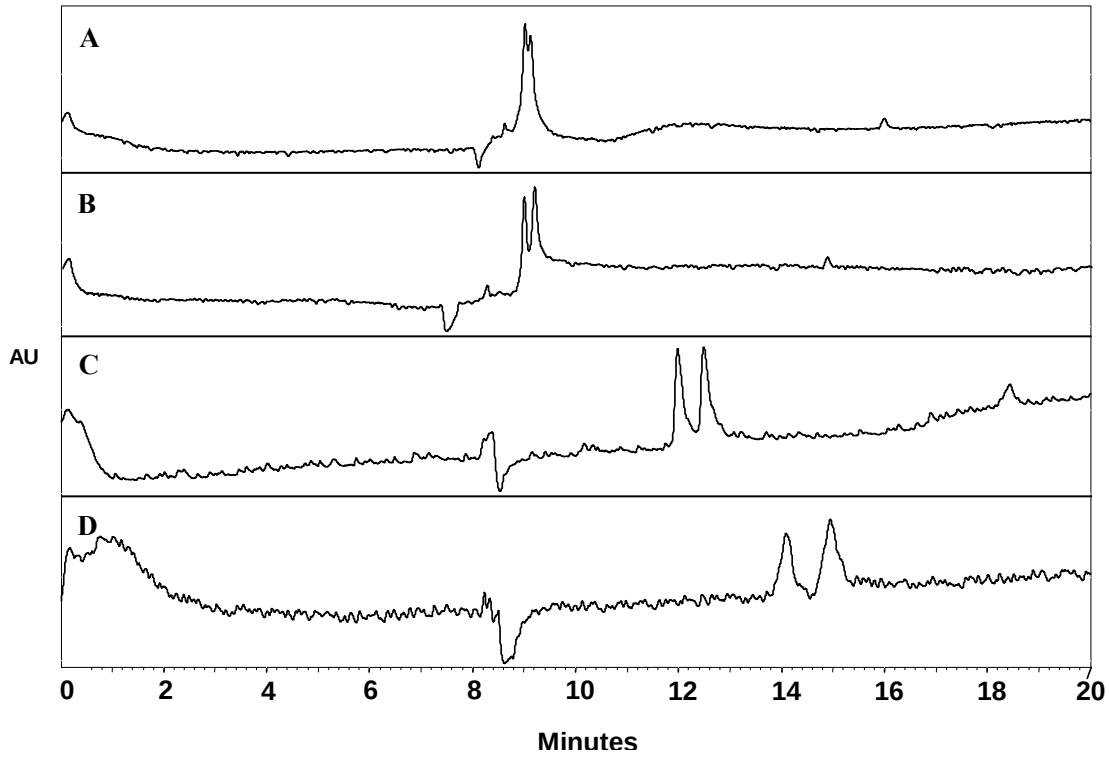

Figure 4.9 Influence of poly-L-SULV concentration in the mobile phase on the resolution of aminoglutethimide using mixed mode separation technique. Conditions: All PEM coatings were constructed using 2 bilayers of 0.5% (w/v) PDADMAC and 0.75% (w/v) p-SULV (A-D). A. Mixed Mode: MEKC: 0.25% (w/v) p-SULV; B. Mixed Mode: MEKC: 0.5% (w/v) p-SULV; C. Mixed Mode:, MEKC: 1.00 %(w/v) p-SULV; D. Mixed Mode: MEKC: 1.50% (w/v) p-SULV Buffer: 50 mM phosphate, pH 7.5; Analyte concentration: 0.2 mg/ml, Capillary Length: 57 cm total (50 cm effective length); Capillary I.D.: 50 μm; Temperature: 15 ºC; Voltage: 30 kV, Injection: 5 psi for 5 s; Detection: 254 nm

4.3.6 Optimum Separation Conditions of Three Chiral Analytes (Benzoin, Benzoin Methyl Ether and Coumachlor) Using MEKC, PEM Coatings, and Mixed Mode Separation Technique

Three additional chiral analytes were investigated to demonstrate the effectiveness of the mixed mode separation. Table 4.1 is a compilation of the separation conditions that achieved the highest resolution for 3 chiral analytes using each separation mode. Using MEKC, resolution values of 0.96, 0.78 and 1.26 were obtained for benzoin, benzoin methyl ether (BME) and coumachlor respectively. The separation of benzoin and coumachlor occurred in approximately 14 minutes, whereas BME had an elution time of 6.13 minutes. Since baseline resolution could not be achieved using MEKC, further experiments were performed using PEM coatings to determine its influence on the resolution of these analytes. However, the results obtained using PEM coatings were inferior to those of MEKC. Though less molecular micelles were consumed, both resolution and migration times decreased using coated capillaries. Therefore, the mixed mode separation approach was implemented to overcome the limitations of each method when used separately. As shown in Table 4.1, the resolution of each chiral analyte increased using mixed mode separation and all three analytes were baseline resolved within 9 minutes. In addition, the capacity factors increased for all 3 analytes when the mixed mode separation was used and the selectivity attained was similar to those of MEKC. These values are great indicators of the effectiveness of these chiral columns.

4.4 Concluding Remarks

Mixed mode separation has been implemented to separate both achiral and chiral analytes. This method can be employed to separate analytes of various compound classes that are difficult to resolve using MEKC or PEM coatings alone. All results are indicative of an increase in resolution when mixed mode separation is used. Baseline resolution was achieved for 8 achiral aryl ketones as well as temazepam, aminoglutethimide, benzoin, benzoin methyl

ether, and coumachlor. Increasing the molecular micelle concentration in the mobile phase resulted in increased chiral resolution. The separation conditions were optimized for the specific analytes investigated; however, mixed mode separation has the potential to be applied to a wide range of analytes. The selectivity of the system can be tailored by altering the molecular micelles used, the bilayer number, as well as cationic and anionic polymer concentrations, among others. Overall, this approach would be of great benefit for quick pharmaceutical screening as well as in areas that require the difficult separation of achiral or chiral analytes.

Table 4.1 Optimum separation conditions of three chiral analytes using MEKC, PEM coatings and mixed mode separation technique. Conditions: All PEM coatings were constructed using 0.5% (w/v) PDADMAC. Temperature: 15 ºC; Buffer: 50 mM phosphate, pH 7.5; Analyte concentration: 0.2 mg/ml, Capillary Length: 57 cm total (50 cm effective length); Capillary I.D.: 50 μm; Injection: 5 psi for 5 s; Detection: 254 nm; Analyte: Benzoin, Benzoin Methyl Ether (BME), Coumachlor

Separation Mode	Analyte	[Poly-L-SULV] (%w/v)	Volt (kV)	EOF (min)	MT1	MT2	Rs1	K1	K2	α
MEKC	Benzoin	1.00	15	7.45	13.22	13.58	0.96	0.77	0.82	1.06
3bilayers-PEM	Benzoin	1.00	30	4.12	4.27	4.43	0.34	0.04	0.08	2.08
2bilayers-MM	Benzoin	0.75/1.50	30	3.34	7.26	7.51	1.50	1.17	1.25	1.06
MEKC	BME	1.00	30	3.04	6.00	6.13	0.78	0.97	1.01	1.04
3bilayers-PEM	BME	1.00	30	3.88	3.97	4.25	0.51	0.02	0.10	4.11
3bilayers-MM	BME	0.75/1.50	30	3.24	8.19	8.43	1.51	1.53	1.60	1.05
MEKC	Coumachlor	1.00	15	7.41	12.84	3.18	1.26	0.73	0.78	1.06
3bilayers-PEM	Coumachlor	0.75	30	4.20	6.13	6.80	0.82	0.46	0.62	1.35
3bilayes-MM	Coumachlor	0.75/1.50	30	3.25	6.77	6.99	1.52	1.08	1.15	1.06

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CHAPTER 5

ACIDIC PROTEIN SEPARATIONS USING POLELECTROLYTE MULTILAYER COATINGS IN OPEN TUBULAR CAPILLARY ELECTROCHROMATOGRAPHY AND GRADIENT ELUTION MOVING BOUNDARY ELECTROPHORESIS

5.1 Introduction

Proteins are biomolecules of great interest as biomarkers for diseases, markers for stage development of organisms, and additives to foods [1 Many studies have been dedicated to the separation and identification of milk proteins such as α-lactalbumin, β-lactoglobulin A, β-lactoglobulin B, bovine serum albumin (BSA) because of immunogenic properties [1-3]. These bovine whey proteins are also widely used as test mixtures for many techniques to determine the quality of nutrients in processed milk [2, 4]. In this manuscript, this protein system was selected as a model for the development of a protein separation approach using CEC and gradient elution moving boundary electrophoresis (GEMBE).

