CORE-BASED HYPERBRANCHED POLYETHYLENEIMINE COATING IN CAPILLARY ELECTROPHORESIS

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Cheerapa Boonyakong

Dr. Sheryl A. Tucker, Dissertation Supervisor

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

CORE-BASED HYPERBRANCHED POLYETHYLENEIMINE COATING IN CAPILLARY ELECTROPHORESIS

Presented by Cheerapa Boonyakong

A candidate for the degree of Doctor of Philosophy

And hereby certify that in their opinion it is worthy of acceptance.

____

Professor Sheryl Tucker

____

Professor Silvia Jurisson

____

Professor Michael Greenlief

____

Professor Thomas Mawhinney

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ABSTRACT

In capillary electrophoresis (CE), chemical and dynamic modification of a fused-silica surface can be used to control the electroosmotic flow (EOF), to minimize the interaction of analytes with the capillary surface and to modify the selectivity. Polyvalent, core-based hyperbranched polyethyleneimine (CHPEI) polymer was utilized as either a static or dynamic coating in this study. A CHPEI-coated capillary was simply constructed in a rinsing fashion or by adding CHPEI in a running buffer. Two generations of CHPEI, CHPEI5 and CHPEI25, were investigated with the concentration ranges from 5-20% (w/v) for the static-coating method and 0.6-1.25% (w/v) for the dynamic-coating method.

In CHPEI static-coated capillaries, several parameters were studied as follows: (1) EOF as a function of buffer pH; (2) effect of coating media (NaCl solution) concentration; (3) effect of buffer concentration; and (4) stability and reproducibility of the coating. The performance of a static-coated capillary was investigated in the separation of a wide variety of compounds, such as phenols, basic amino acids, B vitamins, aniline and its derivatives. From these studies, CHPEI25 was selected as a dynamic coating. Performance of CHPEI25 dynamic-coated capillaries were investigated by varying the polymer concentration. Test analytes were B vitamins, aniline and its derivatives. Migration-time repeatability can be problematic in static-coated capillaries due to a coating depletion. Therefore, utilization of a hybrid-coating technique was proposed and examined.

Further investigation of the dynamic-coating method was undertaken with a 1.25% CHPEI25 dynamic-coated capillary and a commercial eCAP™ capillary from Beckman Coulter. Although primary-amine surface groups are predominant in CHPEI coating and tertiary amines are the major surface groups in eCAP™, the separation performance of both capillaries is comparable. For basic drugs and related compounds, as well as, with B vitamins analysis, it is evident that CHPEI25 dynamic-coated capillary provides significantly improved peak resolution under identical separation conditions compared to that in eCAP™.

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ABBREVIATIONS

Abbreviation	Definition
BFS	Bare fused silica capillary
BPEI	Branched polyethyleneimine
CHPEI	Core-based hyperbranched polyethyleneimine
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CIEF	Capillary isoelectric focusing
CITP	Capillary isotachophoresis
cmc	Critical micelle concentration
CZE	Capillary zone electrophoresis
EOF	Electroosmotic flow
GC	Gas chromatography
HBP	Hyperbranched polymer
HPLC	High performance liquid chromatography
MEKC	Micellar electrokinetic chromatography
PB	Poybrene
PDMAC	Poly (dimethyldiallylammonium chloride)
PEI	Poly (ethyleneimine)
RSD	Relative standard deviation
TDA	Tetradecylammonium ion
TMP	Trimethoprim

CHAPTER 1

Introduction

Capillary electrophoresis (CE) is a hybrid technique that combines features of gel electrophoresis and high-performance liquid chromatography (HPLC) in which the separations occur in free solution based on differential migration in an electric field without the laborious step of casting a gel, as in gel electrophoresis, and the detection is accomplished as the separation progresses [1]. The applicability of CE to separate a wide range of compounds including inorganic ions, organic molecules, and large biomolecules contributes the advantages of CE over gas chromatography (GC), HPLC, thin layer chromatography (TLC), and slab gel electrophoresis (SGE) [2]. Although CE is not a chromatographic technique as it does not have a stationary phase, there are several terms used in CE that can be compared to HPLC as shown in Table 1.1.

1. Instrumentation

Schematic diagram of a standard CE instrument is shown in Figure1.1. It consists of a high voltage power supply, fused silica, two electrodes connected to the power supply, two buffer reservoirs are located at the capillary ends and electrodes tips, and an on-column detector.

Table 1.1 Terminology for HPLC and CE [3]

HPLC	CE
Chromatogram	Electropherogram
Column	Capillary
Pump	High-voltage power supply
Mobile phase	Background electrolyte or buffer
Retention time	Migration time
Column retention factor	Electrophoretic mobility

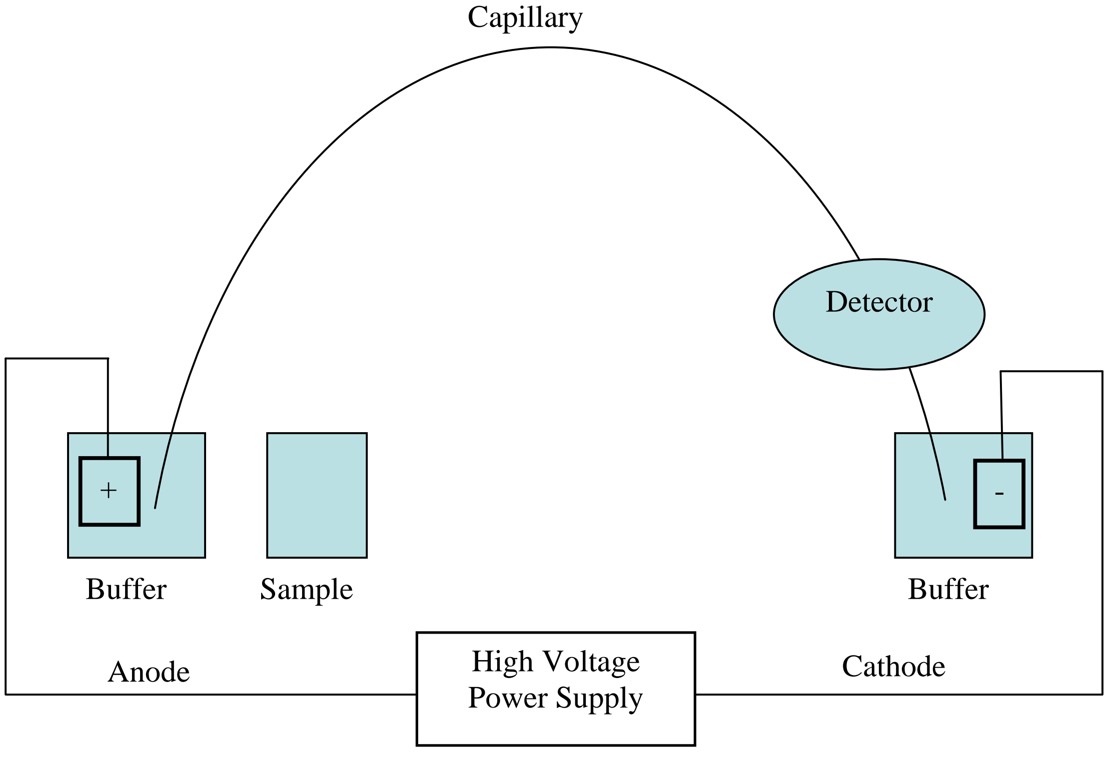


Figure 1.1 Schematic diagram of a CE instrument

1.1 Power supply

Standard power supplies that can deliver constant voltage at high precision up to 30 kV are commercially available. High voltage is applied at the capillary inlet (injection) and the outlet (detector) is at ground potential. When separations are performed in bare fused silica capillaries (BFS), EOF migrates from the inlet (anode) toward the outlet (cathode) and sweeps the solutes along with it. However, each solute possesses its own migration velocity, which makes a difference in the apparent velocity. Separations conducted in positively charged polymer coated capillaries may require reversed polarity. Polarity reversal can be accomplished through software control or manual manipulation [4].

1.2 Sample introduction

In CE, only small quantities (nL) of sample are introduced onto the capillary. There are two main injection modes in CE as shown in Figure 1.2. Hydrodynamic injection is the most widely used sample introduction method in CE. It can be accomplished by three different procedures: (a) application of pressure at the injection end of the capillary, (b) vacuum at the detector end of the capillary and (c) siphoning injection by elevating the injection reservoir relative to the exit reservoir for a short period of time. The quantity of sample in hydrodynamic injection is nearly independent of the sample matrix. The second method is electrokinetic or electromigration injection. It can be performed by replacing the injection reservoir with the sample vial and applying a small voltage across the capillary. In electrokinetic injection, the analyte enters the capillary by both its migration and the pumping action of EOF.

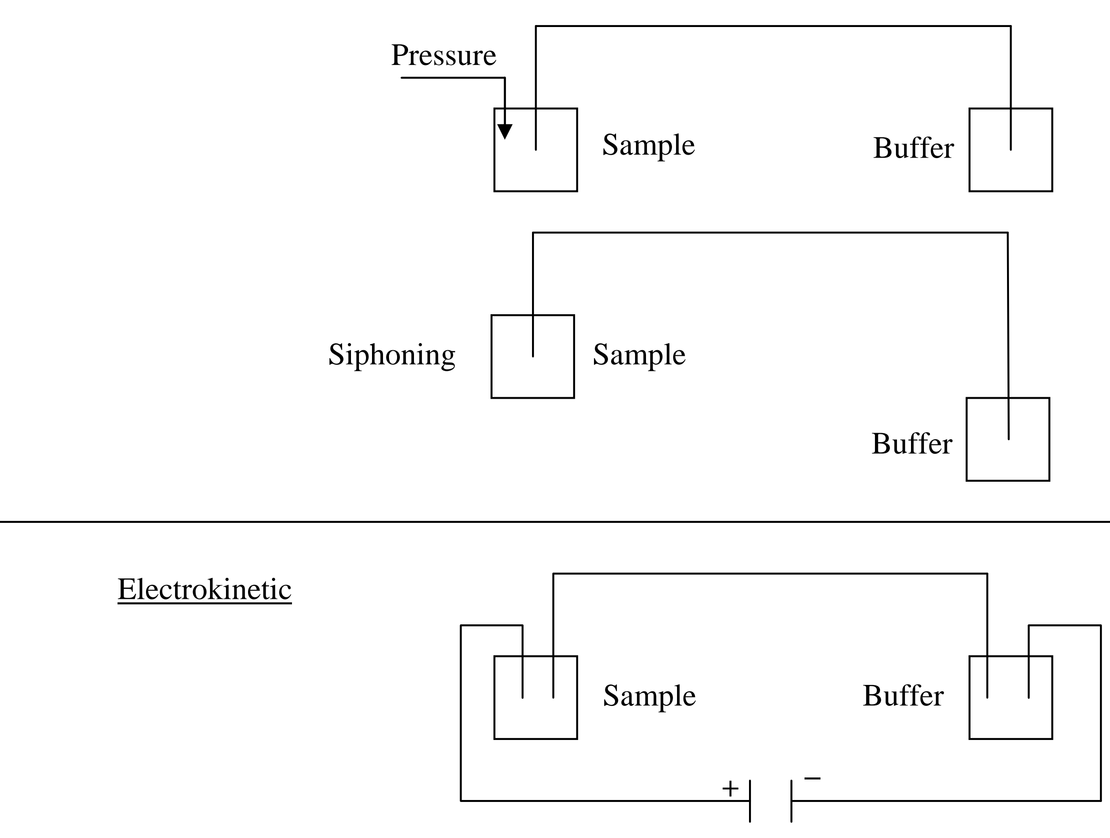


Figure 1.2 Sample introductions in CE

1.3 Detectors

The small diameter of the capillary and the nanoliter sample volume make detection in CE a significant challenge. Detection methods employed in HPLC include direct UV-absorption, indirect UV detection, lamp and laser based fluorescence detection, electrochemical, refractive index (RI), radioisotope and mass spectrometry.

1.3.1 Absorption detection

Ultraviolet/visible absorption is the most widely used detection technique. Several types of absorption detectors are available in commercial instruments including:

- Fixed-wavelength detectors (with mercury or cadmium lamps) using filters for wavelength selection
- Variable-wavelength detectors (with deuterium or tungsten lamps) using a monochromator for wavelength selection
- Photodiode array detectors that can provide both spectral and electrophoretic information

In on-column detection, the light path length is the internal diameter of the capillary. In accordance with Beer's law, the sensitivity of the detector is directly proportional to the path length. Therefore, the sensitivity of a CE detector is lower than that of an HPLC detector, which has a 1 cm path length. In order to improve detection sensitivity in a CE detector, "bubble cell" capillaries or "Z-cells" can be used to extend the light path length and can increase the sensitivity 3 times and 20-50 times, respectively [5].

1.3.2 Fluorescence detection

Fluorescence detection provides high sensitivity and improved selectivity for certain compounds. This detection method requires that the solutes exhibit native fluorescence or contain a group that can be attached to a fluorophore by chemical derivatization. However, derivatization is limited by some disadvantages, such as slow reaction kinetics, complicated reaction or cleanup conditions, poor yields, interference by matrix components, derivative instability, and interference by reaction side products [5].

Design of a fluorescence detector in CE faces some technical problems in which it is necessary to focus sufficient excitation light on the detection window to obtain acceptable sensitivity. This difficulty can be overcome by using a laser light source. The most popular laser used in laser-induced fluorescence (LIF) detection is the argon ion laser. It provides light at a 488 nm that is close to the excitation wavelength for several common fluorophores. In addition, the argon laser is stable and relatively inexpensive [5].

1.3.3 Mass spectrometry

Coupling of CE to mass spectrometry (MS) was first reported by Olivares *et al*. in 1987 [6]. This coupling provides both molecular weight and structural information of the separated components. There are two compatible ionization techniques: electrospray (ESI) and fast-atom bombardment (FAB). However, sufficient fluid flow to maintain stable spray, compatible volatile buffers and additives, including sufficient injection quantity must be considered as challenges in using ESI as an interface from CE into a mass spectrometer [5].

1.4 Capillary

Bare fused silica capillaries are universally used in CE. The inner diameter and outer diameter vary from 20 to 200 μm and 150 to 360 μm, respectively. The outer surface of the capillary is coated with polyimide to provide mechanical strength. This polymeric coating must be removed at the detection point by heating a small segment in a flame or dripping in hot concentrated sulfuric acid [5]. There are several factors to consider for proper capillary diameter selection. Higher resolution and better heat dissipation are achieved by reducing capillary inner diameter; whereas, greater sensitivity and higher capacity are achieved with a larger inner diameter. A 50 μm inner diameter is optimal for most applications but a larger diameter may enhance sensitivity for micropreparative applications [5]. However, a larger internal diameters exhibit poor heat dissipation and requires low buffer concentrations and low field strengths to decrease Joule heating effects which occur from the resistance of the solution to the current flow. Poor heat dissipation through the capillary wall results in higher temperatures at the center of the capillary compared to the edge. These temperature gradients cause viscosity differences in the buffer and give rise to zone deformation.

2. Theory of CE

Electrophoresis is the migration of charged molecules under an applied electric field. Therefore, the separation by electrophoresis is based on differences in the speed of migration of the charged molecules. The migration velocity, ν, can be expressed by Equation 1 as:

$$\nu = \mu_{ep} E \qquad (1)$$

where μ_{ep} is the electroosmotic mobility (cm^2/Vs), E is the electric field and it is a function of the applied voltage (V) and capillary length (L).

$$E = V / L \tag{2}$$

A charged molecule experience two types of forces under an electric field. First is an applied electric force (F_{el}) which is directly proportional to the charge of the molecule (q) and the electric field strength (E). Second is the frictional force (F_{fr}), which is caused from the viscosity of the solution, velocity and size of the molecule. The expressions of these two forces are as follows:

$$F_{el} = qE \tag{3}$$

$$F_{fr} = 6\pi\eta r v_i \tag{4}$$

A steady state between F_{el} and F_{fr} can be obtained during electrophoresis in which the two forces are counterbalanced. Therefore, the charged molecule briefly reaches equilibrium with a velocity of

$$\nu = qE / 6\pi\eta r \tag{5}$$

The relationship between μ_{ep} , the charge and size of the molecule including the solution viscosity is expressed as follows:

$$\mu_{ep} = qE = q / 6\pi\eta r \tag{6}$$

It is demonstrated from Equation 6 that larger and lower charged molecules will migrate slower than smaller and higher charged molecules. Therefore, molecules with different sizes and charges can be separated under the electrophoretic condition.

3. Electroosmotic flow

Electroosmotic flow (EOF) is the movement of the bulk solution in a capillary. The capillary is first treated with NaOH solution to ionize the silanol surface groups to the charged silanoates ($Si\text{-}O^-$). Silanols are weak acids and ionize at pH ~ 2-3 [5]. The negatively charged silanol and positively charged ions from a buffer solution form an immoveable layer at solid-liquid interface (Figure 1.3). When an electric field is applied, the outer layers of cations, which are hydrated, migrate toward the cathode. The movement of the hydrated cations in the mobile layer drags the entire bulk solution through the capillary towards the detector end [4, 5].