Polyelectrolyte multilayer (PEM) coatings have been used in open tubular CEC (OT-CEC) to separate a number of achiral [5, 6] and chiral analytes [7-9], as well as proteins [10-12]. PEM coatings are constructed by alternating layers of cationic and anionic polymers on the inner walls of a deprotonated silica capillary. Each layer of the cationic and anionic polymer is termed, a bilayer [5, 6]. Successful protein separations using PEM coatings are achieved through three major mechanisms. Firstly, protein adsorption is reduced since the exposed negatively charged silanol groups are covered by the PEM coatings. Secondly, these coatings are used to control or modify the EOF. The third major use of PEM coatings is to provide a medium for protein interactions [10].

Both UV and LIF detectors may be employed in protein separations. UV detectors are commonly used in CE for the detection of a wide range of analytes including proteins [13]. Separations performed using UV detection are typically simple and require little or no analyte

derivations, pre-, on- or post-column [14]. Compared to other detection methods, UV detection is inexpensive, simple, and easily automated. Wang *et al.* used CE with UV detection to achieve high resolution, fast separations of various proteins in capillaries coated with multilayers [14]. The results indicated the ability of on-line concentration with pH junctions for the detection of trace proteins. Recently, Luces *et al.* used PEM coatings with UV detection to report the differences in protein selectivity for basic proteins when varying the coating polymers [10]. Coated columns coupled with UV detection have been used to simultaneously separate both acidic and basic proteins and many papers have been published in this area [15-20].

Although, UV detection has been successfully used for many protein and peptide mixtures, it has proven to be troublesome for biological samples with concentrations in the sub-μM ranges [21]. In contrast, LIF detection is by far the most sensitive detection mode used for CE affording the lowest LOD. LIF enables the detection of few or even single molecules [21]. Ramsay *et al.* detected attomolar, femtomolar, and zeptomolar protein concentrations by CIEF with LIF detection [22, 23]. Some proteins are fluorescent in their native state if their primary structure contains the amino acids tryptophan, tyrosine or phenylalanine [24]. It should be noted that the UV excitation required for these amino acids makes this method inconvenient, therefore, CE-LIF with native protein fluorescence is less common than LIF detection of derivatized proteins [25, 26]. In cases where proteins do not natively fluoresce, pre-[27], on- [28] or post-column [29, 30] fluorescent derivatizations are necessary for use with LIF detection.

In pre-column derivatization, the most commonly used, proteins are labeled with a fluorescent dye before introduction to the capillary. Covalent bonds may form between the dye and the amino, carboxylate, thiol, or other functional groups on the proteins [21]. There are currently many commercial dyes that are used to fluorescently label proteins through covalent bonds between the proteins' reactive thiol groups of cystein residues and the dye. Fluorescent

labeling to the thiol groups have proven to produce cleaner electropherograms when compared to other labeling groups [21]. Thiol labeling does not interfere with the charged groups on the protein i.e. $-NH_3^+$ and $-COO^-$ and therefore maintains the net charge of the protein unaltered.

Protein separations using PEM coatings with LIF detection are not limited to only CE techniques; these coatings can also be used with GEMBE. Shackman *et al.* were the first to demonstrate GEMBE, which promotes high resolution separations in short, narrow separation microchannels [31]. GEMBE has the advantage of allowing continuous sample injection into the separation channel which eliminates conventional injection methods such as electrokinetic and hydrodynamic injections. Experiments using GEMBE are performed by carefully controlling a hydrodynamic counterflow, which allows analytes to enter the separation channel. Separations are achieved due to differences in the electrophoretic mobilities of the analytes. Therefore, only analytes with an electrophoretic mobility that is greater than the counterflow will enter the separation channel.

In this manuscript, we report on protein separations using PEM coatings with UV and LIF detection in OT-CEC. The influence of polymer type, bilayer number, pH of the background electrolyte, capillary effective length and internal diameter were investigated to determine their effect on protein resolution and migration time. In addition, the application of PEM coated capillaries for protein separations using GEMBE with LIF detection was explored to demonstrate the coatings' versatility.

5.2 Materials and Methods

5.2.1 Materials

The cationic polymer, poly-L-ornithine hydrobromide, as well as the proteins, α-lactalbumin (bovine milk), β-lactoglobulin A (bovine milk), β-lactoglobulin B (bovine milk), albumin (chicken eggwhite), myoglobin (equine heart), and deoxyribonuclease I (bovine

pancreas) were purchased from Sigma Chemical Company (St. Louis, MO). The chemicals used to synthesize surfactant monomers *N*-hydroxysuccinimide, undecylenic acid, chlorosulfonic acid, dicyclohexylcarbodiimide, and sodium bicarbonate were purchased from Fluka (Milwaukee, WI). The dipeptide (L,L)-leucyl-alaninate was purchased from Bachem Bioscience, Inc. (King of Prussia, PA). Sodium hydroxide, ethyl acetate, and tetrahydrofuran were purchased from Sigma-Aldrich (Milwaukee, WI). The background electrolyte, sodium phosphate dibasic, methanol, and acetone were purchased from Fisher Scientific (Fair Lawn, NJ). All materials were used as received.

5.2.2 Instrumentation

For OT-CEC separations, fused silica capillary (Polymicro Technologies; Phoenix, AZ) with a 5 mm detection window was used with at varying lengths (10 cm, 30 cm, 40 cm) and internal diameters (30 μm, 50 μm). OT-CEC experiments were conducted using a Beckman P/ACE MDQ capillary electrophoresis system, equipped with a photodiode array detector and LIF detector (488 nm argon-ion laser) (Fullerton, CA). For UV detection, the acidic proteins were detected at 200 nm. All analytes were injected at 0.5 psi for 5 s. Liquid coolant was used to maintain the temperature at 15 °C. The applied voltage ranged from 15 kV to 30 kV. For GEMBE separations, a 3 cm fused silica capillary with a 5 mm detection window was used. One end of the capillary was placed into a 360 μm hole in the analyte reservoir with an electrode. The other end of the capillary was attached to a polypropylene syringe that contained ~1 mL of buffer. The syringe was grounded and connected to a ±69 kPa (10 psi) precision pressure controller (Series 600, Mensor, San Marcos, TX), which used helium as the gas supply. The syringe plunger accommodated the high voltage supply and controlled the pressure. All experiments were performed on a fluorescence microscope (DMLB, Leica Microsystems, Bannockburn, IL), with 10 × objective (numerical aperature, NA = 0.3), Hg arc lamp, color CCD

camera, (DXC-390, Sony, New York, NY) and appropriate fluorescence filter sets. Instrumental control and data acquisition used Java 5.0 software (Sun Microsystems, Santa Clara, CA). Raw data were transformed using Savitzky Golay smoothing.

5.2.3 Synthesis of Molecular Micelles

The chiral dipeptide molecular micelles poly-L-SULA and poly-L-SULV were synthesized according to a procedure previously described by Wang and Warner [32]. ^{60}Co γ-ray irradiation was used to polymerize 100 mM monomer solutions. The molecular structures of the molecular micelles as well as the cationic polymer used in this study are presented in Figure 5.1.

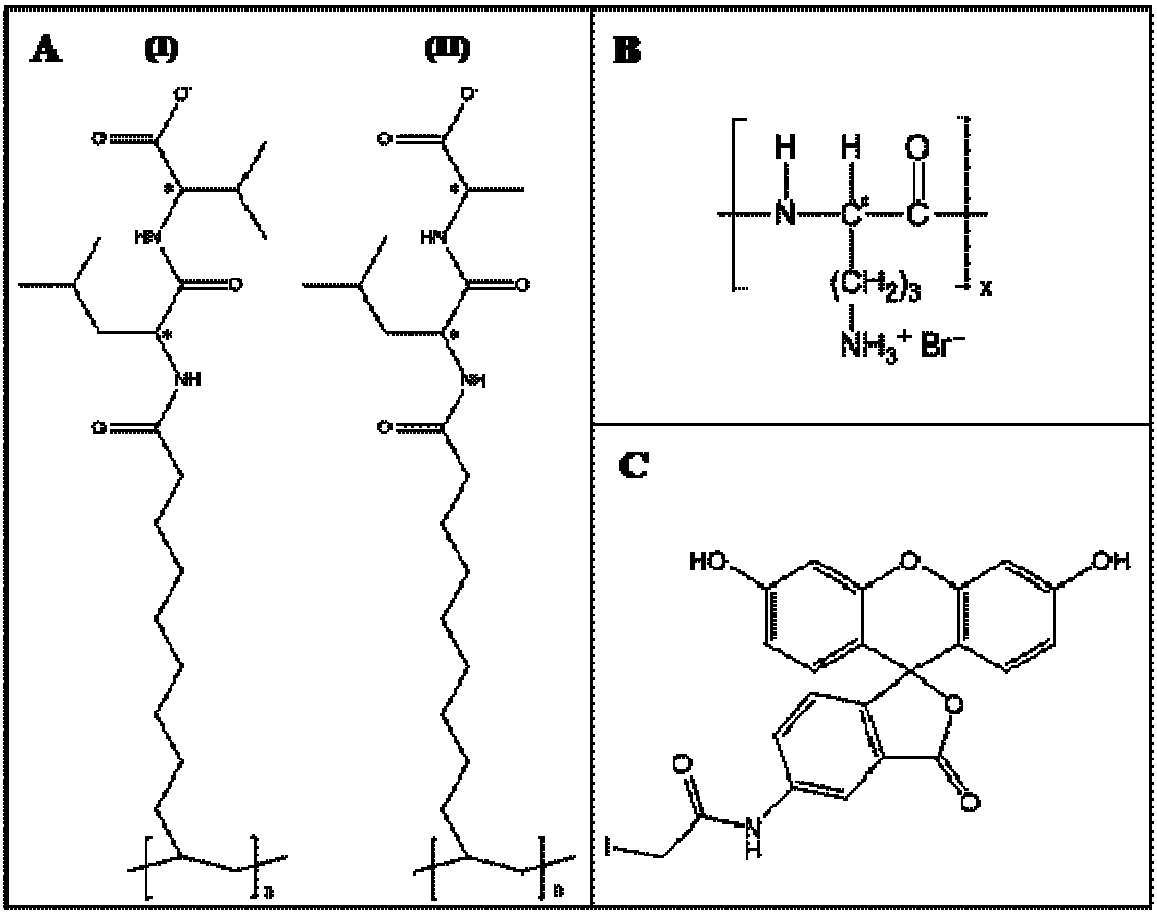

Figure 5.1 Representative molecular structures of compounds used for protein separations: A: Anionic polymer: (I) Poly-L-SULV; (II) Poly-L-SULA; (* indicates the chiral center; B: Cationic Polymer: Poly-L-ornithine hydrobromide; C: 5-Iodoacetamidofluorescein (5-IAF)

5.2.4 Sample and Buffer Preparation

The pH of the background electrolyte for all studies was adjusted using 1 M NaOH and 1 M HCl. All buffers were filtered using 0.45 μm polypropylene nylon filters and sonicated for 15 minutes before use. Stock solutions of the acidic proteins were prepared in 40 mM phosphate buffer at pH 7, 8, 9, and 10. The chiral cationic polymer, poly-L-ornithine hydrobromide, and the chiral molecular micelles (poly-L-SULA and poly-L-SULV) were set at concentrations of 0.03% (w/v) and 0.5% (w/v) respectively.

5.2.5 PEM Coating Procedure

The capillary walls were deprotonated using 1 M NaOH by flushing the capillary for 30 minutes followed by a 15 minute deionized water rinse. First, the cationic polymer was flushed through the capillary for 10 minutes followed by a 5 minute rinse with deionized water. The bilayer was completed by a 5 minute rinse with the anionic polymer, followed by a 5 minute deionized water rinse. Subsequent bilayers consisted of alternate 5 minute rinses of the cationic and anionic polymers; however, the last anionic layer was always flushed for 10 minutes. All rinses were performed using the rinse function of the CE instrument, with applied pressure of 20psi. For the GEMBE technique, the capillary was coated using the CE instrument and then cut into 3 cm pieces.

5.2.6 Fluorescent Labeling of the Acidic Proteins

5-Iodoacetamidofluorescein (5-IAF), a fluorescein derivative was chosen to fluorescently label the acidic proteins [Molecular Weight: 515.26; Excitation Wavelength: 490-495 nm; Emission Wavelength: 515-520 nm (green); Molar Extinction Coefficient at 492 nm: 80,000-85,000 M-1 cm-1; Formula: $C_{22}H_{14}INO_6$. (Figure 5.1C) The required amount of each protein (1-10 mg) was dissolved in 1 mL of 0.1 M phosphate buffered saline (PBS), 0.15 M NaCl, pH 7.2. A mass of 1 mg of 5-IAF was dissolved in 100 μl of DMF. 5-IAF was added to each protein

solution in a concentration 10 times the protein concentration. The reaction was then thoroughly mixed and allowed to react for 2 hours in the dark at room temperature. Then, the excess (free) dye was removed by use of a desalting column. Aliquots of fluorescently labeled proteins were stored at 4 ^{o}C in the dark.

5.3 Results and Discussion

5.3.1 Influence of the pH of the Background Electrolyte on the Separation of 6 Acidic Proteins Using PEM Coated Columns with UV Detection

The purpose of this study was to investigate the effect of the pH of the background electrolyte on six (6) acidic proteins. The results reported in this section were obtained using UV detection. A 2 bilayer PEM coating was constructed using 0.03% (w/v) of poly-L-ornithine and 0.5% (w/v) of poly-L-SULA and 0.03% (w/v) of poly-L-ornithine and 0.5% (w/v) of poly-L-SULV (Figure 5.2). The background electrolyte, 40 mM phosphate was prepared at three different pH levels, 7, 8, and 9. Figure 5.2A, using poly-L-SULA, illustrates all proteins being eluted before 13 minutes when a pH 7 background electrolyte was used, however, myoglobin and α-lactalbumin (proteins 2 & 3) co-eluted. Also, only partial separation was attained between β-lactoglobulin B and albumin. When, poly-L-SULV was used as the anionic layer, also at pH 7, no resolution was attained between the last three proteins (β-lactoglobulin A, β-lactoglobulin B, and albumin). At pH 7, proteins have a net negative charge; however, there may still be some areas of exposed positive charges. Therefore, baseline resolution of the last three proteins may not have been achieved due to some adsorption to the capillary wall. This hypothesis is consistent with the peak tailing observed in the electropherogram. In Figure 5.2B, all 6 acidic proteins were baseline resolved within 17 minutes at pH 8 when both poly-L-SULA and poly-L-SULV were used. In both electropherograms, the efficiencies of deoxyribonuclease (peak 3) and albumin (peak 6) were low. However, any increase in the analyte concentration resulted in co-elution with the preceding peak. At pH 9, myoglobin and α-lactalbumin co-eluted when both

poly-L-SULA and poly-L-SULV (Figure 5.2C) were used. As indicated, when poly-L-SULA was used, broad protein peaks may demonstrate too many interactions with the PEM coating. All further experiments were performed using a 40 mM phosphate background electrolyte at pH 8 as a result of baseline resolution for all proteins.