In standard CE configuration, the anode is the injection end of the capillary and the cathode is located at the detector end. The EOF moves from anode toward cathode as depicted in Figure 1.3. The velocity of the EOF (V_{EOF}) is controlled by the dielectric constant of the electrolyte (ε), the electrolyte viscosity (η), the electric field (E) and the zeta potential (ξ) as shown in Equation (7) [7].

$$V_{EOF} = \varepsilon\xi E / 4\pi\eta \qquad (7)$$

The EOF mobility of the running electrolyte is given by

$$\mu_{EOF} = \varepsilon\xi / 4\pi\eta \qquad (8)$$

The factors affecting the zeta potential are surface charge density, silanoates, pH and electrolyte concentration. Among those parameters, pH has the most significant effect on the magnitude of the EOF. Buffer pH also affects the solute charge and mobility. Low pH buffers will result in protonation of the capillary surface and solute while in high pH buffers both silanol groups and solute are deprotonated [5].

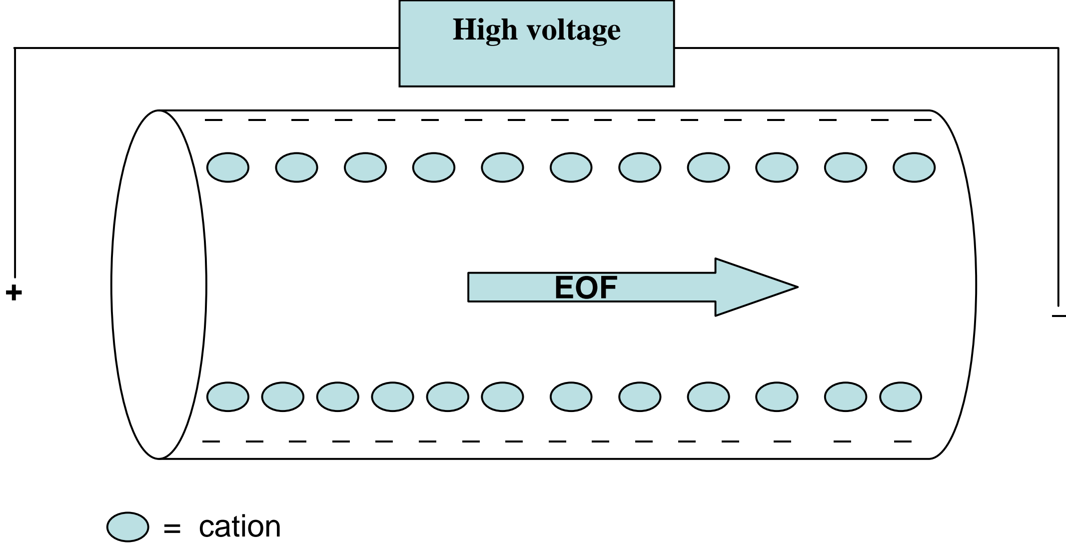


Figure 1.3 Electroosmotic flow (EOF) generated in bare fused-silica capillary

4. Apparent mobility

The apparent mobility (μ_{app}) of an ion is the sum of the electrophoretic mobility of the ion (μ_{ep}) plus the electroosmotic mobility (μ_{EOF}) of the background electrolyte.

$$\mu_{app} = \mu_{ep} + \mu_{EOF} \qquad (9)$$

For a positively charged species moving in the same direction as the EOF, μ_{ep} and μ_{EOF} have the same direction, so μ_{app} is greater than μ_{ep}. In contrast, electrophoresis drives negatively charged species in the opposite direction from the EOF, so the two terms in equation 9 have opposite signs.

The apparent mobility of particular species can be experimentally determined by:

$$\mu_{app} = \left(\frac{L_d}{t}\right)\left(\frac{L_t}{V}\right) \qquad (10)$$

where L_d and L_t are effective length and total length of the capillary respectively, V is the applied voltage and t is the migration time. EOF is measured by adding a neutral solute to the sample and measuring its migration time ($t_{neutral}$) since the neutral species migrates at the same velocity as the EOF.

$$\mu_{app} = \frac{L_d L_t}{V t_{neutral}} \qquad (11)$$

5. Efficiency

The high efficiency in CE is a consequence of the following factors:

1. Unlike in HPLC, band broadening due to resistance to mass transfer is eliminated because of an absence of stationary phase [8].

2. In pressure driven systems such as in HPLC, the frictional forces of the mobile phase interacting at the wall result in a greater flow rate at the center compared to the wall of the column. This is known as hydrodynamic flow profile, and it results in band broadening and as a consequence lower separation efficiency [4, 8].

In electroosmotic driven systems, there is a flat flow profile that results from uniform distribution of all analyte ions as they migrate at the same rate along cross-sectional path of the capillary [5]. Figure 1.4 illustrates flow profiles and peak width resulting from hydrodynamic and EOF.

6. Separation modes in CE

6.1 Capillary zone electrophoresis (CZE)

CZE also known as free solution capillary electrophoresis is the simplest mode of CE, and it is straightforward to perform. Inlet and outlet reservoirs are filled with the same background electrolyte. The sample is injected at the inlet end of the capillary, and the solutes migrate toward the detection end according to their mass-to-charge ratio. In conventional CZE, cations will reach the detector first followed by neutral solutes and lastly anions, as shown in Figure 1.5. Even though anions are attracted to the anode, the higher magnitude of EOF will carry them to the cathode. It should be noted that neutral molecules migrate as the same velocity at the EOF and will not be separated since their charges are zero.

CZE offers several advantages over other CE separation modes. The technique is relatively inexpensive since low-cost buffers and salts are used. In addition, separation selectivity can be varied by buffer pH manipulation or use of additives.

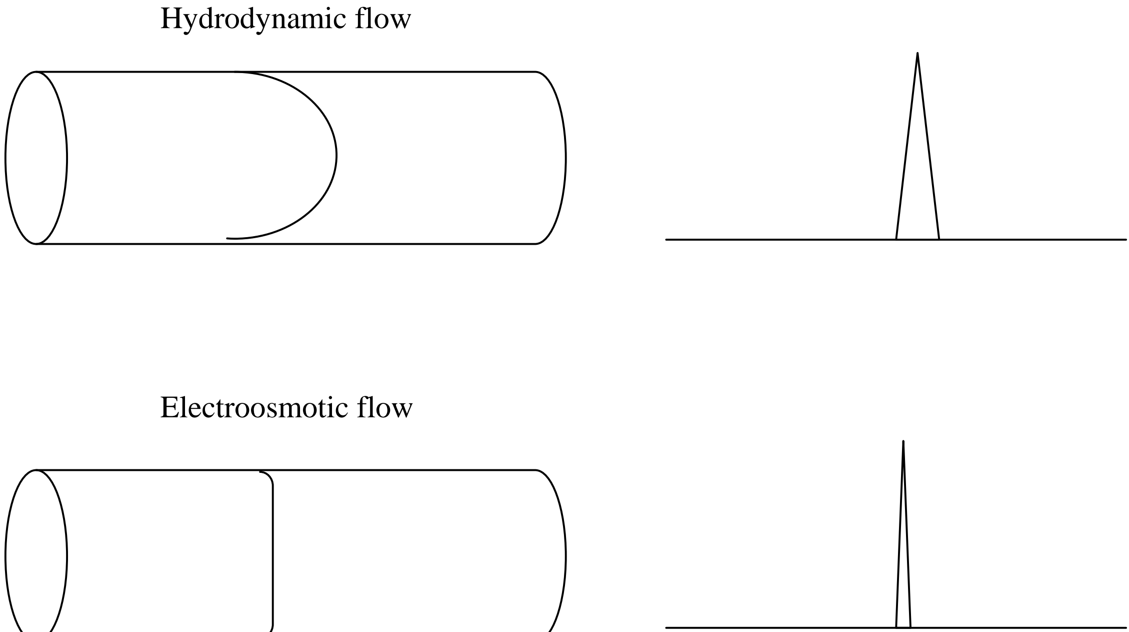


Figure 1.4 Comparison of flow profiles in HPLC and CE

6.2 Capillary isoelectric focusing (CIEF)

Isoelectric focusing is mostly applicable to proteins and peptides, which possess isoelectric points [4]. In CIEF, not only sample compositions can be determined but also their isoelectric points can be measured. The pH gradient is produced by carrier ampholytes (zwitterions) when the span of their isoelectric points encompasses the isoelectric points of the analytes [9]. To perform CIEF, the capillary is filled with a mixture of sample and carrier ampholytes. The samples will be charged depending on their pI values. When an external voltage is applied, the ampholytes and solutes migrate to a position where they become neutral (pH = pI). Charged molecules migrate toward an opposite charged electrode. In most cases, CIEF separations are carried out in the absence of EOF or with low EOF.

6.3 Capillary isotachophoresis (CITP)

In CITP, a sample zone migrates between two different electrolytes: leading electrolyte at the front and trailing electrolyte at the end. The leading electrolyte contains a co-ion that migrates faster than the analyte ions. The trailing electrolyte contains a co-ion that migrates slower than the analyte ions. Under an external electric field, the components of the sample separate and form discrete zones between leading and trailing electrolytes. Each zone contains only one component and the common counterion. The discrete zones of analytes elute in order of decreasing mobility and the length of each zone is related to the analyte concentration. It should be noted that anions and cations cannot be separated simultaneously and the analyses are usually performed in constant-current mode [9].

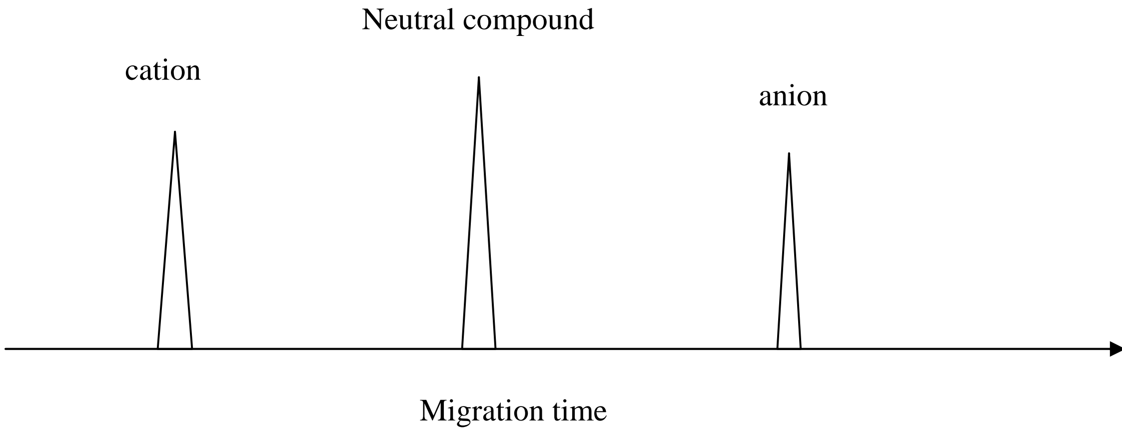


Figure 1.5 Elution order of analytes in classical CZE

6.4 Capillary electrochromatography (CEC)

In CEC, the packed capillary, coated capillary and capillary with replaceable media are utilized to separate analytes of interest [8]. However, there is no pressure-driven pump, as in HPLC, but the EOF acts as an electropump. The separation mechanism in CEC depends on both the electrophoretic mobility of the solutes and the interaction between the solutes and packing material. It is illustrated that CEC with coated capillary works best for charged solutes, since they can migrate and the chromatographic interaction will affect the selectivity [8]. While porous silica beads derivatized with hydrophobic ligands such as C18 have been used in packed capillary for applications including reversed-phase type, entangled polymer has also been used as a replaceable media to separate neutral solutes for applications in the area of size separations.

6.5 Micellar electrokinetic chromatography (MEKC)

MEKC is similar to CZE in which surfactants (micelles) are buffer additives to facilitate the separation of neutral solutes based on differential portioning between two phases. Surfactant monomers of an ionic surfactant aggregate to form micelles, when the surfactant concentration is greater than the critical micelle concentration (cmc). The hydrophobic tail of the surfactant monomers are oriented toward the center of the micelles and the hydrophilic head groups face outward into the aqueous phase as shown in Figure 1.6. Hydrophobic neutral molecules will spend different amounts of time in the micellar phase depending on their hydrophobicity, whereas, hydrophilic molecules with no interaction with the micelle will migrate in the aqueous phase at the same rate as the

EOF. When an ionic surfactant, such as sodium dodecylsulfate (SDS) is used, the micelles migrate toward the anode as a result of electrophoretic mobility. However, the magnitude of the EOF toward the cathode is higher than the mobility of the micelle, thus the micelles are slowly swept toward the detector.

7. History of electrophoresis

Electrophoretic and chromatographic methods are routine separation techniques used to analyze a variety of analytes ranging from small ions to large biomolecules. Although both methods can be applied to similar types of analytes, the separation mechanism is different, resulting in complementary and distinct separation patterns. The first report of using electrophoresis was made by Erne Tiselius in 1932. He developed the moving boundary" also called "zone electrophoresis" to separate serum proteins in solution [10]. He applied a mixture of proteins in a U-shaped cell containing a buffer solution. The electrodes were immersed at each end of U-tube. Under an applied voltage, the protein samples migrated at different velocities toward the anode or cathode depending on their charges and the movement of boundaries can be detected at both ends using Schlieren optics [10, 11]. With the significance of Tisselius's work, he was awarded the Nobel Prize for chemistry in 1948.

The first example of CE in 5 mm narrow tubes was described in 1958 [11] and was cited in a thesis written by Stellen Hjerten in 1967 [12]. To overcome band broadening causes from Joule heating, Hjerten's instrument employed a rotated capillary to diminish the thermal gradient. There has been an attempt to improve performance in CE separation by reducing the diameter of the capillary. In 1979, Mikkers reported the

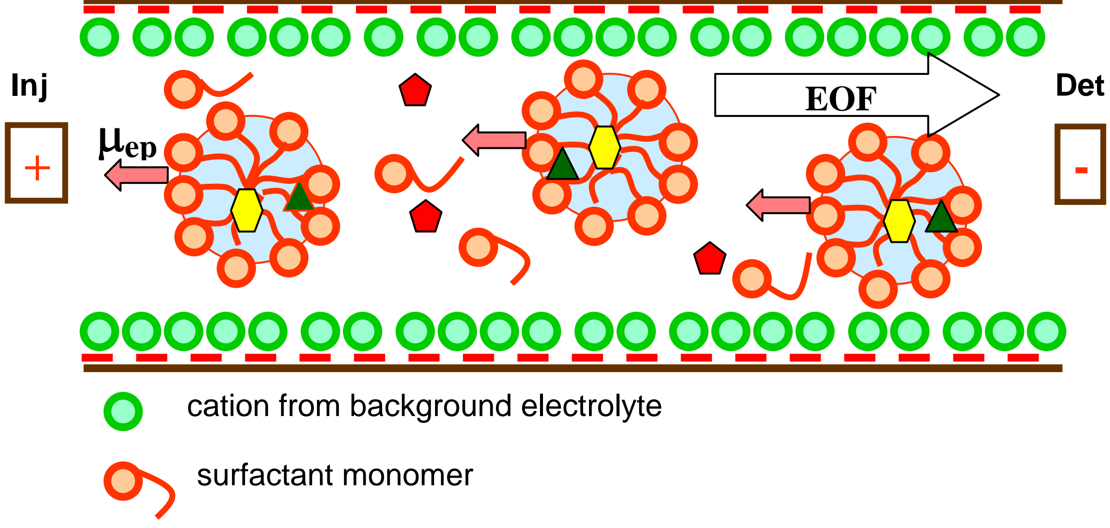


Figure 1.6 Separation in MEKC

use of 200 μm i.d. capillaries in high performance zone electrophoresis with UV and conductimetric detection [13].

An introduction of the use of polyamide coated fused silica capillary used in GC as a standard capillary in CE was the pivotal point in CE development [14]. Several properties of fused silica proved useful in CE: the ability to manufacture the capillary with precision, the ease of handling, the inert surface compared to glass, and excellent UV transparency when the coating is removed. In 1981, Jorgenson and Lukacs published an article with the first used of a 75 μm i.d. pyrex glass capillary and a 30 kV for separation of derivatized amino acids, peptides and amines [14]. In 1984, CE applications were extended when Terabe introduced micelles as buffer modifiers to separate neutral and charged compounds based on relative affinity for the hydrophobic interior and/or ionic exterior of a micellar pseudo-stationary phase [15, 16].

8. Capillary wall modification

Adsorption of compounds to a solid surface is a common phenomenon occurring in separation science. However, the adsorption of proteins, peptides and basic compounds on the fused silica capillary wall usually causes serious problems such as lower efficiency, peak tailing, unstable baseline, non-reproducible migration time and shorten capillary lifetimes. According to the strength of adsorption, the adsorbed phases can be categorized into two groups: dynamically adsorbed and physically adsorbed phases [17-19]. Dynamical adsorption is a weak modification at the capillary wall. In this case, the modifier is added to the background electrolyte. In contrast, physical adsorption is a

strong modification of the capillary wall. In this case, the addition of the modifier in the background electrolyte is not necessary.