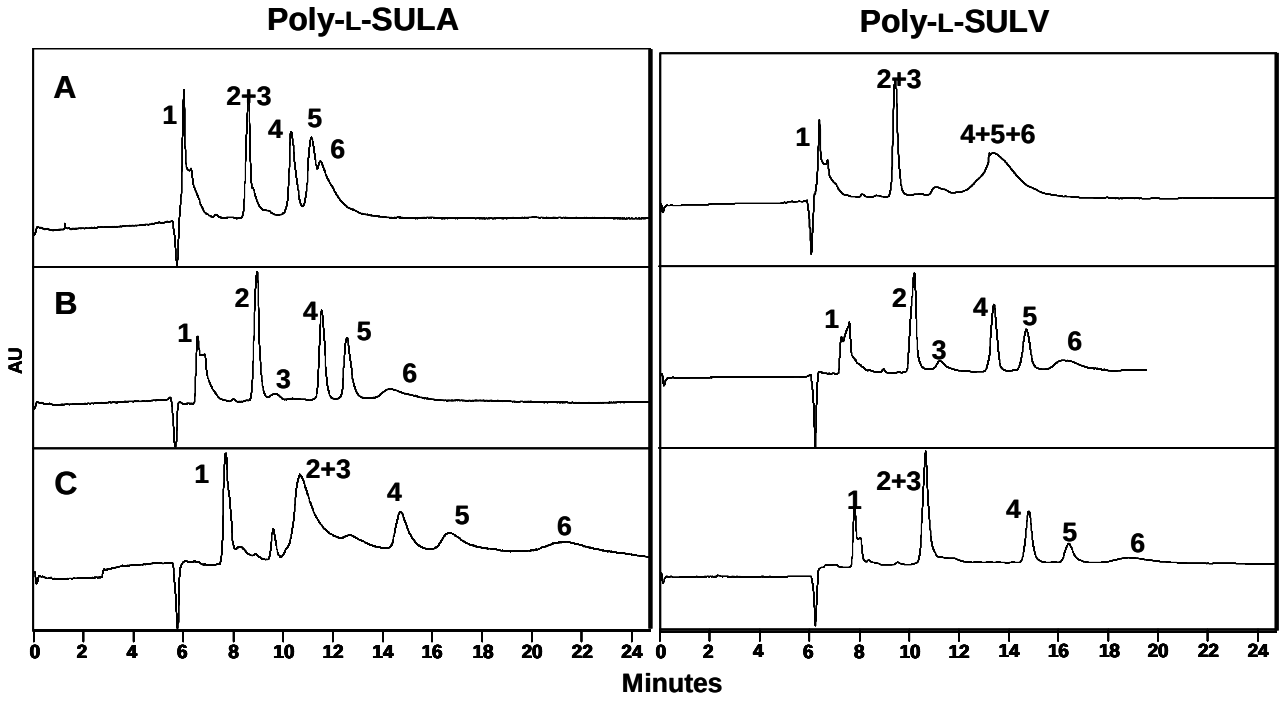

Figure 5.2 Influence of pH on the separation of 6 acidic proteins using PEM coated columns and UV detection. Conditions: Number of Bilayers: 2 bilayers; Cationic Polymer: 0.03% (w/v) poly-L-ornithine, Anionic Polymer: 0.5% (w/v) poly-L-(I)SULA/(II)SULV; Buffer: 40 mM phosphate; pH (A) 7; (B) 8; (C) 9; Analyte concentration: 0.5 mg/ml; Capillary length: 50 cm total (40 cm effective length); Capillary i.d.: 50 µm; Injection: 0.5 psi for 5 s; Temperature: 15 ˚C, Voltage : 15 kV. Detection: 200 nm; Analytes: 1. Myoglobin, 2. α-lactalbumin, 3. Deoxyribonuclease; 4. β-lactoglobulin A, 5. β-lactoglobulin B, 6. Albumin

5.3.2 Influence of the Number of Bilayers on the Separation of 6 Acidic Proteins Using PEM Coated Columns with UV detection

Three different bilayer numbers were investigated using poly-L-ornithine as the cationic polymer with poly-L-SULA and poly-L-SULV (Figure 5.3) as the anionic polymers. The bilayer numbers varied from 1 to 3. When 1 bilayer was constructed using either one of the anionic polymers, complete resolution of the proteins was not achieved (Figure 5.3A). It was suspected

that the exposed negatively charged silanol groups were not completely covered; therefore, yielding to protein adsorption to the capillary walls, was consistent with the observed low peak efficiency. Also, the co-elution of myoglobin and α-lactalbumin as well as β-lactoglobulin B and albumin may indicate no or limited protein-PEM interactions. When 2 bilayers were formed, baseline resolution was achieved when either anionic polymer was used to form the PEM coatings due to protein-PEM interactions [10] (Figure 5.3B). When 3 bilayers were formed, current breakdown as well as irreproducible results was observed (Figure 5.3C). Furthermore, peak identification was unattainable. The optimum number of bilayers required for separating this protein system was determined to be 2 bilayers.

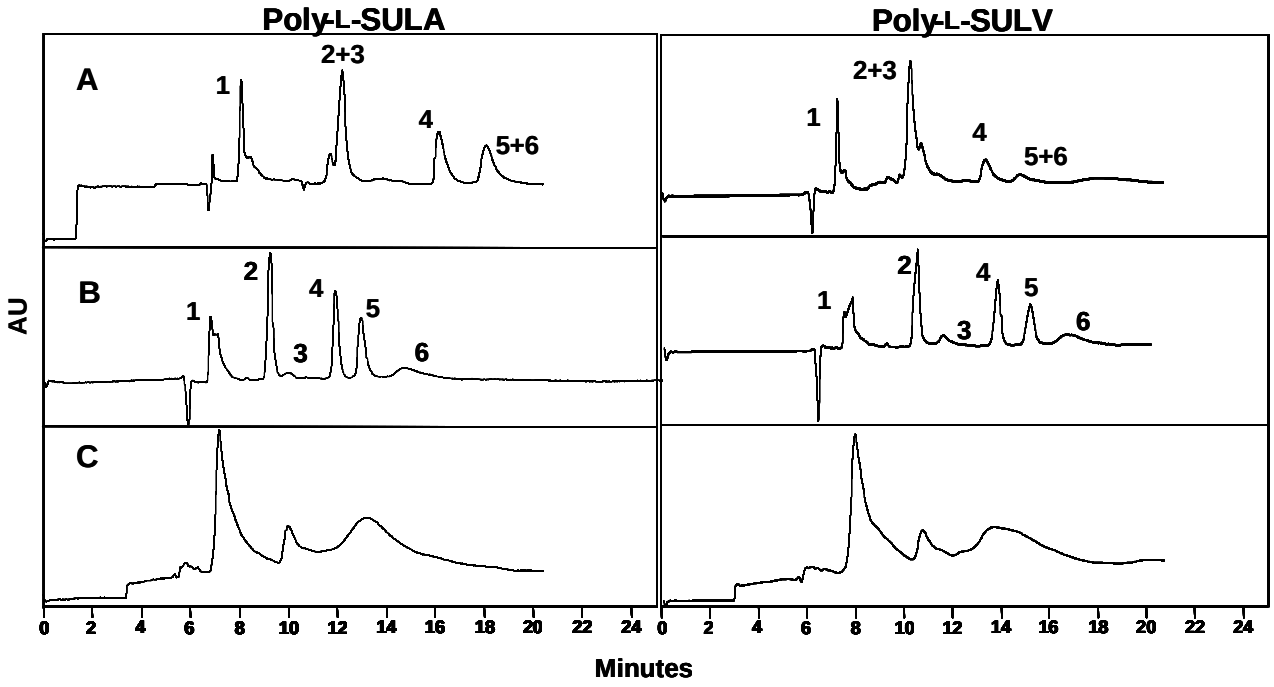

Figure 5.3 Influence of the number of bilayers on the separation of 6 acidic proteins using PEM coated columns and UV detection. Conditions: Number of Bilayers: (A) 1 bilayer; (B) 2 bilayers; (C) 3 bilayers; Cationic Polymer: 0.03% (w/v) poly-L-ornithine, Anionic polymer: 0.5% (w/v) poly-L-(I)SULA/(II)SULV; Buffer: 40 mM phosphate, pH 8; Analyte concentration: 0.5 mg/ml; Capillary length: 50 cm total (40 cm effective length); Capillary i.d.: 50 μm; Injection: 0.5 psi for 5 s; Temperature: 15 ˚C, Voltage : 15 kV, Detection: 200 nm; Analytes: 1. Myoglobin, 2. α-lactalbumin, 3. Deoxyribonuclease, 4. β-lactoglobulin A, 5. β-lactoglobulin B, 6. Albumin

5.3.3 Influence of pH of the Background Eectrolyte on the Separation of 3 Acidic Proteins Using PEM Coated Columns with LIF Detection

Three acidic proteins, β-lactoglobulin A, β- lactoglobulin B and albumin were fluorescently labeled with 5-IAF. These three proteins were chosen because they have free groups for labeling. A required step when proteins have no free groups to label is denaturation to produce free groups. Previous experiments revealed inferior resolution of denatured proteins when PEM coatings were used (results not shown). Therefore, only proteins that did not require denaturation were labeled. All results in this section were attained using the LIF detector in the CE instrument. The PEM coating was created using 0.03% (w/v) poly-L-ornithine and 0.5% (w/v) poly-L-SULA. The background electrolyte was used at three different pH values, 8, 9 and 10. Similar experiments were conducted using UV detection; however, the purpose here was to investigate the influence, if any, of the fluorescent dye. All three proteins were baseline resolved within 14 minutes with pH 8 buffer (Figure 5.4C). As seen with UV detection, protein resolution of the third protein (albumin) was lost at pH 9 (Figure 5.4B) and pH 10 (Figure 5.4A) buffer. High efficiency peaks were attained at all pHs investigated. The other peaks observed in the electropherograms are associated with impurities and degradation products. These peaks were also observed with UV detection; however, the peaks were several orders of magnitude larger using the more sensitive LIF detection method. The optimum pH for the protein separations using both UV and LIF detection was 8. It appears that the fluorescent dye, 5-IAF, did influence the migration time and resolution of the three proteins investigated through different protein-PEM interactions. The migration time of β-lactoglobulin A was ~12 minutes without any fluorescent labeling decreased to ~4 minutes when it was labeled with 5-IAF. Similar decreases in migration times were observed with β-lactoglobulin B and albumin. In addition, increased protein resolution was evident the fluorescently labeled proteins.

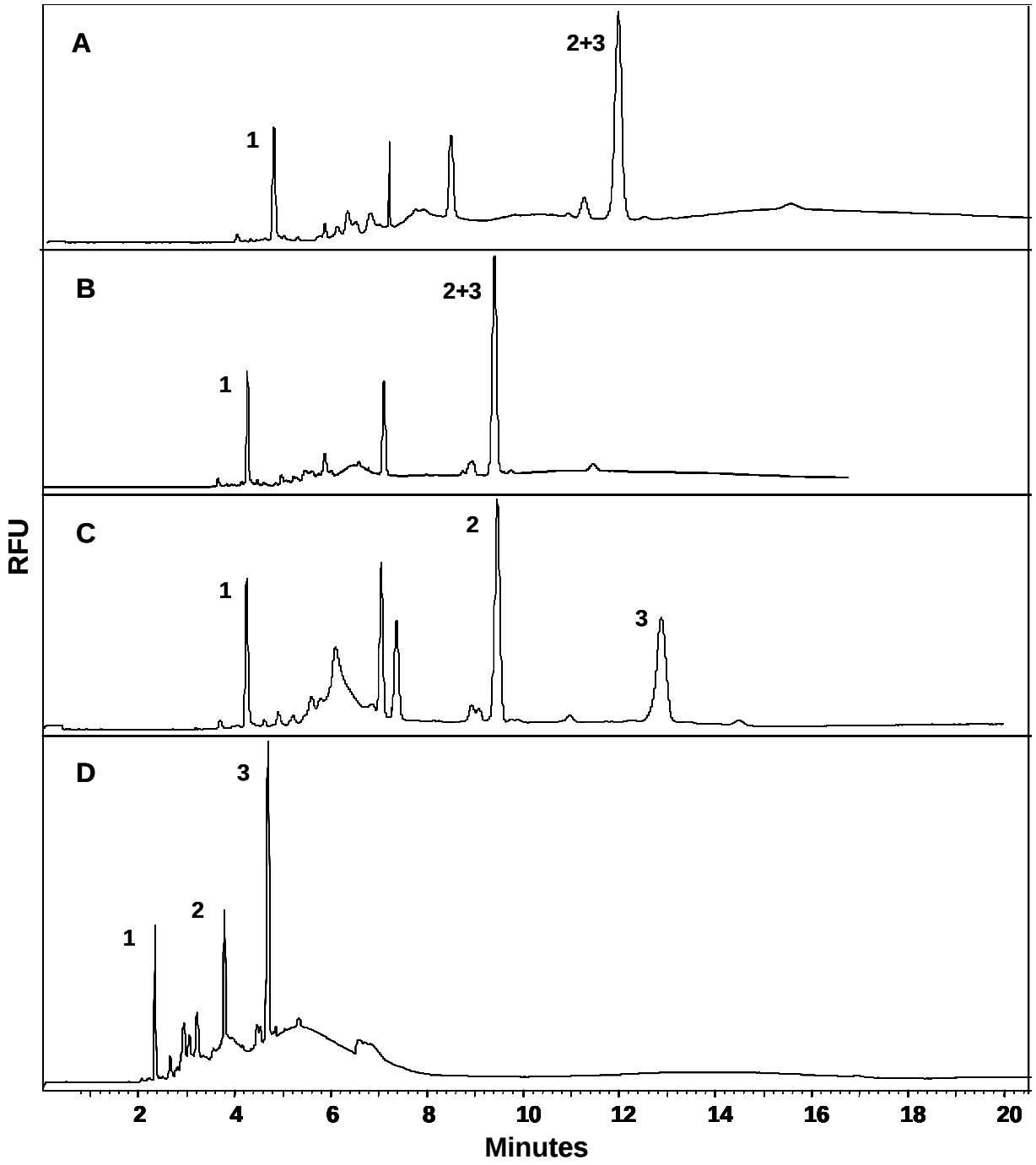

Figure 5.4 Influence of pH of the background electrolyte and internal diameter on the separation of 3 acidic proteins using PEM coated columns with LIF detection. Conditions: Number of Bilayers: 2 bilayers; Cationic Polymer: 0.03% (w/v) poly-L-ornithine, Anionic Polymer: 0.5% (w/v) poly-L-SULA; Buffer: 40 mM phosphate pH (A) 10, (B) 9, (C) 8, (D) 8; Capillary length: 50 cm total (40 cm effective length); Capillary i.d.: (A-C) 50 µm, (D) 30 µm; Injection: 0.5 psi for 5 s; Temperature: 15 ˚C, Voltage : 15 kV, Analytes: 1. β-lactoglobulin A, 2. β-lactoglobulin B, 3. Albumin