Several problems are associated with conventional capillaries: differences in capillary-to capillary EOF, variation of the magnitude of EOF from day-to-day or run-to-run basis, adsorption of solutes to the capillary wall, deterioration of reproducibility and separation efficiency. Capillary wash procedures, dynamic and static coatings often used to minimize these effects. For many zone electrophoresis separations, capillary coatings will solve most reproducibility problems [20, 21]. Utilizing coated capillaries to reduce analyte adsorption on the capillary inner wall was reported by Hjerten [22].

Reproducibility of separations in CE is a consequence of properly controlling the EOF. At pH above 2-3, the silanol groups on the capillary surface become ionized, and the charge density on the wall increases with increasing pH. At pH ~10 all silanol groups are fully dissociated [5]. The charged character of the capillary wall results in two phenomena. First, the charged surface can have electrostatic interactions with cationic samples or cationic group containing samples such as proteins and peptides. Analyte-wall interactions can result in band broadening, tailing, and reduced detector response. Second, changes in chemical nature of the wall during an analysis or run-to-run can alter the magnitude of EOF as a consequence variation in peak migration times and peak areas. One of the strategies used to minimize analyte-wall interactions, besides the use of extreme pH [23, 24], high ionic strength buffer [25] and zwitterionic additives [26], is the use of coated capillaries. The coatings prevent access of the samples to the capillary wall and as a consequence minimize analyte-wall interactions and also modify the EOF. Coatings can be categorized into two groups as:

8.1 Dynamic coatings

Dynamically coating the capillary wall is a desirable coating method because it eliminates chemical derivatization reactions in the capillary lumen. A dynamic-coated capillary is simply prepared by rinsing the capillary with a buffer containing a coating agent to shield the capillary wall [12, 22]. The buffer additive equilibrates with the surface of the capillary and alters its properties. Due to the fact that the interaction between the coating and the wall is based on adsorption, a low concentration of coating is usually added to the running buffer to regenerate the coating on the wall and to extend the coating lifetime. The major disadvantages of using dynamic coatings are a decrease in detector signal and incompatibility with mass spectrometric detection.

8.2 Covalent or permanent coatings

Covalent coatings are attached to the wall, via chemical bonds, and are considered permanent. Neutral hydrophilic polymers, such as polyethylene glycol [27], polyvinyl alcohol [28] and alkylated celluloses [12], are typically used as permanent coatings. A capillary is rinsed with a solution of the polymer prior to use. To prevent deterioration of capillary performance during repetitive runs, low concentration of the polymer might be added to the running electrolyte so that the coating is continuously replaced. Charged polymers, such as polyamine compounds, have also been used to bind with charged silanol groups on the capillary wall [29]. Advantages of covalently-attached coatings include the coated capillaries exhibit longer lifetimes and require less maintenance [30]. However, most covalent binding procedures require several steps, making the capillary coating time-consuming, irreproducible and unstable under hash conditions [5, 30].

9. Polymers as capillary wall coatings

Several natural and synthetic, charged and neutral polymers have been examined in seeking the best capillary wall coating. Various coating methods have been developed for different types of analytes.

9.1 Charged polymers

The adsorption of charged polymers onto capillary wall generates positively or negatively charged surface. The charged surface results in changing the direction and magnitude of the EOF, as well as the analyte-wall interaction. Charged polymeric coatings have been developed to solve a specific separation problem, such as accelerating the separation of positively charged analytes. Cationic coatings, such as poly (dimethyldiallylammonium chloride) (PDMAC) [31-34], poly (ethyleneimine) (PEI) [35], polybrene (PB) [36, 37], are often used effectively in separations of proteins and peptides, including drug samples [3]. A novel method of using polyelectrolyte multilayer coating has been recently developed by successively rinsing the capillary with positively and negatively charged polymers. The resulting capillaries were employed in separations of basic proteins and revealed its stability under extreme pH and ionic strength [3, 38-40].

9.2 Neutral polymers

Neutral polymers have been introduced to use in capillary coatings to reduce or eliminate EOF and analyte-wall interactions. Their hydrophobic properties mainly result in reducing analyte-wall interactions in biopolymer separations. Since polymer coatings are adsorbed onto charged capillary wall, increasing hydrophobicity may reduce the

stability of the adsorbed coatings [11]. Cellulose-based adsorbed coatings, such as hydroxypropylmethylose, can easily be washed off from the capillary wall because of their hydrophilicity; therefore, these compounds must be added to the buffer to replenish the polymer lost from the surface [41, 42]. Poly (vinyl alcohol), PVA, binds more strongly to silica surface than those cellulose-based coatings, thus PVA coated capillaries offer improved stability [43]. Poly (acrylamide), PAA, is a very hydrophilic polymer and used primarily for permanent wall coating. Although it is a hydrophilic polymer, PAA does not bind well with the silica surface [41]. Thus, novel copolymers of acrylamide, such as poly (acrlamide-co-allyl α-D-glucopyranoside), were synthesized. The resulting copolymers are highly hydrophilic and improve the adsorption to the silica surface due to an introduction of epoxy groups from the polymer backbone [42].

10. Dendritic polymers in CE

Dendrimers and hyperbranced polymers (HBPs) are categorized into the same group of well-defined, highly branched macromolecules that their branches derive from a central core [44]. They possess a densely branched structure and a large number of reactive surface groups. However, there are differences between dendrimers and HBPs. Dendrimers are monodisperse hyperbranched polymers obtained from divergent or convergent synthesis process; whereas, ordinary HBPs are described as polydisperse polymers that can be obtained from one-pot polymerization. Dendritic polymers may find usefulness in the area where structural uniqueness and multi-functionality are required. There are several advantages of dendritic polymers over traditional linear and branched polymers:

- They are highly reactive due to the large number of functional end-groups
- Their viscosities are significantly lower than those of traditional polymers due to the lack of chain entanglement and higher degree of branching
- Their polarities can be adjusted by their functional end groups and selective solvent
- Their highly defined globular structures allow them to serve as branching scaffolds for other architectures, [45-47].

A number of applications of dendritic polymers in CE have been reported. Tomalia demonstrated that the starburst dendrimer (SBD) has the same topological distinction of a micelle [48]. The fundamental difference between a dendritic polymer and micelles is that the structure of a dendrimer is static, with all end groups covalently bonded to a central core, whereas, the structure of micelle is dynamic. In addition, the hydrophobicity of the interior of an SBD can be modified by the use of relatively hydrophobic alkyl diamines to mimic an aqueous micelle [44]. Aggregation numbers of micelles may range from 62-100 depending on the surfactant [49]. In contrast, active functional surface groups on the dendrimers play an important role,similar to aggregation number in micelles.

Dendritic macromolecules have been utilized as substitutes in micellar electrokinetic chromatography (MEKC) for micelles. The mode of separation utilizing dendritic polymers in CE has been termed dendrimer electrokinetic capillary chromatography (DECC) [50]. Tanaka et al. were the first to report the use of starburst dendrimers (polyamidoamines) as a pseudo-stationary phase in electrokinetic chromatography for the separation of substituted benzene and naphthalene compounds in

aqueous buffer and for the separation of polycyclic aromatic hydrocarbons (PAHs) in buffer modified with methanol [51]. Poly (propyleneimine) has been employed in DECC to separate substituted benzyl alcohol [52], and polyamidoamine starburst dendrimers with terminal carboxylate groups were used as a pseudo-stationary phase in DECC for the separation of aromatic amino acids [53]. Novel diaminobutane dendrimer (DABD) was synthesized and employed as a hydrophobic modifier in the separation of PAHs in which selectivity has been found substantially different from that in micelles of sodium dodecyl sulfate (SDS) [54].

Since applications of dendritic polymers in CE have gained great interest and dendrimers are relatively more costly and more complicated to synthesize compared to HPBs, HPBs have become candidates for capillary coating materials. Chongqi et al. reported the use of hyperbranched polyester physically coated capillaries for basic protein analysis [55]. A physical adsorption of synthesized hyperbranched poly (amine-ester) onto capillary surface has shown great reduction of the EOF and suppression of protein adsorption [56].

11. Polythyleneimine in capillary electrophoresis

Branched polyethyleneimine (BPEI) is a cationic polymer (Figure 1.7a) that contains approximately 1:2:1 primary:secondary:tertiary amine ratio [58-60]. High molecular weight BPEI (Mr $6x10^5$- $1x10^6$) was used by several research groups as a buffer additive or capillary coating agent [59-62]. BPEI adsorbs strongly on the silica surface and causes a reversal of the EOF due to its positively charged surface. It can

modify the separation selectivity of small and large ions and can mask the silanol groups and as a consequence lower the EOF and eliminate analyte-wall interaction [35, 62].

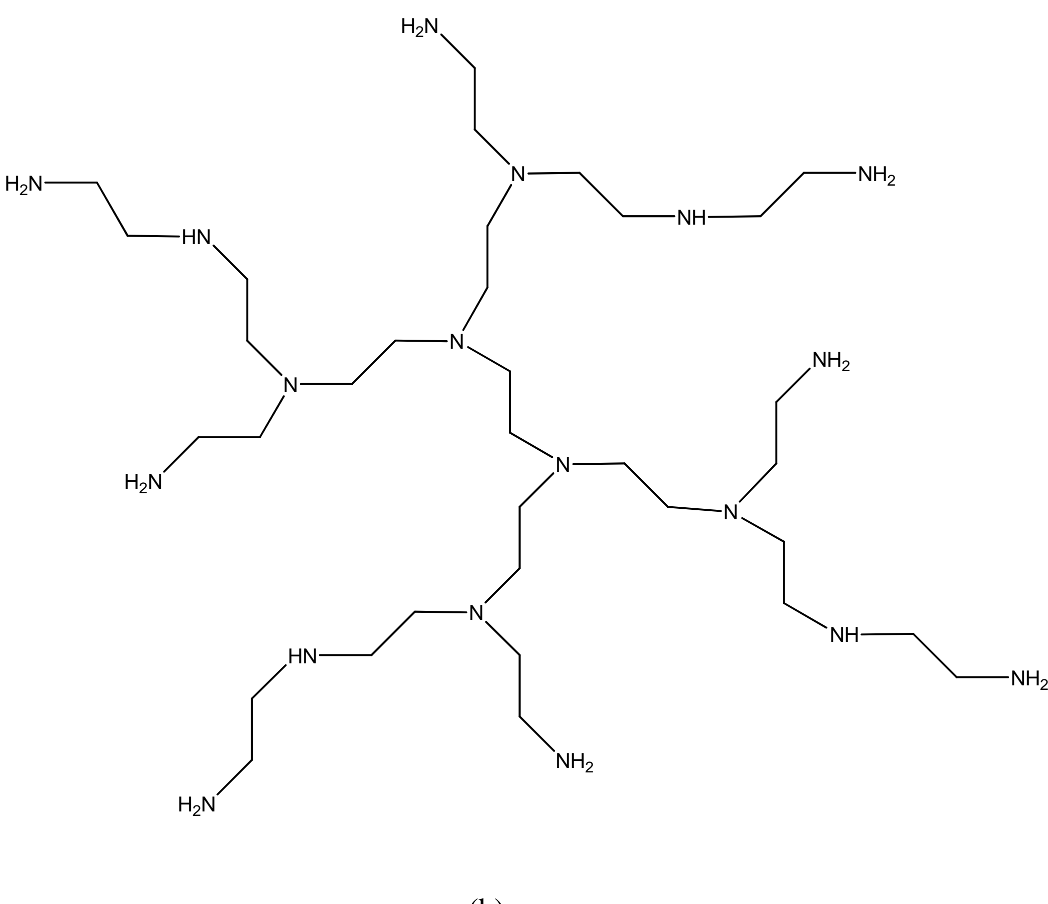
Hyperbranched polyethyleneimine (HPEI) is an amine-functional hyperbranched polymer with a globular, dendritic-like structure (i.e., "core") and branching degree of 65-75 %. It is a clear viscous liquid [63]. Such core-based hyperbranched polymers like core-based hyperbranched polyethyleneimine (CHPEI) have been used for long time for industrial purposes and in biomedical applications as well as unimolecular inverted micelles for anionic dye encapsulation [61].

The structure of CHPEI is illustrated in Figure 1.7b. Two generations of CHPEI; generation 5 (CHPEI5) and generation 25 (CHPEI25) have approximately 35 and 174 amine groups at the peripheral surface, respectively [64], as illustrated in Table 1.2.

Traditional branched polyethylenimine (BPEI) has been used as a replaceable cationic pseudo-stationary phase in electrokinetic chromatography for analysis of neutral compounds (benzyl alcohol and phenols) with varying percentage of methanol or acetonitrile [60]. The addition of an organic modifier decreases retardation of analytes which result in a drastic loss of resolution. Applications of BPEI have been extended to static- [35, 62] and dynamic-capillary coatings [35, 65-67]. Satisfactory separation was obtained when BPEI was used as a dynamic coating for peptide analysis in buffer containing ethylenediamine and 1,7 diaminoheptane [65]. Dynamic BPEI coated-capillaries have successfully separated seven generations of poly (amidoamine) (PAMAM) at neutral pH [67]. Since the polymer coating process is rather laborious and time-consuming, performing physical coating might be an alternative method. Physically adsorbed capillaries of high molecular mass BPEI (6×10^5 to 1×10^6) have been

$$H_2N-\left[CH_2CH_2\underset{\displaystyle CH_2CH_2NH_2}{\underset{|}{N}}\right]_n-\left[CH_2CH_2NH\right]_m-$$

(a)



(b)

Figure 1.7 Structure of (a) branched polyethyleneimine (BPEI) and (b) core-based hyperbranched polyethyleneimine (CHPEI)

Table 1.2 Properties of Core-based hyperbranched polyethyleneimine [63, 64]

Polymer	MW	Core size	Surface groups	Polydispersity
CHPEI5	5000	C2	35	1.3
CHPEI25	25000	C2	174	2.5

employed in analysis of peptides and proteins. Electroosmotic flow profile of the capillary was obtained from pH 3 to 11. The coating produced efficient separations of basic proteins. However, the separation performance of acidic proteins was poor due to interaction with the coating [35].

12. Characterization of coated capillaries

Due to the fact that the capillary surface is very small and relatively inaccessible, the characterization of coated capillaries is extremely difficult. Therefore, characterization of the properties of coated capillaries is usually achieved by measuring the pH-dependence EOF [68]. There are several methods used to measure the EOF:

- Photometric monitoring of the electrophoretic pattern of neutral marker [69]
- Fluorescent neutral maker zone monitoring using CCD camera [70]
- Measuring liquid streaming potential [27]
- Probing the coated inner surface by using Atomic Force Microscopy (AFM) [71, 72]

The most common method for the measurement of the EOF involves the migration time measurement of a neutral marker using photometric detection. This method was used in this work. The neutral marker will migrate at the same velocity with the EOF. This method assumes that there are no interactions between the marker and the capillary wall [73].

Although CHPEIs have never been employed as capillary coatings, their unique architectural structure and abundance of the functionalities may be useful as

polyelectrolyte adsorption layer to modify the capillary wall. In addition, their low cost and one-pot synthesis are advantages over the aforementioned dendrimers. In this research work, CHPEI5 and CHPEI25 were selected to employ as capillary coatings. Different coating methods, dynamic, static including hybrid, were investigated. Characteristics of CHPEI coated capillaries were examined by measuring the EOF in the pH range from 4-9. Separation efficiencies of the coated capillaries obtained from different coating methods were compared in the separations of neutral and charged molecules.

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CHAPTER 2

Materials and Methods

1. Materials

Bare fused silica capillaries (Polymicro Technology, Phoenix, AZ) of 75 μm id/365 μm od and 64 cm total length (56.5 cm effective length) were used in EOF determinations and analyte separations. The detection window was constructed by burning out about 2-3 mm of the outer polyimide coating. The window then was cleaned with acetone to remove coating residue or fingerprints.

An eCAP™ capillary is a polyamine-treated capillary that provides a strong positively charged surface. The eCAP™ capillary (pre-burned detection window) and regenerator solution were purchased from Beckman Coulter Inc, (Fullerton, CA). The capillary total length and the effective length were adjusted to 64 cm and 56.5 cm, respectively, to fit with the capillary holder.

Reagent-grade sodium acetate, sodium phosphate monobasic, sodium phosphate dibasic, sodium tetraborate and potassium hydrogen phthalate for buffer preparation were obtained from Sigma-Aldrich (St. Louis, MO). 6-Aminohexanoic acid and 2,4-dihydroxybenzoic acid used to prepare a buffer for organic acid analysis were purchased from Fluka (St. Louis, MO). The following analytical reagent-grade chemicals were obtained from Sigma: pyridoxine, riboflavin, nicotinamide, phenolic compounds, 2-chlorophenol, 3-chlorophenol, 4-nitrophenol, aniline, 3-chloroaniline, tryptophan, lactic acid, succinic acid, imidazole, 4-aminobenzoic acid, 3,4,5-trimethoxybenzoic acid.

Tyrosine, phenylalanine, 3-nitroaniline, trimethoprim sodium chloride and tartaric acid (all analytical reagent-grade) were purchased from Fluka. Glacial acetic acid was obtained from Fisher Scientific (Pittsburgh, PA). Analyte solutions of 100 ppm were prepared for all the separations. Acetone was obtained from Fisher Scientific. A 100 ppm acetone solution was used as an EOF marker. Purchased from HyperPolymers (Germany), CHPEI5 (MW 5000) and CHPEI25 (MW 25000), were and used without further purification.

Aqueous stock solutions of 25% (w/v) CHPEI5 and 30% (w/v) CHPEI25 were prepared with type-I grade water from Aqua Solutions System (Aqua Solutions, Jasper, GA). Working solutions (5, 10, 20% (w/v) of each generation of CHPEI were diluted with said water from the stock solutions and utilized as coating solutions to prepare six coated capillaries. Those capillaries were characterized by measuring EOF as a function of buffer pH. Optimized coating conditions were found at 10% CHPEI25. For further investigations, 10% CHPEI25 coating solutions were prepared with an addition of an appropriate concentration of NaCl.

2. Instrumentation

Separations were carried out on Waters Quanta 4000E Capillary Electrophoresis System equipped with a UV detector set at 254 nm. Millennium software (Waters corporations, Milford, MA) and Logger Pro (Vernier Software and Technology, Beaverton, OR) were used for data acquisition. Samples were introduced onto the capillaries by hydrodynamic injection (10 cm height for 5 seconds).

3. Coating procedures

A new fused silica capillary was first conditioned with 1 M NaOH for 30 min and then rinsed with water for 15-20 min. For static coating, core-based hyperbranched polymers (CHPEI5 and CHPEI25) were attached to the capillary inner surface by flushing the treated capillary with different concentrations of each CHPEI solution [5, 10, 20% (w/v)] for 30 min. The coated capillaries were then left to stand overnight and then flushed with working electrolyte for 15-20 min before performing the separations.

For the dynamic coating method, the desired concentration of CHPEI25 was added to a running buffer. A newly treated BFS capillary was rinsed with the buffer containing CHPEI25 for 30 min before performing a separation and 2 min between each run.

4. eCAP™ capillary conditioning

The standard manufactures recommended procedure for the eCAP™ capillary is to initially condition the treated capillary and then use the regenerator (polymer coating solution) continuously to obtain stable runtime as follows:

a) Flush the capillary 2 min with 1 M NaOH
b) Flush the capillary with regenerator for 2 min
c) Inject the sample
d) After each run and at the end of the day, flush the capillary with 1M NaOH and regenerator for 2 min each.

5. Buffer solutions for EOF determination

The EOF mobilities at different pH were measured in the same capillary using six buffer solutions, which were made in the usual fashion: (i) 20 mM acetate buffer pH 4.0, (ii) 20 mM phthalate buffer pH 5.0, (iii) 20 mM phosphate buffer pH 6.0, (iv) 20 mM phosphate buffer pH 7.0, (v) 20 mM phosphate buffer pH 8.0 (the desired pH values of phosphate buffers were adjusted with 0.1 M NaOH or 0.1 M phosphoric acid in the usual fashion), and (vi) 20 mM phosphate-borate buffer pH 9.0. All buffer solutions were prepared with Type I grade water and filtered through 0.45 μm polypropylene membrane filter (Whatman Inc, Florham Park, NJ).

6. Buffer solutions for separations

Separations of neutral and charged molecules were performed in the following buffer solutions: (i) phenolic compounds were separated in 20 mM phosphate buffer pH 7.0; (ii) separations of B vitamins were performed in 20 mM acetate buffer pH 4.0; (iii) separations of organic acids were carried out in a buffer composed of 20 mM 6-aminohexanoic acid and 5 mM 2,4-dihydroxybenzoic acid pH 4.9; (iv) basic amino acids could be separated in 20 mM phosphate buffer pH 7.0; (v) separations of aniline and its derivatives using dynamic and physical coated capillaries were carried out in 0.6%, 1% and 1.25% (w/v) CHPEI25 in 20 mM acetate buffer pH 4.0 and 20 mM acetate buffer pH 4.0, respectively. The desired pH value of phosphate buffer was adjusted with 1 M NaOH or 1 M phosphoric acid and the final pH of acetate buffer was adjusted with 1 M NaOH or 1 M acetic acid. All buffer solutions were prepared with Type I grade water from Aqua

Solutions System (Aqua Solutions, Jasper, GA) and filtered through 0.45 μm membrane filters.

7. Precision of the measurements

Precision of electroosmotic flow (EOF) measurements and migration-time repeatabilities are expressed as the coefficient of variation (CV) or percent relative standard deviation (RSD) as follows:

$$\%\,\mathrm{RSD} = 100\left(\frac{\mathrm{SD}}{\overline{\mathrm{X}}}\right) \qquad (12)$$

where SD is standard deviation and $\overline{\mathrm{X}}$ is mean of the measurements. The RSD allows standard deviations of different measurements to be compared more meaningfully [1].

An F test was used to compare the precision associated with two sets of experimental conditions or two different populations by assuming that the two normal populations (A and B) are equal (i.e. $\sigma_A{}^2 = \sigma_B{}^2$). This assumption is called the null hypothesis. The alternative assumes that $\sigma_A{}^2 \neq \sigma_B{}^2$ is called the alternative hypothesis. The F_{calc} value is defined as the ratio of two variances:

$$F_{calc} = \frac{S_A{}^2}{S_B{}^2} \qquad (13)$$

where $S_A{}^2 =$ the first estimated variance with $(n_A - 1)$ degrees of freedom for population A and $S_B{}^2$ = the second estimated variance with $(n_B - 1)$ degrees of

freedom for population B. The F_{calc} is then compared with the $F_{critical}$ from the table at the 95% confidence limit; if $F_{calc} < F_{critical}$ then the null hypothesis is accepted [2, 3].

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CHAPTER 3

Preparation and Characterization of Core-based Hyperbranched Polyethyleneimine Static-coated Capillaries

1. Introduction

The capillary wall has an important influence on separation efficiency, resolution, speed and accuracy in capillary electrophoresis (CE). In fused silica capillary electrophoresis, there are two phenomena that occur at the capillary wall. The first is an induction flow called the electroosmotic flow (EOF), which is caused by the composition of the capillary wall at the solid-liquid interface, pH and composition of the buffer. The second is the electrostatic interaction between high positively charged analytes and the negatively charged of deprotonated silanol groups on the capillary wall [1]. Although the EOF can provide simultaneous separation of negative and positive charged analytes, analyte-wall interactions will deteriorate the separation quality of positively charged compounds, such as basic proteins. Manipulation of the EOF by utilizing coated capillaries has been proposed by numerous research groups [2-8]. A coating can be permanently or dynamically attached to the inner surface of a capillary [9]. A capillary with permanent coating can be achieved by forming a covalent bond between an ionized silanol group and a polymeric material [10].

Preparation of covalently modified capillaries is time-consuming and laborious [11, 12]; thus, static or dynamic coating, rinsing the capillary with coating agent solution or addition of a small amount of coating agent to the mobile phase, has been proposed as an

alternative method [13, 14]. The coatings are attached to the capillary wall via hydrogen bonding or electrostatic interactions between oppositely charged molecules. In the same manner, a multilayer-coated capillary can be constructed by successively rinsing the capillary wall with multiple ionic polymer solutions [15, 16].

Hyperbranched polymers have successfully been employed as capillary coatings in CE. Shou *et. al.* investigated the use of hyperbranched polyester coated capillaries to separate basic proteins [8]. It was illustrated that the coated capillary could reduce both the magnitude of the EOF and adsorption of proteins on the capillary wall in the pH range of 3-7 [8]. While a high-molecular-weight branched polyethyleneimine (BPEI) has been utilized as a capillary coating in the separation of basic proteins and peptides, phenolic compounds and organic acids in beverages [2, 4, 8, 17, 18], the newer core-based hyperbranched polyethyelenimines (CHPEI) have not and are studied in this work. CHPEI is an attractive coating material due to its properties such as abundance of highly reactive surface groups, low viscosity due to the lack of chain entanglement, and hydrophilic properties of the surface groups.

The major aim of this work was to understand how the structured molecular architecture of CHPEI affects its properties as a coating material compared to BPEI. Therefore, the effects of pH, CHPEI concentration, coating media concentration, and ionic strength on the stability and reproducibility of the static CHPEI coated capillaries were studied. The difference in repetitive migration time was reported in terms of percent relative standard deviation (% RSD).

2. Results and discussion

2.1 EOF of CHPEI5 and CHPEI25 coatings

It is expected that CHPEI coating can suppress and reverse the direction of the EOF. Therefore, the EOF of the coated capillaries constructed from different generations and concentrations of CHPEI were investigated at various pH values using acetone as an EOF marker. Figures 3.1 and 3.2 show pH dependence of the EOF mobility on the concentration of CHPEI5 (EOF difference < 4% RSD with n = 5) and CHPEI25 (EOF difference < 2% RSD with n = 5), respectively. Both CHPEI5 and CHPEI25 exhibit negative EOF at acidic pH indicating that residual surface amine groups of the coatings are in protonated form and a reversed EOF compared to the BFS surface can be obtained. When the buffer pH was higher than 6.0, the contribution of deprotonated amine and silanol groups results in negatively charged surface as a consequence a positive or normal EOF compared to the bare fused-silica capillary (BFS) was observed. However, there is no significant difference in the EOF mobility from those two generations of CHPEI except at pH 7. In addition, in Figure 3.1, the coated capillary exhibits pH independence when CHBPEI5 concentrations were 10% and 20% (w/v) in neutral and basic pH ranges. However, with the CHPEI25 coated capillary only 10% (w/v) CHPEI25 coating exhibits pH dependence in those ranges. At pH greater than 6, positive EOF was observed indicating a negatively charged surface due to excess silanol groups and

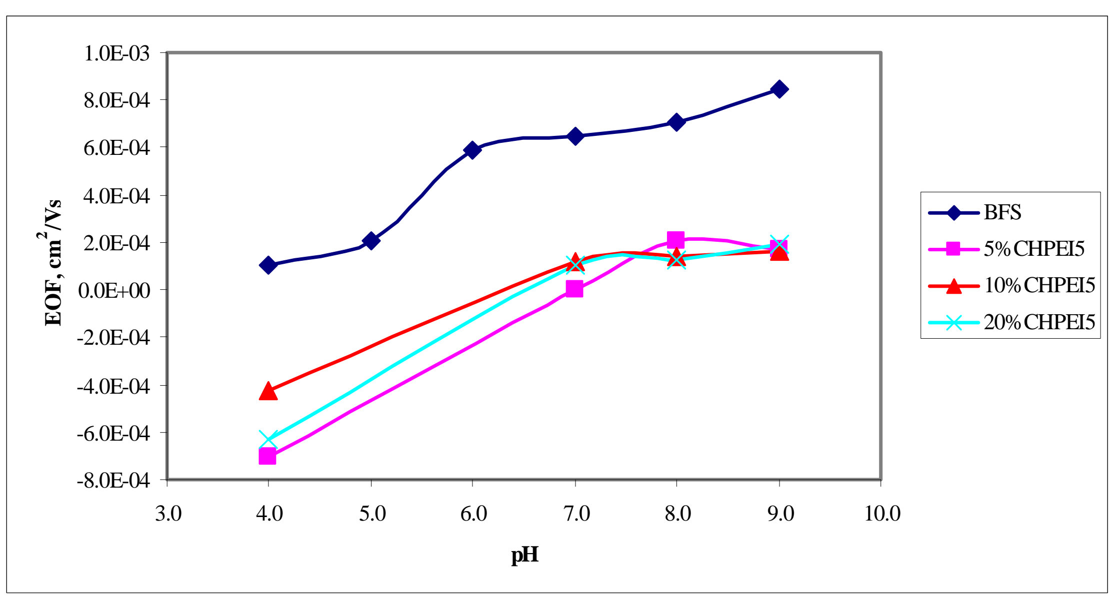

Figure 3.1 Effects of CHPEI5-coating concentration on the EOF versus pH. Applied voltage: ± 15 kV, hydrodynamic injection: 10 cm for 5 sec, detection: 254 nm, and acetone was an EOF marker. (The reported EOF values were from 5 replicate runs and the SD values are smaller than the symbols).

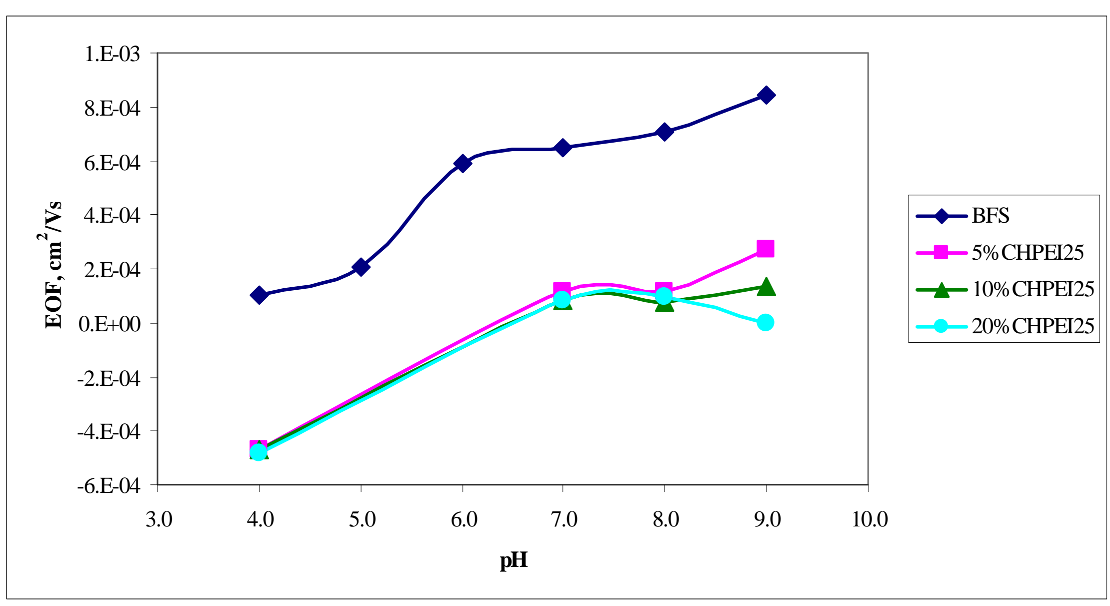

Figure 3.2 Effects of CHPEI25-coating concentration on the EOF versus pH. Applied voltage: ± 15 kV, hydrodynamic injection: 10 cm for 5 sec, detection: 254 nm, and acetone was an EOF marker. (The reported EOF values were from 5 replicate runs and the SD values are smaller than the symbols).

deprotonated amine groups of CHPEI near neutral pH [11, 19]. With regard to the concentration of CHPEI25, it should be noted that 20% (w/v) CHPEI25 was not a suitable coating solution because of its high viscosity, difficulties in handling and capillary blockage. From the findings, 10% CHPEI25-coated capillary was selected for further investigation because it exhibits higher EOF precision (better %RSD) compared to that of CHPEI5. In addition, the higher number of surface groups of CHPEI25 can act as a stronger polyvalent coating to mask silanol ions on the capillary wall.

2.2 Effect of coating media concentration

In general, polymer coatings can be deposited on a variety of hydrophilic media [20]. In addition, variables such as polymer concentration, deposition time and molecular weight have small impact on film thickness [21]. The major role of salt additives in controlling polymer adsorption, via an ion-exchange competition, was proposed by Decher [22]. The presence of salt as a coating media can enhance film thickness and reproducibility of the EOF [3]. Figure 3.3 demonstrates the magnitude of the EOF and its dependence on pH when the coating was deposited using water and salt solutions as coating media. Coated capillary constructed using water and 10 mM NaCl reveal similar magnitude of the EOF in acidic and neutral phosphate buffer solution. Moreover, the coating in 10 mM NaCl exhibits pH-independence in basic pH range. The coating in 20 mM NaCl shows relatively higher EOF mobility in buffer pH 6 to 7. In addition, pH dependence was obtained in wider pH range than that of lower salt concentration media. Coating material that dissolved in salt concentration greater than 20 mM have similar magnitude of the EOF but the pH independence range was only at pH 6-8.