5.3.4 Influence of Internal Diameter and Effective Length of the Capillary on the Separation of 3 Acidic Proteins Using PEM Coated Columns with LIF Detection

In this study, the effect of the internal diameter (ID) of the capillary was investigated. PEM coatings (2 bilayers) were constructed in two capillaries, 30 μm ID and 50 μm ID using poly-L-ornithine and poly-L-SULA. The total length of both capillaries was 50 cm (40 cm effective length). When 3 acidic proteins were injected into the 30 μm ID capillary, high efficiency and baseline resolved proteins were observed within 5 minutes (Figure 5.4D). However, similar experiments in the 50 μm ID capillary resulted in a longer separation time of 13 minutes for baseline resolution of the proteins (Figure 5.4C). The faster separation times obtained in the 30 μm ID capillary were due to the higher electric field, therefore resulting in faster elution through the capillary. In both cases, the applied voltage was 30 kV. Since electric field is influenced by voltage and the area of the capillary, a smaller area yields a higher electric field. Special attention was given to achieving a more stable baseline, however, the electropherograms illustrated in Figure 4 were the best obtained.

In order to observe the effect on protein resolution, three capillaries of varying effective lengths, 10 cm, 30 cm and 40 cm were coated with 2 bilayers of 0.03% (w/v) poly-L-ornithine and 0.5% poly-L-SULA. Short-end injection was used to obtain a shorter elution time and also to overcome the constraints of the minimum capillary length possible in commercially available CE cartridges (Figure 5.5A). In short-end injections, voltage is applied at the outlet of the capillary in reversed polarity to obtain the EOF moving in the direction of the detection window. Therefore, the effective length was 10 cm and the total capillary length, 40 cm. Figure 5.5A shows very fast, complete resolution of the 3 acidic proteins within 2 minutes. Again, this was the best baseline obtained after varying several parameters. High efficiency and high resolution peaks were obtained within 5 minutes for acidic protein separations using a 30 cm (effective length) capillary (Figure 5.5B). In Figure 5.5C, 3 proteins were separated with the longest

migration time of approximately 12 minutes. In this system, the electric field was smaller; therefore, the analytes migrated through the column at a slower rate. Also, as a result of increased protein-PEM interactions, protein peaks had lower efficiency than in Figure 5.5A and 5.5B.

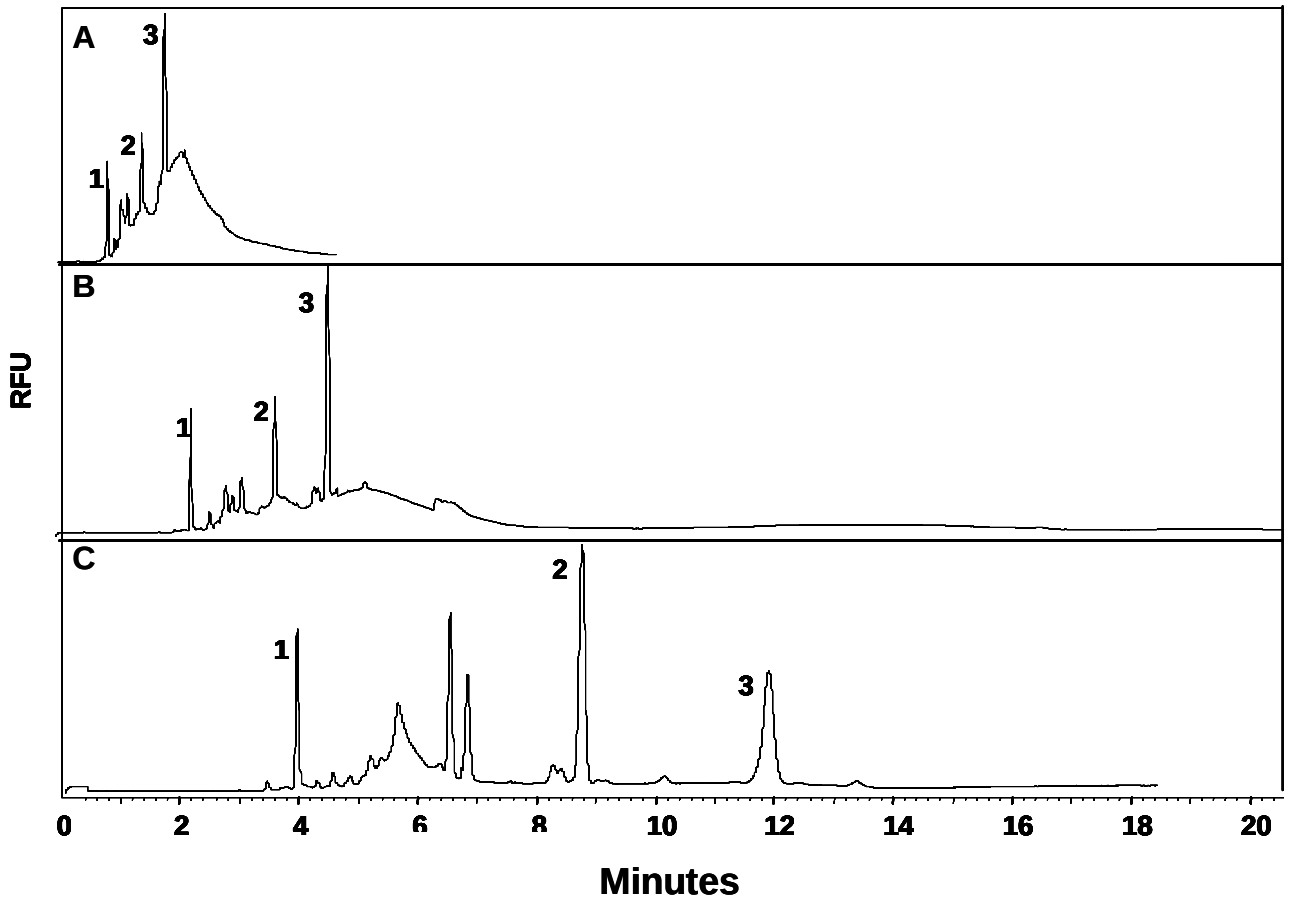

Figure 5.5 Influence of effective length of the capillary on the separation of 3 acidic proteins using PEM coated columns with LIF detection. Conditions: Number of Bilayers: 2 bilayers; Cationic Polymer: 0.03% (w/v) poly-L-ornithine, Anionic Polymer: 0.5% (w/v) poly-L-SULA; Buffer: 40 mM phosphate pH 8; Capillary length: (A) 10 cm (short-end injection); (B) 30 cm, (C) 40 cm effective length, (50 cm total length); Capillary i.d.: 30 µm; Injection: 0.5 psi for 5 s; Temperature: 15 ˚C, Voltage : 15 kV Analytes: 1. β-lactoglobulin A, 2. β-lactoglobulin B, 3. Albumin;