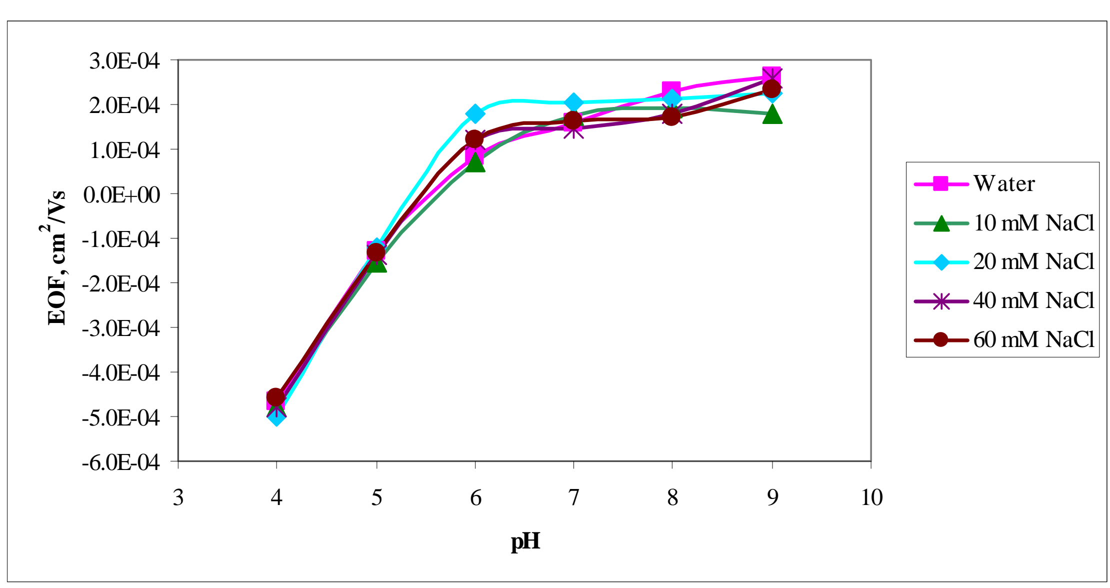

Figure 3.3 Effects of coating media on the EOF of 10% CHPEI 25 coated capillary at different pH. Applied voltage: ± 15 kV, hydrodynamic injection: 10 cm for 5 sec, detection: 254 nm, and acetone was an EOF marker. (The reported EOF values were from 5 replicate runs and the SD values are smaller than the symbols).

2.3 Effect of ionic strength

In uncoated BFS, an increase in the ionic strength of the buffer decreases the magnitude of the EOF because of the decrease of the zeta potential at the solid-liquid interface. A study of the effect of ionic strength on the EOF mobility would indicate if there are other mechanisms the limit the effective surface charge. Preliminary measurements were performed by varying the buffer concentration from 20-60 mM (Figure 3.4). The effect of the ionic strength on the EOF mobility in CHPEI25 coated capillary was investigated at both acidic and basic ranges. The EOF at those two pH values were fairly constant (<5% difference) with increasing buffer concentration, showing that the ionic strength did not have a significant effect on the EOF mobility.

2.4 Stability of the EOF

One of the crucial factors to consider when constructing coated capillaries is stability of the coating. In this study, the stability of the coating was investigated by 25 consecutive runs at pH 4.0. The buffer was refreshed every single run to prevent problems with buffer depletion. The comparison of the EOF obtained from both CHPEI25 coated capillary and BFS is shown in Figure 3.5. The EOF mobility in CHPEI25 coated capillary was incredibly stable for continuous injection. This suggests the strong attachment of the coating to the ionized silanol groups. The variations in the EOF were only 0.3% RSD (n = 5) in the coated capillary and less than 3% RSD (n = 5) in BFS. Such stable EOF is a great benefit that will provide reproducible migration behavior.

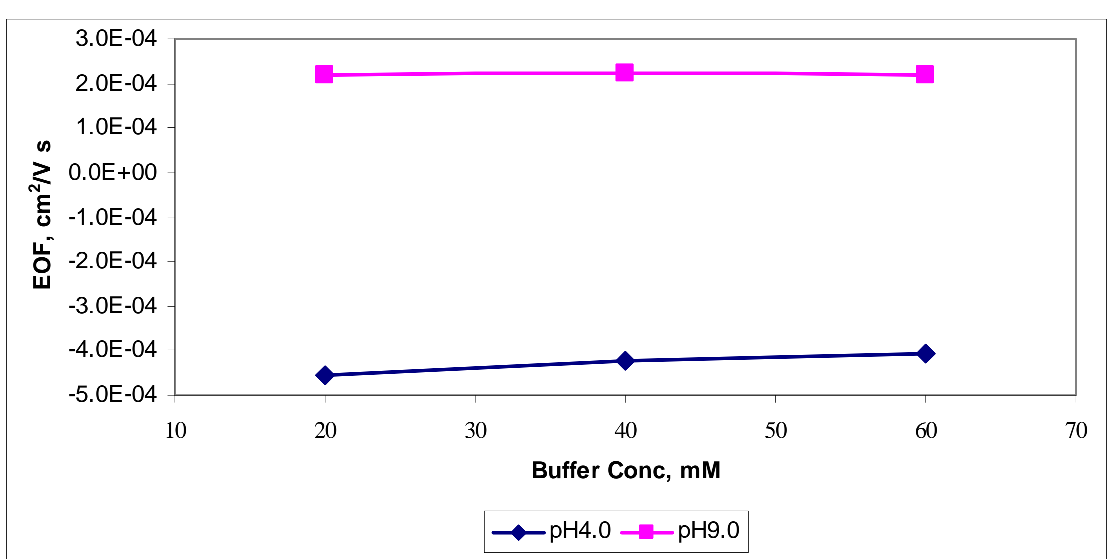

Figure 3.4 Effect of ionic strength on the EOF of 10% CHPEI25 static-coated capillary using acetate buffer pH 4.0 and phosphate-borate buffer pH 9.0. Applied voltage: ± 20 kV, hydrodynamic injection: 10 cm for 5 sec, detection: 254 nm, and acetone was an EOF marker.

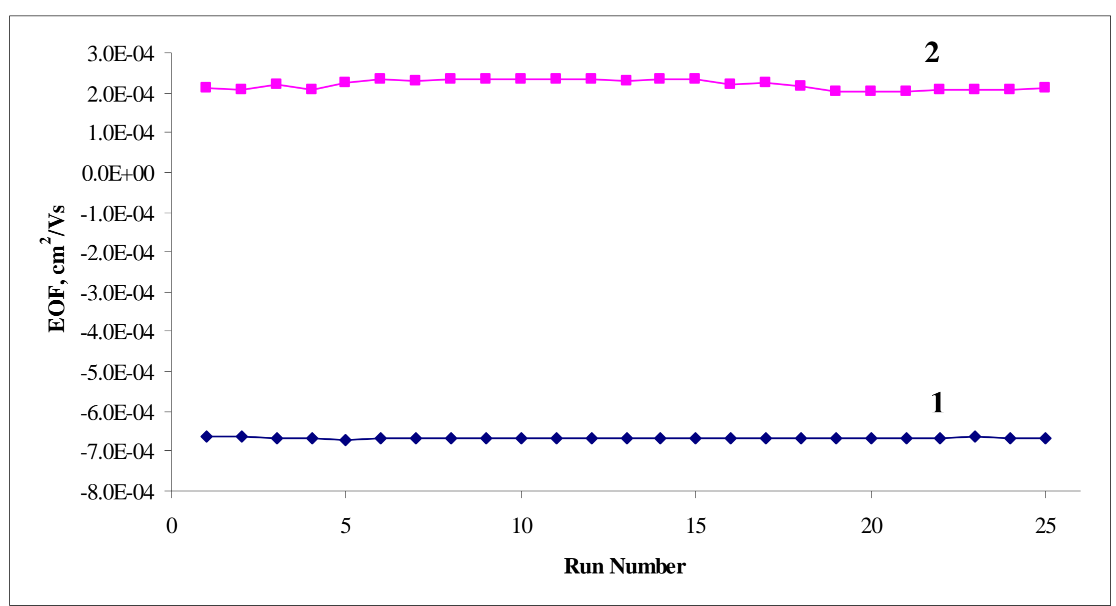

Figure 3.5 Stability of the EOF in 1)10% CHPEI25 static-coated capillary and 2) bare fused silica using 20 mM acetate buffer pH 4.0. Applied voltage: ± 20 kV, hydrodynamic injection: 10 cm for 5 sec, detection: 254 nm, and acetone was an EOF marker.

2.5 Reproducibility of EOF upon changing the pH

Method development in separation science is carried out by varying separation parameters to obtain optimum separation quality. In CE, one of parameters that typically is varied is buffer pH. Stability and reproducibility of the coating after alternatively exposing it to different buffer pH is very crucial. Further study was conducted by measuring the EOF of different pH cycles. The reproducibility of the EOF then was evaluated by calculating the difference in the EOF in term of percent (%) degradation as follows [15, 16]:

$$\%\,\text{Degradation} = 100\left(\frac{\text{EOF}_1 - \text{EOF}_2}{\text{EOF}_1}\right) \qquad (14)$$

where EOF_1 was measured in acidic pH and EOF_2 was measured in neutral or basic pH. Upon altering the buffer pH from acidic-to-neutral-to-acidic and acidic-to-basic-to-acidic in Figure 3.6, it was found that in both cases (pH 4-7-4 cycle and pH 4-9-4 cycle) the coating was relatively stable with the degradation ratio ~6%. Moreover, the overall reproducibility of the coating was very impressive with the degradation ratio only ~0.4%, as can be seen from pH 4-7-4-9-4 cycle. In addition, the coated capillary exhibits pH independence in neutral and basic pH ranges which, corresponds to the results in Figure 3.2.

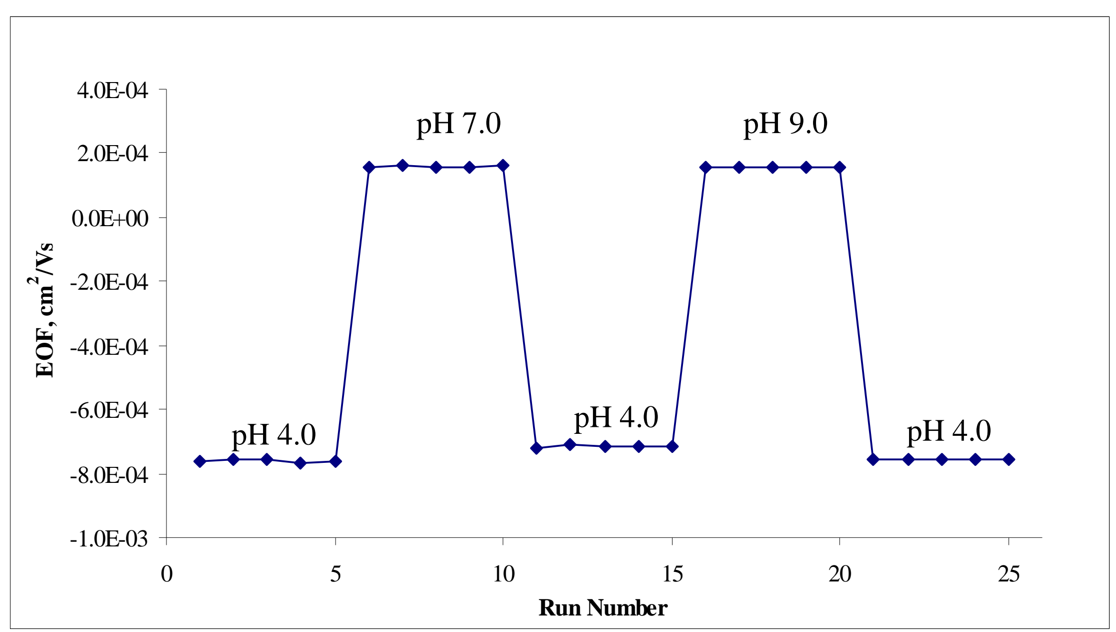

Figure 3.6 Reproducibility of the EOF upon changing the pH for a 10% CHPEI25 static-coated capillary. Applied voltage: ± 20 kV, hydrodynamic injection: 10 cm for 5 sec, detection 254 nm, and acetone was an EOF marker.

3. Conclusions

CHPEI coatings were immobilized on the inner wall of the capillary in a dynamic fashion. Owing to the abundance of surface groups, CHPEI coatings provide polyvalent interactions between anionic silanol and the reactive amine surface groups. In addition, their hydrophilic functional end groups make them completely soluble in water; therefore, the use of organic solvent in separations can be minimized. CHPEI5 and CHPEI25 coatings successfully decrease the EOF of BFS by at least 3-fold. The EOF mobility can change reversibly from positive to negative depending on the pH of the background electrolyte. Reversal of the EOF was observed due to the contribution of excess ionized silanol groups and deprotonated amine surface groups at pH greater than 6. Reproducibility, stability and pH independent EOF over a broad pH range were obtained. The static coating does not require a refreshment or further conditioning procedure; therefore, its potential application to CE separations of a variety of compounds will be examined.

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CHAPTER 4

Performance of Core-based Hyperbranched Polyethyleneimine Static-Coated Capillaries

1. Introduction

Several reasons for capillary wall modification in capillary electrophoresis (CE) have been discussed previously. Objectives are reduction or elimination of analyte-wall interactions, modification of adsorption phenomena and alteration of electroosmotic flow (EOF) to manipulate separation parameters such as shortening analysis time, improved reproducibility and enhanced resolution [1, 2]. The hydrophilicity of the coating is a very crucial factor for separation of biomolecules; whereas, other applications may require hydrophobic coatings. Ideal properties of a capillary wall coating may include lack of coating depletion under separation conditions or under subsequent runs, stability over a broad range of pH, endurance under a change of condition, and reasonable lifetime.

High molecular mass polyethyleneimine (PEI) is one of cationic polymers employed as dynamic adsorption or permanent capillary modification to reverse the EOF and eliminate analyte-wall adsorption [3-12]. Core-based hyperbranched polyethyleneimine (CHPEI) is a commercially available and valuable cationic polyamine. It has been widely used as an efficient drug carrier and a gene delivery system [13]. However, there is no report on an application of CHPEI in capillary wall coating. Because of its high density of functional terminal groups, this hyperbranched polymer

might be found useful to provide hydrophilic character and contribute to interactions between analyte and capillary wall for separation of different types of analytes in CE.

Due to an inaccessibility and low inner surface area of the capillary, conducting wall coating reactions is an area of active work. Homogeneity and reproducible application of the coating are always an important issue. Investigation of efficiency of the coating can be achieved not only from measuring the EOF of a coated capillary but also from an assessment of separation efficiency of neutral and charged molecules. CHPEI generation 25 (CHPEI25) was selected as a coating material in this work because from the characterization of CHPEI-coated capillaries, CHPEI25 demonstrated better EOF stability compared to CHPEI5. In addition, CHPEI25 has higher number of functionalities to serve as a polyvalent coating. To demonstrate the performance of static-, dynamic- and hybrid-coated capillaries in CE separations, several groups of compounds, phenols, B vitamins, organic acids, basic amino acids including aniline and its derivatives, have been separated and compared with the separations in BFS. Migration time variations of intra-capillary and inter-capillary were investigated. Separations in dynamic-, static- and hybrid-coating methods were also studied.

2. Results and discussion

2.1 Separations of neutral compounds

Four phenols were used as sample components: phenol, 2-chlorophenol, 3-chlorophenol and 4-nitrophenol. Considering their pK_a values, which are between 7.2 and 9.9 [6], these phenols are uncharged under the separation condition (pH 7.0). Charge state of these phenols becomes apparent with the separation in bare fused silica capillary

(BFS) (20 mM phosphate buffer pH 7.0) as shown in Figure 4.1a. All four phenols co-migrate and appear as a single peak, as they migrate with the same velocity as the EOF on BFS. When the separation was performed in CHPEI25-coated capillary, as illustrated in Figure 4.1b, the sample zone and EOF of CHPEI25 coated capillary, at pH higher than 6.0, move in the same direction toward the cathode. Therefore, the separation of phenols occurs under the co-current flow. This co-current flow leads to an acceleration of the neutral phenols, which then migrate with a velocity faster than that of the EOF. The molecular interaction between analytes and CHPEI25 is sufficiently strong and selective to separate the analytes except phenol and 2-chlorophenol, which are partially resolved. The migration order corresponds to the different positions of the substituting group. Phenol exhibits the weakest interaction and reaches the detector first. Among the solutes containing two substituent groups, ortho-positioned substitution (2-chlorophenol) is less retarded than meta- (3-chlorophenol) and para-positioned substitution (4-nitrophenol). The stability of the coating and repeatability of the separation were examined by performing five replicate separations and measuring analyte migration times (Table 4.1). The percent relative standard deviations (%RSD) of all analytes were less than 2.3 (n = 5). This consistency of migration times is a consequence of improved EOF stability in the coated capillary.

2.2 Separation of organic acids

Small organic acids have the same magnitude of electrophoretic mobilities but in the opposite direction to the EOF. This generates counter-flow separation that

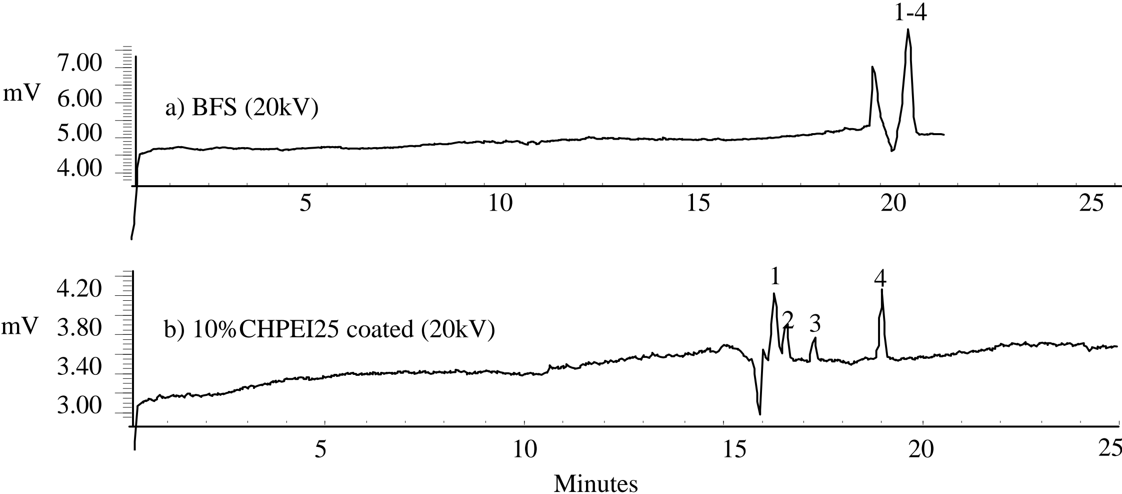

Figure 4.1 Separation of phenolic compounds using 20 mM phosphate buffer pH 7.0, a) in bare fused silica and b) in 10% CHPEI25 static-coated capillary. Applied voltage: 20 kV, detection: 254 nm, hydrodynamic injection: 10cm for 5 sec. Migration order: 1) phenol, 2) 2-chlorophenol, 3) 3-chlorophenol, and 4) 4-nitrophenol.

Table 4.1 Migration times and %RSD from separations of phenolic compounds in 10% CHPEI25 static-coated capillary. Other conditions as in Figure 4.1

Peak No.	Analyte	Avg. migration time (min)	%RSD (n = 5)
1	phenol	16.55	1.30
2	2-chlorophenol	16.82	1.31
3	3-chlorophenol	17.50	1.45
4	4-nitrophenol	19.14	2.28

results in excessive migration times of the anions [9, 10]. Best separation can be accomplished by reducing or reversing the EOF through an addition of an EOF modifier or using cationic polymer coated capillary. Since small carboxylic acids do not absorb ultraviolet (UV) light, indirect UV detection is applied by the addition of a chromophore to the running buffer. In the present study, 10% CHPEI25 static-coated capillaries have been employed for the separation of small organic acids without using any EOF modifier in the buffer. Figure 4.2 illustrates separations of four carboxylic acids in BFS (Fig. 4.2a) and 10% CHPEI25 static-coated capillary (Fig. 4.2b), respectively. Owing to the pK_a of the acids and the pH dependent EOF of the coating, buffer pH 4.9 was selected as optimal for the separation [18]. With the choice of utilizing zwitterionic electrolyte, 6-aminohexanoic acid, a high anodic EOF was obtained, thus analyte migration times decrease with satisfied resolution for all analytes.

2.3 Separation of basic amino acids

One of primary goals of utilizing polymer coated capillaries is for basic proteins and peptides analysis. The use of a positively charged polymer coating can overcome the problem of having electrostatic interactions between the positively charged proteins and the negatively charged wall. Amino acids act as building blocks of proteins and peptides. The presence of basic amino acids in proteins and peptides can contribute to analyte-wall interactions and deteriorate separation performance.

In this study, three basic amino acids, phenylalanine, tyrosine and tryptophan, were chosen. Figure 4.3 shows a comparison of the CE separations under identical conditions using BFS and 10% CHPEI25 static-coated capillary at pH 7.0. All analytes

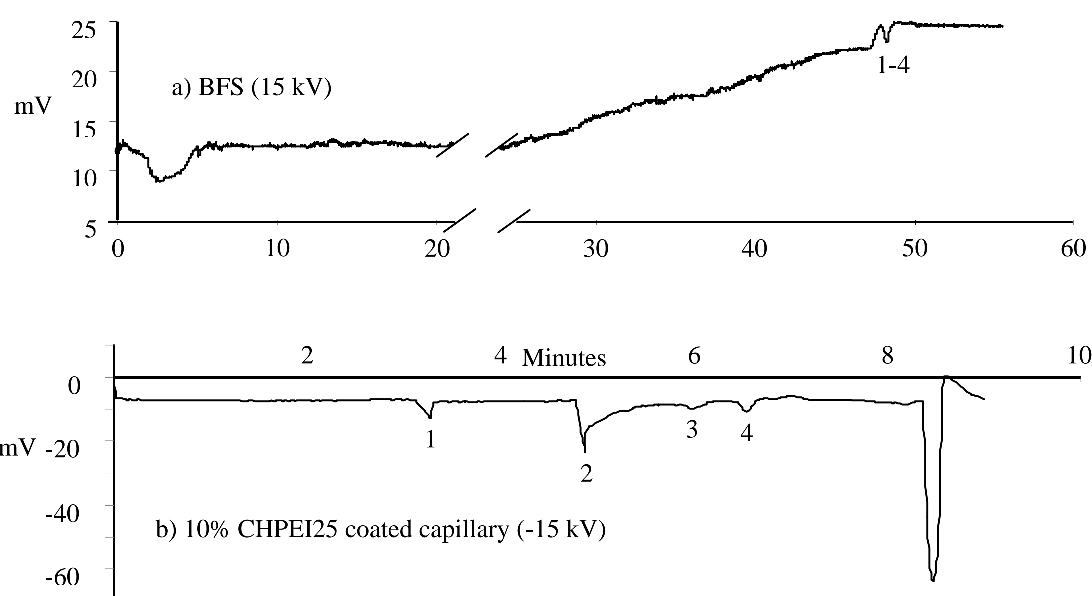

Figure 4.2 Separation of organic acids using 20 mM 6-aminohexanoic acid and 5 mM 2, 4-dihydroxybenzoic acid pH 4.9, a) in bare fused silica and b) in 10% CHPEI25 static-coated capillary. Applied voltage: -15 kV, indirect detection: 254 nm, hydrodynamic injection: 10 cm for 5 sec. Peak order: 1) succinic acid, 2) lactic acid, 3) tartaric acid, 4) citric acid.

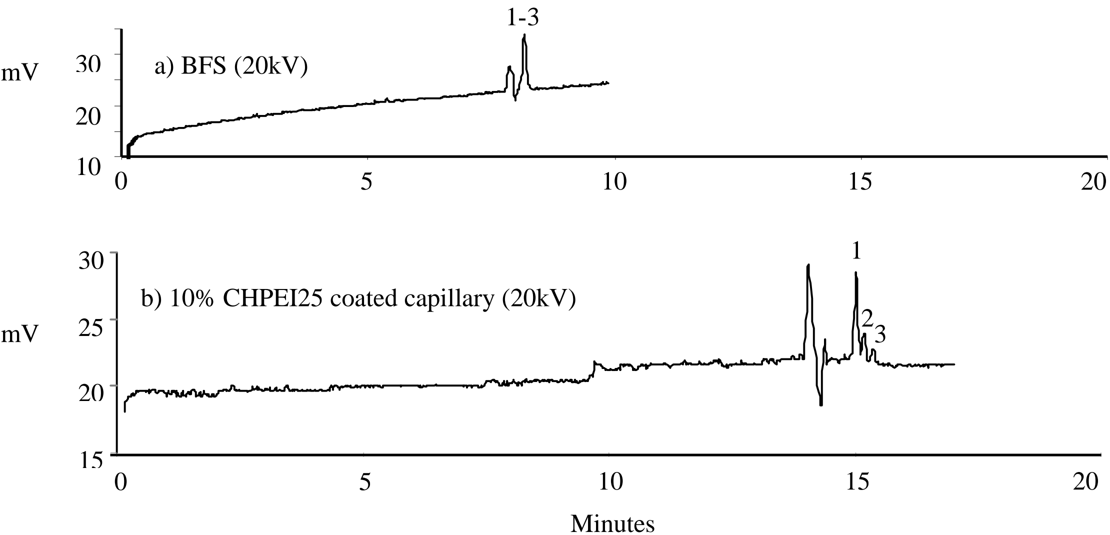

Figure 4.3 Separation of basic amino acids using 20 mM phosphate buffer pH 7.0, in bare fused silica and b) in 10% CHPEI25 static-coated capillary. Applied voltage: 20 kV, detection: 254 nm, hydrodynamic injection: 10 cm for 5 sec. Peak order: 1) tryptophan, 2) phenylalanine, 3) tyrosine.

are negatively charged at this pH thus, positive polarity was chosen making the separation a counter-flow fashion. Although a low resolution is observed in 10% CHPEI25 static-coated capillary, no separation was achieved in BFS at around physiological pH value. Unfortunately, there is no separation in 10% CHPEI25 static-coated capillary under very acidic conditions such as pH below 3.0, probably due to the detachment of the coating when both silanol and amine surface groups are protonated, which weakens the electrostatic interaction between the coating and silanol surface.

2.4 Separation of basic drugs and related compounds

Capillary electrophoresis (CE) using BFS can cause poor resolution in the assay of a basic drug because of a high EOF and analyte-wall interactions. Several studies to overcome those difficulties, including separation at low pH to reduce the EOF, introduction of buffer additives to manipulate the EOF, changing the CE mode and utilizing polymer coated capillaries to remove the high negative charge have been reported [14]. The use of polymer coated capillaries is helpful in capillary zone electrophoresis (CZE) analysis of biological samples because it enhances separation efficiency and resolution [14, 15]. In this approach, four basic drugs and related compounds containing basic, acidic and neutral functionalities were selected for separation in 10% CHPEI25 static-coated capillary as is illustrated in Figure 4.4. The separation in buffer pH 4.0 and anodic EOF reveals complete baseline resolution for all four analytes with reasonable run times of less than 10 minutes. Migration time repeatability was also impressive with less than 2% RSD (n = 5) as illustrated in Table

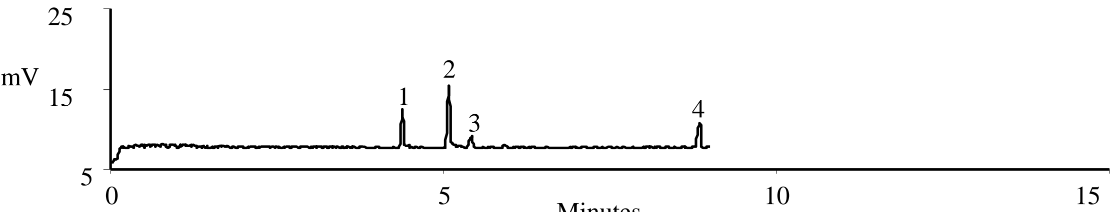

Figure 4.4 Separation of basic drugs and related compounds using 20 mM acetate buffer pH 4.0 in 10% CHPEI25 static-coated capillary. Applied voltage: -20 kV, detection: 254 nm, hydrodynamic injection: 10 cm for 5 sec. Peak order: 1) 3, 4, 5-trimethoxybenzoic acid, 2) 4-aminobenzoic acid, 3) imidazole, 4) trimethoprim.

4.2. Interestingly, selectivity of the coating reveals that acidic compounds reach the detector prior to basic ones. This is because the positive coating interacts with the acidic functionality weaker than that of basic compounds. Imidazole and 4-aminobenzoic acid, relatively small molecules, migrate with similar velocity. Since the latter molecule contains an acidic functionality, it migrates slightly faster than the basic functionality containing molecule. Contribution of analyte mobility and analyte-coating interactions results in the most retardation of trimethoprim, the largest and a basic functionality containing molecule. It should be noted that there is no separation of those analytes in BFS with positively applied voltage.

2.5 Dynamic versus static coating

2.5.1 Separation of B vitamins in CHPEI25 static-coated capillaries

Water-soluble vitamins: riboflavin (vitamin B_2: MW 376.36 g/mol), nicotinamide (vitamin B_3: MW 122.12 g/mol) and pyridoxine (vitamin B_6: 169.18 g/mol) have previously been separated by electrokinetic chromatography using tetradecylammonium ions (TDA^+) as a pseudostationary phase, and in the presence of acetonitrile [16]. In this study, the water-soluble vitamins were separated in 10% CHPEI25 static-coated capillary with negatively applied voltage (Figure 4.5). Since the analytes are uncharged in the separation medium, they migrate at the same velocity as the EOF and toward the anode. Due to the fact that their molecular weights are sufficiently different, all bands are

Table 4.2 Migration times and %RSD from separations of basic drugs and related compounds in CHPEI 25 static-coated capillary. Other conditions as in Figure 4.4

Peak#	Analyte	Avg. migration time (min)	%RSD (n = 5)
1	3, 4, 5-trimethoxybenzoic acid	4.42	1.89
2	4-aminobenzoic acid	5.04	1.20
3	imidazole	5.96	1.60
4	Trimethoprim (TMP)	9.10	1.87

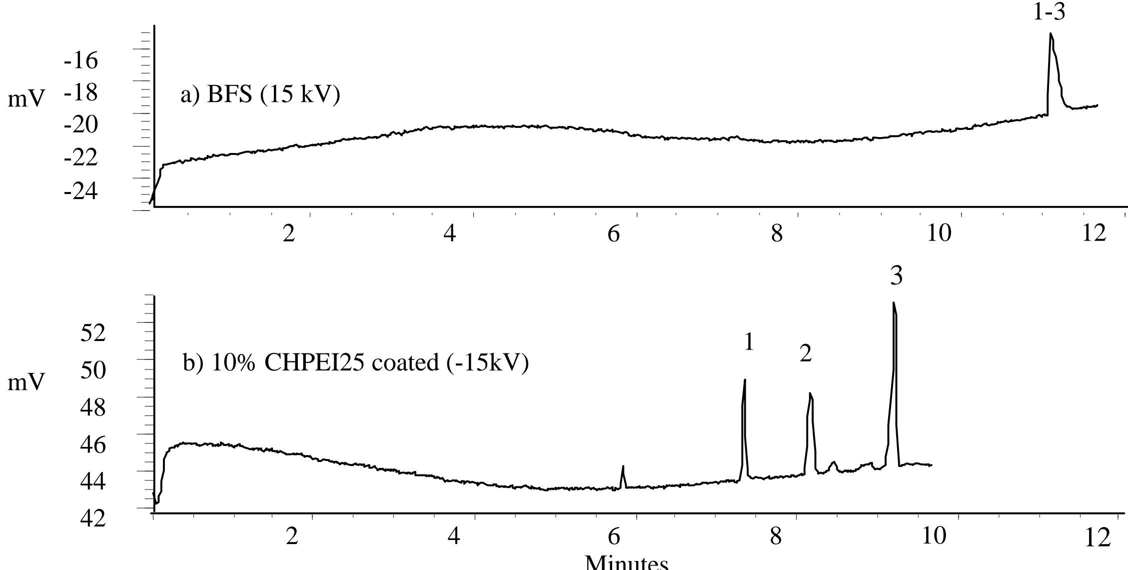

Figure 4.5 Separation of B vitamins using 20 mM acetate buffer pH 4.0, a) in bare fuse silica and b) in 10% CHPEI25 static-coated capillary. Applied voltage: -15 kV, detection: 254 nm, hydrodynamic injection: 10 cm for 5 sec. Migration order: 1) riboflavin, 2) pyridoxine, 3) nicotinamide.

completely resolved. The peak order in CHPEI25 static-coated capillary corresponds to the decrease in analyte molecular weight. Although riboflavin is the most bulky molecule, it reaches the detector first because of lower interaction with the hydrophilic coating. Pyridoxine and nicotinamide contain hydroxyl and amide groups, respectively, which can hydrogen bond with amine surface groups of the coating. This interaction retards the migration of the two analytes. The separations in the coated capillary reveal very impressive repeatability of migration time (Table 4.3) with %RDS less than 0.7 (n = 5). Although the results were reasonably remarkable, a crucial test of this approach is the reproducibility of the coating procedure. The inter-capillary, migration-time repeatability of B vitamins separations on three different capillaries was determined. The reproducibility of the coating procedure expressed as % pooled RSD of all three analyte migration times are shown in Table 4.4 and were better than 2%. It can be concluded that the coating procedure was routinely reproducible.