5.3.5 Separation of 3 Acidic Proteins Using GEMBE

GEMBE technique combines the electrophoretic migration of each analyte with the variation of the hydrodynamic bulk counterflow of the solution in short capillaries or microfluidic devices. In general, high resolution separations are achieved using the GEMBE

technique. Analytes with an electrophoretic mobility greater than the velocity of the counterflow will enter the separation channel. As the velocity of the counterflow is varied, each analyte enters the separation channel at a different time. To our knowledge, this is the first report of the use of GEMBE for protein separations. PEM coatings were first constructed using the rinse function in the CE instrument. After the coating was completed, the capillary was removed from the instrument and cut into 3 cm pieces to be used in the GEMBE setup. The capillary was placed in the setup as described in Section 2.2. Figure 5.6 illustrates the effect of voltage on protein separation using the GEMBE technique. The sample was first introduced into the capillary with a starting pressure of 2000 Pa. At intervals of 5 Pa, the sample moves through the capillary until it is captured by the CCD camera. The voltage was varied from 300 V, 700 V, and 1000 V (Figure 5.6). All three voltages provided a migration time of approximately 20 minutes. From these results, we can conclude that voltage did not have a significant influence on the protein separation time (migration time only varied by 1 or 2 minutes) . Furthermore, the migration time of the proteins are considerably longer using PEM coatings with the GEMBE technique than PEM coatings with OT-CEC. GEMBE usually provides fast separations, however, the migration times obtained in these experiments are increased due to increased protein-PEM interactions.

5.4 Concluding Remarks

In this study, PEM coatings were constructed for use in OT-CEC and GEMBE. The PEM coatings consisted of 0.03% (w/v) poly-L-ornithine and 0.5% poly-L-SULA/SULV. Both UV and LIF detection were used to observe protein separation. It is evident that the fluorescent dye affected both protein resolution and migration time. The anionic polymer, pH of background electrolyte, internal diameter of capillary, effective length of capillary, bilayer number and applied voltage were varied to optimize protein separations. The results indicated that PEM

coatings constructed from 2 bilayers using the anionic polymer, poly-L-SULA and pH 8 background electrolyte, provided high efficiency, high resolution protein separations. The migration time of the proteins were considerably shorter in PEM coated capillaries in OT-CEC than with PEM coated capillaries using the GEMBE technique. Results indicate that PEM coatings can be coupled with the GEMBE technique, however, its presence increases the migration time in a usually fast separation method. PEM coatings provide chromatographic interactions with the analytes in OT-CEC also seem to work in GEMBE. Though, a coating that generally minimizes analyte-wall adsorption may be more suitable for GEMBE. Overall, these studies prove the versatility of PEM coatings with applications for acidic protein separations in OT-CEC and the GEMBE technique as well as basic protein separations that has been previously published [10].

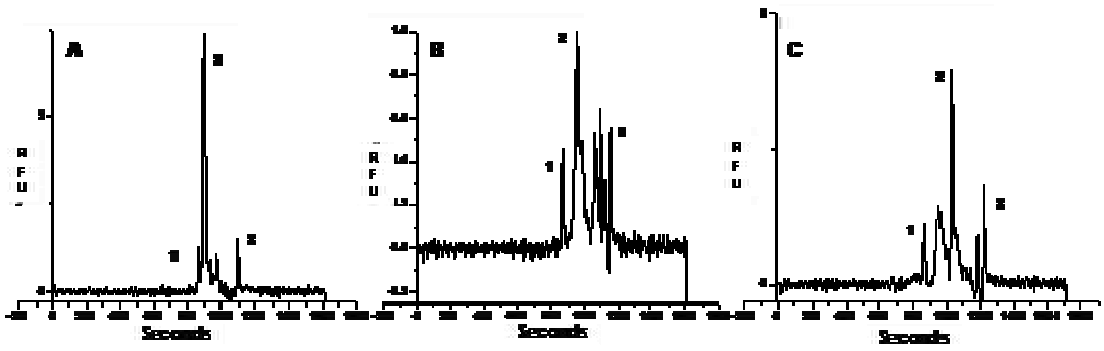

Figure 5.6 Influence of applied voltage on the separation of 3 acidic proteins using PEM coated columns using GEMBE. Conditions: Number of Bilayers: 2 bilayers; Cationic Polymer: 0.03% (w/v) poly-L-ornithine; Anionic Polymer: 0.5% (w/v) poly-L-SULA; Buffer: 40mM phosphate, pH 8; Voltage: (A) 300, (B) 700, (C) 1000 V; Capillary length: 3 cm, 30 µm I.D; Starting Pressure: 3000 Pa; Step interval: 5; Analytes: 1: β-lactoglobulin A; 2. β -lactoglobulin B; 3. Albumin

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CHAPTER 6

CONCLUSIONS AND FUTURE STUDIES

In this dissertation, achiral, chiral and protein separations with molecular micelles using chromatographic techniques are described. These techniques include micellar electrokinetic chromatography (MEKC), open tubular capillary electrochromatography (OT-CEC), and gradient elution moving boundary electrophoresis (GEMBE). Various molecular micelles, both achiral and chiral, were used for novel separations. PEM coatings with molecular micelles were used for the first time for protein separations. In addition, the first use of the new GEMBE technique with molecular micelles for protein separations was also demonstrated. The work completed in this dissertation should have a great impact on the pharmaceutical and biotechnological arenas. Novel methods for protein and chiral separations will provide new insight into their interactions with PEM coatings as well as aid in optimization procedures. The optimized separation conditions outlined in these chapters can serve as a template for the separation of other analytes. A background and introduction of these techniques, as well as the molecular micelles used were detailed in Chapter 1.

In Chapter 2, chiral polymers were used to construct novel PEM coatings for the separation of three chiral analytes. The cationic layer of these coatings consisted of one of four commercially available chiral polymers; poly-L-ornithine, poly-L-lysine, poly-L-lysine-serine and poly-L-glutamic acid-lysine. To complete the bilayer, the molecular micelles, poly-L-SULA and poly-L-SULV were synthesized in our laboratory. After several experiments, 0.5% poly-L-SULA was chosen as the optimal concentration and molecular micelle for the anionic polymer in the PEM coatings. Increased resolution and migration time were observed when the bilayer number was increased. The optimum cationic polymer used in PEM coatings was analyte dependent. Poly-L-ornithine gave the best resolution for the two binaphthyl derivatives while,

poly-L-glutamic acid-lysine provided the best resolution for the β-blocker, labetalol. Robust PEM coated columns endured over 350 runs and resulted in %RSDs of less than 1%.

PEM coatings used for protein separations are described in Chapter 3. The cationic polymers, poly-L-glutamic acid-lysine, poly-L-lysine-serine, poly-L-ornithine, and poly-L-lysine as well as three anionic molecular micelles, poly-L-SULA, poly-L-SULV, and poly-SUS were used to separate α-chymotrypsinogen, lysozyme, ribonuclease A and cytochrome c. Experimental design, specifically Box Behnken was used to optimize the separation conditions. The conditions included operating temperature, applied voltage, type and concentration of cationic polymer, and background electrolyte pH. Though all proteins were resolved using each cationic polymer, the use of poly-L-glutamic acid-lysine in the PEM coating resulted in the highest resolution and poly-L-lysine the lowest. Chapter 3 demonstrated the importance of the use of molecular micelles in PEM coatings since inferior separations were achieved with only a cationic coating. In addition, a comparison of using achiral or chiral molecular micelles in PEM coatings was performed. Results showed that chiral molecular micelles, poly-L-SULA and poly-L-SULV resulted in higher protein resolution than an achiral molecular micelle (poly-SUS). The selectivity of PEM coatings was determined by the choice of polymers used which was displayed by the different elution orders of the proteins investigated.