2.5.2 Separation of B vitamins in CHPEI25 dynamic-coated capillaries

A dynamic-coated capillary is constructed by an addition of CHPEI25 in a running buffer. Anodic EOF in the coated capillary dictates that CHPEI25 binds to the negatively charged surface. Although the same CHPEI25 was used as the coating material, the dynamic-coated capillary exhibits different vitamin B selectivity compared to the static-coated capillary (Figure 4.5 and 4.6). In comparison to the static-coated capillary, dynamic-coated capillary provides better peak resolution when CHPEI concentration is greater than 0.6% and longer analysis time. Increased CHPEI25 concentration in the buffer results in increased peak resolution and run time because the

Table 4.3 Migration times and %RSD from separations of B vitamins in 10% CHPEI25 static-coated capillary. Other conditions as in Figure 4.5

Peak No.	Analyte	Avg. migration time (min)	%RSD (n = 5)
1	riboflavin	7.35	0.52
2	pyridoxine	8.16	0.44
3	nicotinamide	9.24	0.70

Table 4.4 Inter-capillary migration-time repeatability of B vitamins obtained from three different 10% CHPEI25 static-coated capillaries. Other conditions as in Figure 4.5

Peak No.	Analyte	Avg. migration time (min)	%RSD (pooled) (n = 3)
1	riboflavin	7.73	1.15
2	pyridoxine	8.71	1.20
3	nicotinamide	9.87	2.07

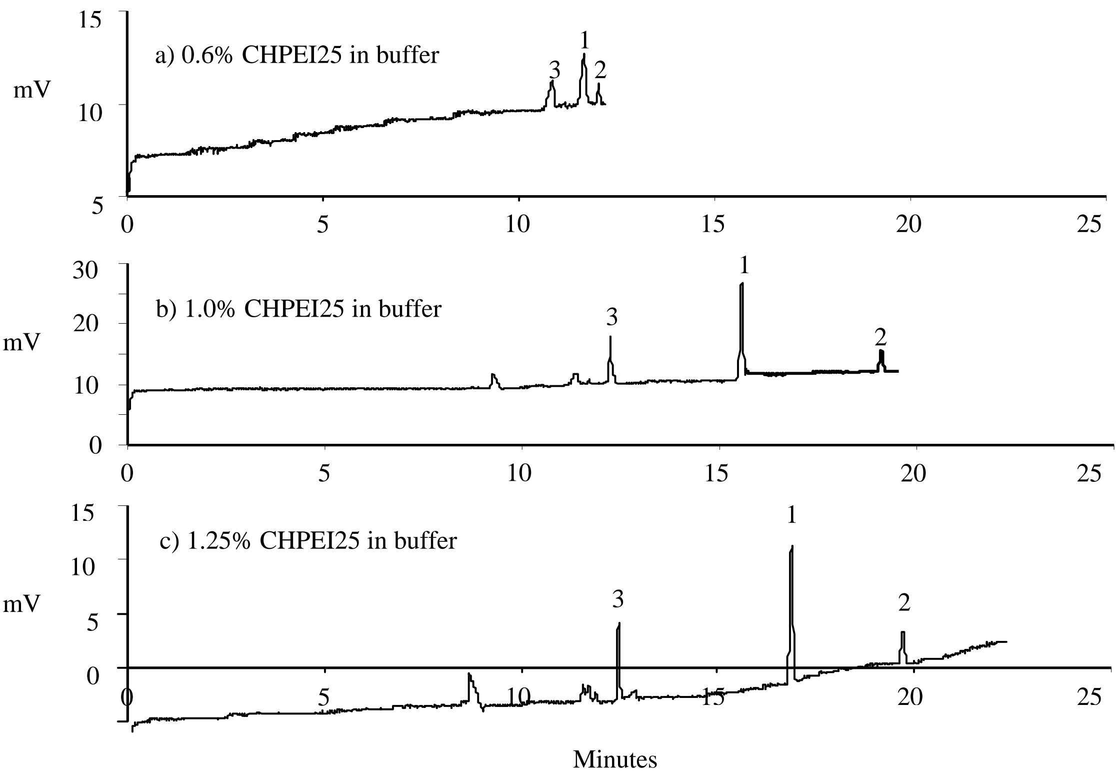

Figure 4.6 Separation of B vitamins in CHPEI25 dynamic-coated capillary (an addition of different CHPEI25 concentrations in 20 mM acetate buffer final pH 4.0). Applied voltage: -15 kV, detection: 254 nm, hydrodynamic injection: 10 cm for 5 sec. Migration order: 1) riboflavin, 2) pyridoxine, 3) nicotinamide.

analytes are retarded by the coating. It is expected that its own mobility is a major contribution of overall migration velocity of nicotinamide (the lowest MW). In contrast, interaction with the coating, via hydrogen boding, is a major role that retards migration velocity of pyridoxine (moderate MW). The weakest interaction between the coating and riboflavin (the highest MW) has previously been described. Although riboflavin mobility is expected to be the lowest, the small magnitude of interaction make it migrates faster than pyridoxine.

2.5.3 Separation of aniline and its derivatives in CHPEI25 static- and hybrid-coated capillaries

Aniline, and mono-substituted aniline derivatives (3-chloroaniline and 3-nitroaniline) were selected to examine the CHPEI25-coated capillary. In this approach, 10% CHPEI25 was used to immobilize onto the NaOH-treated BFS surface. The separations were carried out using 20 mM acetate buffer pH 4.0 and 20 mM acetate buffer containing different concentrations of CHPEI25 to replenish the coating on the capillary surface, and to further alter the magnitude of the EOF. Due to the fact that pK_a values of aniline, 3-chloroaniline and 3-nitroaniline are 4.63, 3.46 and 2.47, respectively [17], they are positively charged under the separation conditions; thus, they migrate in the opposite direction as the EOF mobility. Figure 4.7 illustrates the decrease in analysis time when the concentration of CHPEI25 in the running buffer increases. Interestingly, the presence of CHPEI25 in the buffer media not only prevents the coating depletion, but also accelerates the EOF and as a consequence decreases the analysis time and improves the peak shape with acceptable resolution.

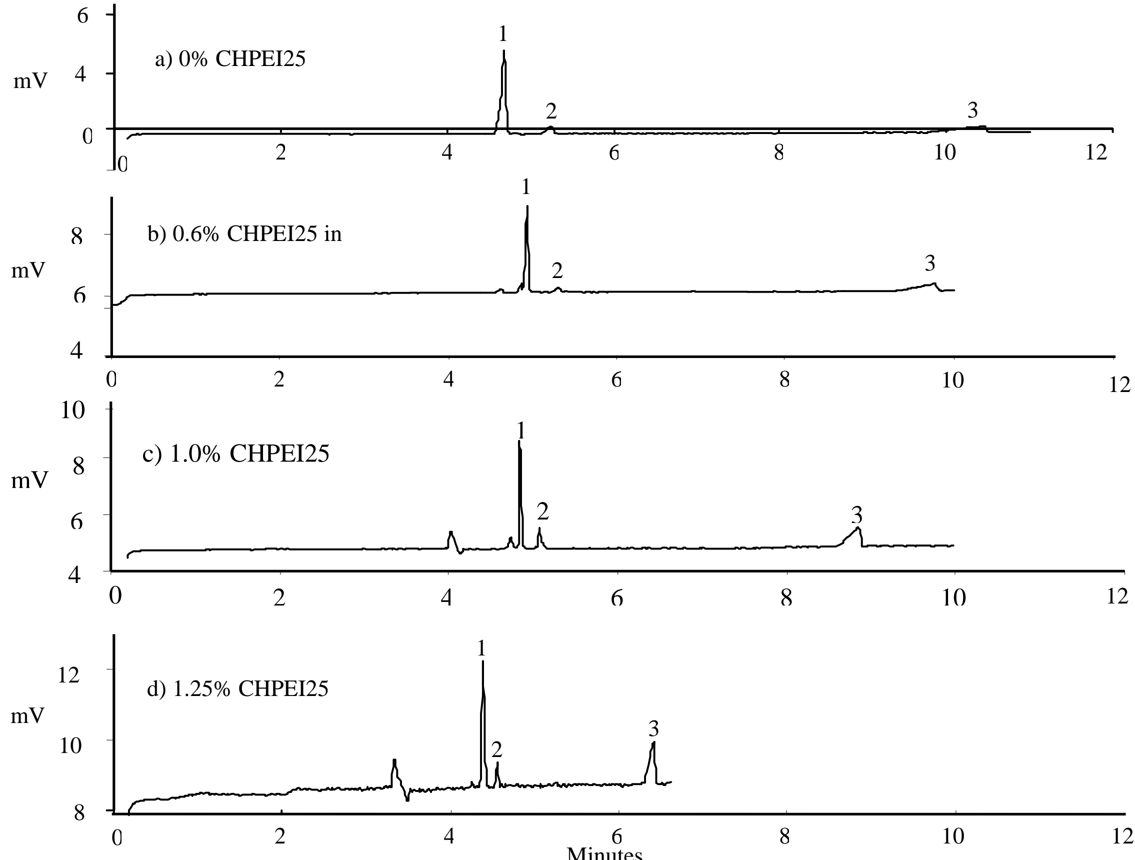

Figure 4.7 Separations of aniline and its derivatives in 10% CHPEI25 static-coated capillary with an addition of different CHPEI25 concentration in 20 mM acetate buffer, final pH 4.0. Applied voltage: -25 kV, detection: 254 nm, hydrodynamic injection: 10 cm for 5 sec. Peak order: 1) 3-nitroaniline, 2) 3-chloroaniline, 3) aniline.

In the static-coated capillary, the asymmetric peak (fronting peak) of aniline might result from faster mobility of aniline than that of background electrolyte. When CHPEI25 was added in the running buffer, the EOF increased, and as a consequence, the band broadening decreased.

The increase of the EOF in buffer containing CHPEI25 can be described. The presence of CHPEI25 in the running buffer causes an excessive positively charged surface that can attract more anions from the bulk solution. These anions move toward the anode under an applied voltage and accelerate the migration of the bulk solution (EOF).

2.5.4 Separation of aniline and its derivatives in CHPEI25 dynamic-coated capillaries

The aforementioned increase of the EOF with increasing CHPEI25 concentration in the buffer has been verified by the results in Table 4.5. A significant increase of the EOF was obtained when adding 1.25% CHPEI25 in the buffer. It should be noted that the negative sign is a conventional specification of the anodic EOF. A similar tendency of the hybrid-coated capillary is clearly observed in dynamic-coated capillary. The analysis time and aniline peak shape were improved with an increased concentration of CHPEI25 in the background electrolyte. However, the background noise increases when the concentration of CHPEI25 in the buffer is greater than 1.25%.

Static-, dynamic- and hybrid-coating methods exhibit very impressive analyte migration-time repeatability with less than 2% RSD, as shown in Table 4.6. It is obvious

Table 4.5 Measured EOF in CHPEI25 dynamic-coated capillaries at pH 4.0 with an applied voltage of -25 kV. Acetone is an EOF marker

Coated capillary	**EOF, cm^2/Vs (%RSD, n = 5)**
0.6% CHPEI25	-5.24×10^{-4} (-1.86)
1.0% CHPEI25	-5.64×10^{-4} (-2.26)
1.25% CHPEI25	-7.15×10^{-4} (-0.99)

Table 4.6 Migration times and %RSD obtained from the separation of aniline and its derivatives in CHPEI25 dynamic-, static- and hybrid-coated capillaries. Other conditions as in Figure 4.7-4.8

Analyte	**Dynamic coating**	**Static coating**	**Hybrid coating**
	Avg. migration time, min (%RSD) (n = 5)	**Avg. migration time, min (%RSD) (n = 5)**	**Avg. migration time, min (%RSD) (n = 5)**
3-nitroaniline	4.26 (0.90)	4.69 (0.93)	4.42 (0.85)
3-chloroaniline	4.43 (1.04)	5.25 (1.13)	4.60 (0.89)
aniline	6.16 (1.89)	10.53 (0.62)	6.46 (1.08)

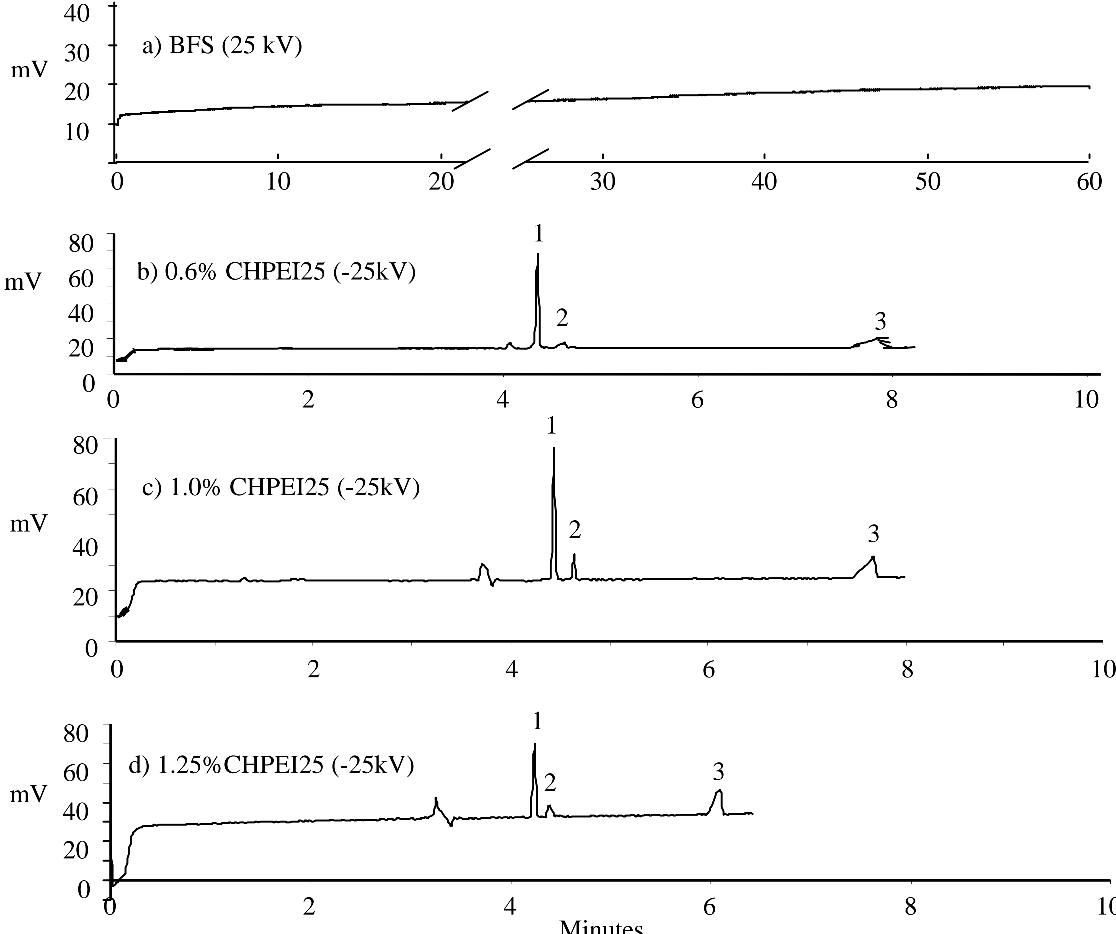

Figure 4.8 Separation of aniline and its derivatives in CHPEI25 dynamic-coated capillary (varying CHPEI25 concentration in 20 mM acetate buffer, final pH 4.0). Applied voltage: - 25 kV, detection: 254 nm, hydrodynamic injection: 10 cm for 5 sec. Peak order: 1) 3-nitroaniline, 2) 3-chloroaniline, 3) aniline.

that the optimum condition for aniline separation can be achieved by separation in the static-coated capillary using 1.25% CHPEI25 in the buffer (Figure 4.8d).

3. Conclusions

This study further demonstrates the performance of CHPEI25-coated capillaries in separations of neutral and charged molecules. The findings indicate that CHPEI25-coated capillaries can be simply constructed by static-, dynamic- and hybrid-coating methods. The capillaries constructed from different coating methods provide highly stable analyte migration times. 10% CHPEI25 static-coated capillaries exhibit anodic EOF in acidic pH and cathodic EOF in basic pH. Moreover, they also exhibit pH-independence EOF in the pH ranges from 7-9. In contrast, CHPEI25 dynamic-coated capillaries provide only anodic EOF in the pH range from 4-9. These different characteristics of the two coating methods may be useful for altering analyte selectivity. Intra-capillary and inter-capillary migration-time repeatabilities in static-coated capillaries are acceptable. The hybrid-coating method is not only to circumvent problems associated with coating depletion, but also to enhance peak symmetry and to provide reasonable analysis time.

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CHAPTER 5

Separations in Core-based Hyperbranched Polyethyleneimine and Commercial Amine Dynamic-Coated Capillaries

1. Introduction

In recent years, several applications of polymers as capillary coatings have shown many advantages, such as improved reproducibility, selectivity and efficiency of the capillary electrophoresis (CE) separations. Branched and hyperbranched polymers have been utilized as a buffer additive or a coating material, to modify the capillary wall [1-10]. Capillary-wall coatings are described as dynamic and static based on the immobilization of the coating onto the capillary surface. A dynamic-wall coating is a simple and attractive coating method. It is typically prepared by rinsing the capillary with a buffer solution containing a small amount of coating agent. The lifetime of the dynamic-coated capillaries can be extended by utilizing a periodic regeneration process. Static coating is an alternate coating method, which eliminates the tedious process of performing dynamic coating prior to each run or periodical rinsing the capillary with a coating solution. A static coating method is mainly used to suppress the EOF and analyte-wall interaction including, reversing the EOF [11].