In Chapter 4, a novel technique that combined PEM coatings and MEKC, mixed mode separation technique, was demonstrated to separate both achiral and chiral analytes. Mixed mode separation technique is important for analytes that cannot be resolved by the use of MEKC or PEM coatings alone. Studies revealed an increase in resolution when mixed mode separation was used. Baseline resolution was achieved for 8 achiral aryl ketones as well as all five chiral analytes. Increasing the molecular micelle concentration in the mobile phase resulted in increased chiral resolution. Mixed mode separation technique can be used to separate a number

of achiral and chiral analytes from various compound classes. The selectivity of the method can be tailored by changing the polymers used in the process.

Future experiments involving altering the cationic polymers used in PEM coatings may result in the resolution of additional analytes. These chiral cationic polymers may include poly-L-glutamic acid-lysine, poly-L-lysine-serine, poly-L-ornithine, and poly-L-lysine. In addition, it would be of great interest to determine the effect, if any, of using different molecular micelles for the mixed mode method since the selectivity and resolution will be altered. For example, coating the wall with a cationic polymer and poly-L-SULA and using poly-L-SULV in the MEKC phase, may increase chiral interactions. In Chapter 3, the elution order of proteins was reversed when two polymers were used. It is suspected that similar results may be revealed when applied to achiral and chiral analytes.

In Chapter 5, PEM coatings were constructed for use in OT-CEC and GEMBE. For the first time in this dissertation LIF detection was used to observe protein separation. A comparison between native proteins (UV detection) and derivatized proteins (LIF detection) was demonstrated. Studies involving fluorescently labeled proteins illustrated increased resolution and migration time. Separation parameters such as choice of anionic polymer, pH of background electrolyte, internal diameter of capillary, effective length of capillary, bilayer number and applied voltage were varied to optimize protein separations. The optimum coating conditions for both native and derivatized proteins were 2 bilayers of poly-L-ornithine and poly-L-SULA. The background electrolyte at pH 8 provided high efficiency, high resolution protein separations. PEM coatings coupled with GEMBE were also demonstrated for the first time. The migration time of the proteins were considerably shorter in PEM coated capillaries in OT-CEC than with PEM coated capillaries using the GEMBE technique. Generally, microfluidic devices are used for the GEMBE method.

The main purpose of the coating in this case will be to prevent protein adsorption to the inner walls The next step in this study should involve constructing PEM coatings in a microfluidic device. The PEM coatings should be thinner than those in a silica capillary due to the smaller micro channels. A thinner PEM coating can be constructed by lower bilayer numbers, lower coating polymer concentrations, and shorter coating rinse times. These coatings will provide less protein-PEM interaction, therefore shorter elution times. These experiments will display the advantages of the GEMBE technique, i.e. fast, high resolution separations.

APPENDIX: LETTER OF PERMISSION

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To whom it may concern,

I am currently writing my dissertation and would like to request your permission to include the information in my publication. The article is listed below.

Candace A. Luces, Sayo O. Fakayode, Mark Lowry, Isiah M. Warner "Protein Separations Using Polyelectrolyte Multilayer Coatings in Open Tubular Capillary Electrochromatography" Electrophoresis, 2008, 29, 889-900.

Thank you for your consideration with this matter.

Sincerely,
Candace A. Luces

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VITA

Candace was born in Port-Of-Spain, Trinidad, November, 1979, to Trevor and Sandra Luces. Her primary education was completed at Tranquility Government Primary School. After excelling in the entrance examination for admission to secondary school, Candace attended Bishop Anstey High School, one of the most prestigious schools in the country. Upon graduation, Candace elected to continue her education at the Sixth Form Government School for two years of post secondary matriculation. Deciding to migrate to the United States for her tertiary education, Candace was granted a Presidential Scholarship to attend Claflin University, Orangeburg, South Carolina. While at Claflin, she attended many conferences and interned at the University of South Carolina and The Rockefellar University during her summers. Always passionate about research, Candace was named the Most Outstanding Summer Researcher by the South Carolina Alliance for Minority Participation Program (SCAMP). She received numerous honors and awards during her time at Claflin. After graduating *Summa Cum Laude* from Claflin in 2003, Candace enrolled at Louisiana State University, Baton Rouge, Louisiana, to pursue her doctorate in chemistry. Under the mentorship of Dr. Isiah M. Warner, Candace began her graduate school career and her drive and motivation did not go unnoticed. In 2008, she was given the honor to attend a workshop, Building Engineering and Science Talent (BEST) Symposium, sponsored by the Dow Chemical Company, which helped to prepare her for a career in the chemical industry. Candace was also honored as Outstanding Researcher in the Chemistry Department at Louisiana State University in 2008. Her dissertation focuses on the development of novel polyelectrolyte multilayer coatings for protein separations as well as of achiral and chiral analytes. The articles she published based on her research are listed below:

Luces, Candace A., Ross, Locascio, Laurie, Lowry, Mark, El Zahab, Bilal, Warner, Isiah, M., "Protein Separations using Polyelectroyte Multilayer Coatings in Open Tubular Capillary Electrochromatography and Gradient Elution Moving Boundary Electrophoresis", Manuscript in Preparation.

Luces, Candace A., and, Warner, Isiah M.; "Achiral and Chiral Separations Using Micellar Electrokinetic Chromatography, Polyelectrolyte Multilayer Coatings, and Mixed Mode Separation Techniques with Molecular Micelles", Manuscript Submitted for Publication in Electrophoresis on 8/26/09.

Luces, Candace A.; Fakayode, Sayo O.; Lowry, Mark; Warner, Isiah M.; "Protein Separations Using Polyelectrolyte Multilayer Coatings in Open Tubular Capillary Electrochromatography" Electrophoresis, **2008**, 29, 889-900.

Candace presented her work at a number of national professional meetings and her presentations are listed below:

Spring 2009 – Oral Presentation, "Protein Separations using Polyelectrolyte Multilayer Coatings in Open Tubular Capillary Electrochromatography and Gradient Elution Moving Boundary Electrophoresis"; **Candace A. Luces**, Ross, D., El Zahab, B., Lowry, M., Locascio, L., Warner, I.M. – National Organization of Black Chemists and Chemical Engineers (NOBCChE), St Louis, MO

Spring 2008 – Oral Presentation, "Protein Separations using Polyelectrolyte Multilayer Coatings with Molecular Micelles in Open Tubular Capillary Electrochromatography"; **Candace A. Luces**, Fakayode, S., Lowry, M., Warner, I.M. – American Chemical Society (ACS), New Orleans, LA

Spring 2008 – Poster Presentation, "Polyelectrolyte Multilayer Coatings with Molecular Micelles in Open Tubular Capillary Electrochromatography"; **Candace A. Luces**, Fakayode, S., Lowry, M., Warner, I.M. – National Organization of Black Chemists and Chemical Engineers (NOBCChE), Philadelphia, PA

Spring 2008 – Poster Presentation, "Separation of Chiral Analytes and Proteins Using Polyelectrolyte Multilayer Coatings with Molecular Micelles in Open Tubular Capillary Electrochromatography"; **Candace A. Luces**, Fakayode, S., Lowry, M., Losso, J., Warner, I.M. – 59th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (Pittcon), New Orleans, LA

Fall 2007 – Poster Presentation, "Protein Separations Using Polyelectrolyte Multilayer Coatings in Open Tubular Capillary Electrochromatography"; **Candace A. Luces**, Fakayode, S., Lowry, M., Warner, I.M. – Federation of Analytical Chemistry and Spectroscopy Societies (FACCS), Memphis, TN.