Polyethyleneimine polymers are cationic functional polymers. Among those ethylene based polymers, branched polyethyleneimine (BPEI) has been employed either as a dynamically or physically adsorbed layer with excellent stability between pH 3 and 11. It offers a wide variety of applications in CE separations [1, 3, 8, 12-15].

A commercial amine coated capillary, eCAP™ is commercially available from Beckman Coulter. This coated capillary can be operated at higher pH for better sample mobility differentiation and to induce the EOF. This amine capillary utilizes a dynamic coating on its surface and requires rinsing with the amine regenerator between runs to assure a uniform capillary coating from run to run. This capillary has been effectively used to separate basic proteins, basic drugs and glycoforms. The surface that is created is a cross-linked polymer with a quaternary ammonium ion providing a uniform positively charged surface and a consistent EOF.

Core-based hyperbranched polyethyleneimine generation 25 (CHPEI25) was employed as a coating material in this work. It bears a primary amine surface, while the coating material in eCAP™ contains tertiary amine functionalities. To compare the performance of the coatings containing primary and quaternay amines in CE separations, several groups of compounds, phenols, B vitamins, organic acids, basic amino acids including aniline and its derivatives, were separated in both capillaries. Migration time variations in the two dynamic-coated capillaries were investigated.

2. Results and discussion

2.1 pH-dependence EOF of dynamic-coated capillaries

One of the major goals of using polymer coated capillaries in CE separations is to suppress or to induce reversed EOF. In this approach, the anodic EOF from eCAP™ and 1.25% CHPEI25 dynamic-coated capillaries were measured using acetone as an EOF marker at -15 kV. Table 5.1 shows the EOF values obtained from both amine coatings in pH ranges from 4-9. A negative sign dictates conventional direction of the EOF that

Table 5.1 Comparison of pH-dependence EOF of eCAP™ and 1.25% CHPEI25 dynamic-coated capillaries measured at -15 kV. Acetone is an EOF marker

pH	EOF from dynamic coated capillaries, cm^2/Vs	
	eCAP™ (anodic)	**CHPEI25 (anodic)**
4	$-8.49x10^{-4}$	$-5.58x10^{-4}$
5	$-7.46x10^{-4}$	$-4.84x10^{-4}$
6	$-3.81x10^{-4}$	N/A[a]
7	$-2.37x10^{-4}$	N/A[a]
8	$-2.56x10^{-4}$	$-2.56x10^{-4}$
9	$-3.07x10^{-4}$	$-1.16x10^{-4}$

[a] EOF in CHPEI25 dynamic-coated capillary cannot be measured in phosphate buffer containing CHPEI25 because of the white precipitation that occurs when the buffer solution was adjusted with 1 M phosphoric acid to the final pH of 6 and 7.

migrates from cathode toward anode. The eCAP^{TM} capillary exhibits significantly higher EOF values than those of 1.25% CHPEI25 dynamic coated capillary except at pH 8.0. Since tertiary amines are the predominant surface group in eCAP^{TM}, this relative hydrophobic surface lessens electrostatic interaction with the anions from the buffer solution. Thus, under an applied voltage anions move faster toward anode, which in turn accelerates EOF mobility. EOF deviation for CHPEI25 dynamic-coated capillary (less than 1.6% RSD, n = 5) is slightly better than that of eCAP^{TM} (less than 2% RSD, n = 5). It is obvious that the EOF in eCAP^{TM} capillary greatly increases in the pH range from 5-6. Unlike 10% CHPEI25 static-coated capillary, CHPEI25 dynamic-coated capillary and eCAP^{TM} do not exhibit cathodic and pH-independent EOF when the buffer pH is greater than 5. Moreover, the EOF values in eCAP^{TM} capillary tend to decrease when the buffer pH increases from 7-9.

Extensive studies on the separation behavior of eCAP^{TM} compared to 1.25% CHPEI25 dynamic coated capillary were carried out in analysis of different groups of compounds: water-soluble vitamins, aniline and its derivatives as well as basic drugs and related compounds.

2.2 Separation of B vitamins

Three B vitamins were successfully separated in CZE with completely resolved peaks both in eCAP^{TM} and 1.25% CHPEI25 dynamic-coated capillaries as depicted in Figure 5.1. Due to the fact that the EOF of eCAP^{TM} is much higher than that of CHPEI25 dynamic-coated capillary, the total runtime in eCAP^{TM} is much shorter. From Table 5.1,

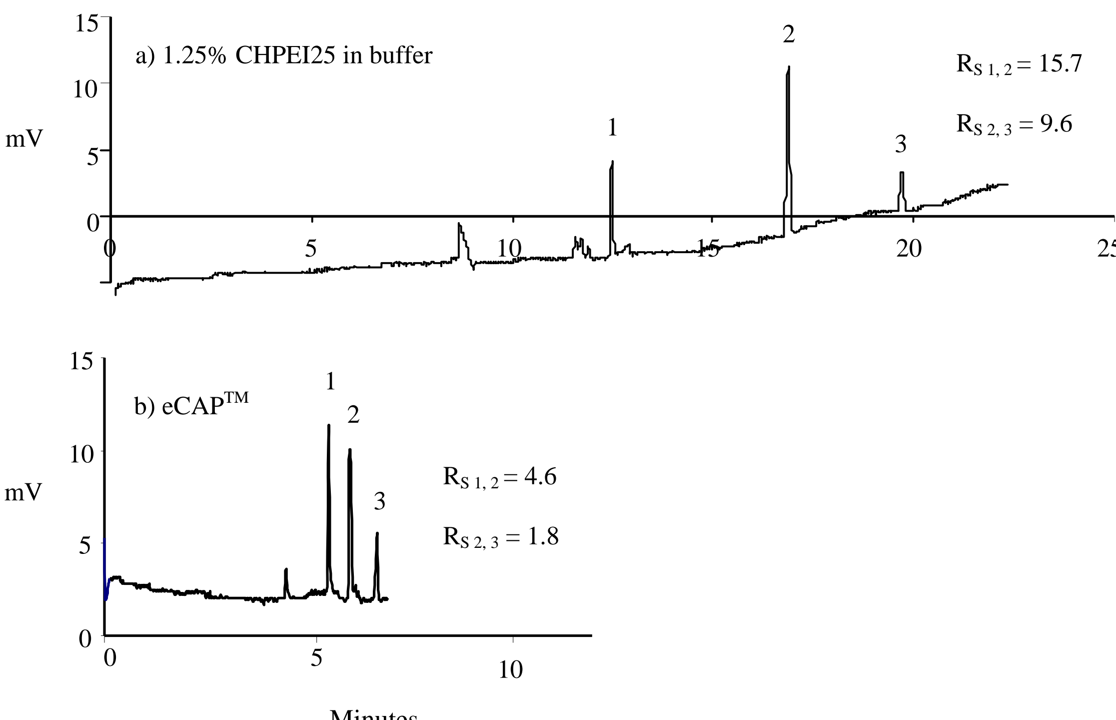

Figure 5.1 Separation of B vitamins a) in CHPEI25 dynamic-coated capillary (1.25% CHPEI25 in 20 mM acetate buffer, final pH 4.0) and b) in eCAP™ capillary using 20 mM acetate buffer pH 4.0. Applied voltage: -15 kV, detection: 254 nm, hydrodynamic injection: 10 cm for 5 sec. Migration order: 1) nicotinamide, 2) riboflavin, 3) pyridoxine.

both capillaries generate anodic EOF and all analytes are neutral at the separation condition (pH 4.0), thus the separation follows co-current mode. Nicotinamide, the lowest molecular weight, migrates faster than riboflavin and pyridoxine and exhibits weak interactions with the coating; thus, it reaches the detector first. Although riboflavin, the largest molecule, should have lower mobility than pyridoxine, the weaker interaction with the coating makes it migrate faster than pyridoxine. Pyridoxine, a medium size molecule, exhibits the strongest interaction with the coating and reaches the detector last. The separations in the eCAP™ capillary reveal satisfactory analysis time and peak resolution. However, enhanced peak resolution with acceptable analysis time was obtained in CHPEI25 dynamic-coated capillary. Migration time repeatability is acceptable in both capillaries with less than 2% and 2.8% RSD (n = 5) for CHPEI25 dynamic-coated capillary and eCAP™, respectively.

2.3 Separation of aniline and its derivatives

Figure 5.2 illustrates CZE separation of aniline and its derivatives in eCAP™ compared to that in CHPEI25 dynamic-coated capillary. Both capillaries exhibit incredible migration time repeatability (less than 1% RSD and 2% RSD, n = 5 in CHPEI25 and eCAP™, respectively). In this approach, separations in both capillaries not only offer fast analysis times but also enhanced peak shape. Aniline (pK_a 4.63) [16] and amine surface groups of the coating are positively charged at the separation condition (pH 4.0). Positively charged aniline will be repelled from the protonated amine surface;

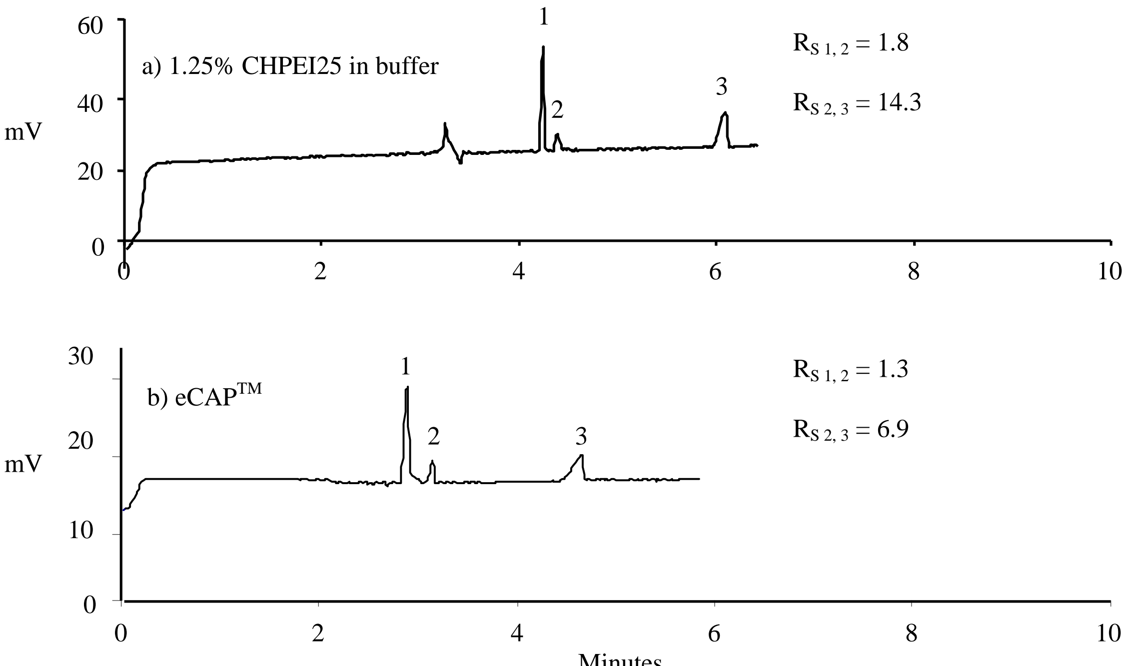

Figure 5.2 Separation of aniline and its derivatives a) in CHPEI25 dynamic-coated capillary (1.25% CHPEI25 in 20 mM acetate buffer, final pH 4.0) and b) in eCAP™ capillary using 20 mM acetate buffer pH 4.0. Applied voltage: -25 kV, detection: 254 nm, hydrodynamic injection: 10 cm for 5 sec. Peak order: 1) 3-nitroaniline, 2) 3-chloroaniline, 3) aniline.

therefore, analyte-coating interactions are diminished. It was mentioned earlier that tertiary amines are the predominant surface group in eCAP^{TM}. Therefore, it is expected that weaker interactions should occur between charged species and relatively hydrophobic coating in eCAP^{TM}. Since protonated, primary amines groups predominate in the CHPEI coating and aniline is positively charged at this condition, there is no electrostatic interaction between aniline and the coating. Aniline derivatives, 3-nitraniline and 3-chloroaniline, are fully protonated because their pK_a values (2.47 and 3.46, respectively) [16] are lower than the separation pH. These positively charged species exhibit weaker interactions with relative hydrophobic coating (eCAP^{TM}) and are repelled from the positively charged coating (CHPEI25); thus, they migrate faster and reach the detector prior to aniline.

2.4 Separation of basic drugs and related compounds

In this approach, an extensive application of positively charged polymer coated capillaries to basic drugs and related compounds was described. Basic drugs and related compounds have been successfully separated in commercial eCAP^{TM} capillary [17]. In comparison, both coated capillaries provide reasonable analysis times and baseline separations except 4-aminobenzoic acid and imidazole in eCAP^{TM} capillary (Figure 5.3). Migration-time repeatability from both capillaries is less than 1.3% and 2.6% RSD (n = 5) for CHPEI25 coated capillary and eCAP^{TM}, respectively. In addition, both capillaries exhibit a different migration order that dictates different selectivity of the two amine coatings. Although CHPEI25 capillary provides significant resolved peak, imidazole, the

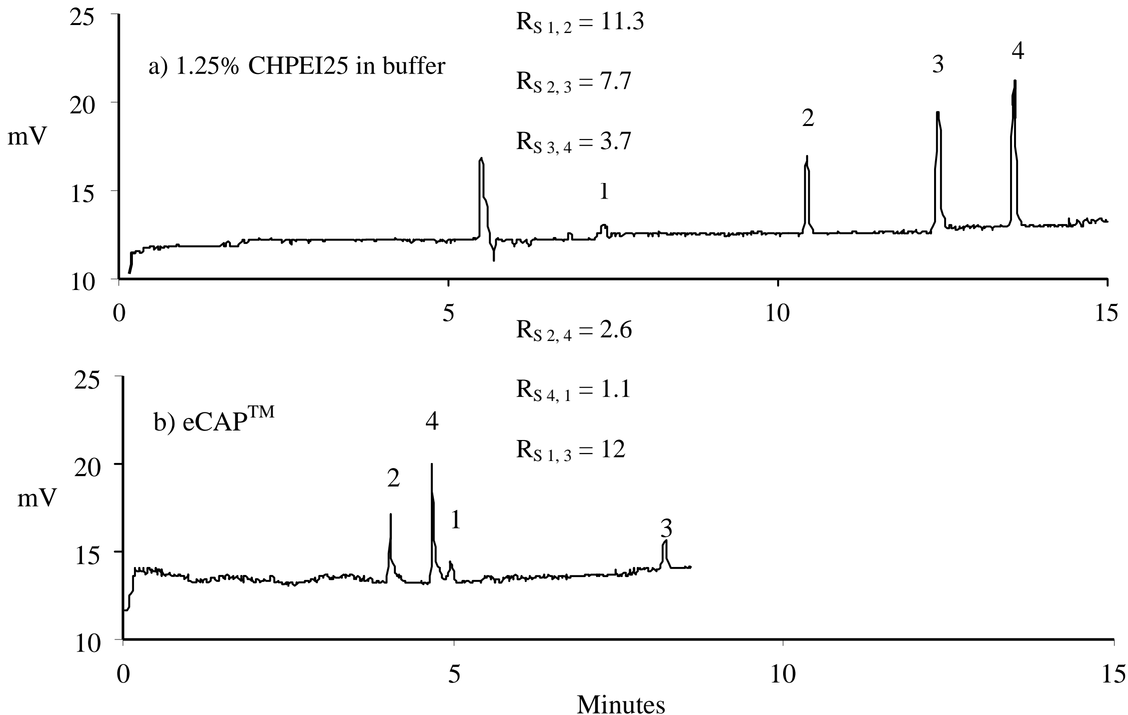

Figure 5.3 Separation of basic drugs and related compounds a) in CHPEI25 dynamic-coated capillary (1.25% CHPEI25 in 20 mM acetate buffer, final pH 4.0) and b) in eCAP^{TM} capillary using 20 mm acetate buffer pH 4.0. Applied voltage: -20 kV, detection: 254 nm, hydrodynamic injection: 10 cm for 5 sec. Peak order: 1) imidazole, 2) 3, 4, 5-trimethoxybenzoic acid, 3) trimethoprim, 4) 4-aminobenzoic acid.

smallest molecule that has the greatest mobility tends to bind with the coating surface. This binding results in low sensitivity and undesired peak shape.

These findings suggest that both coated capillaries could be used for rapid assays of bulk drug as well as pharmaceutical preparations for both active and related compounds. However, robustness and reproducibility of CHPEI25 coated capillary warrants further investigation beyond this proof of concept.

3. Conclusions

In this approach, performance of CHPEI25 dynamic coated capillary has been compared to that of commercial amine coated capillary, eCAP™, from Beckman Coulter. Unlike eCAP™ where the capillary surface mainly consists of tertiary amine, the CHPEI coated capillary surface is composed of primary amines. Although CHPEI25 and eCAP™ contain different amine surface groups, they exhibit the same selectivity in the separation of B vitamins as well as aniline and its derivatives. However, they reveal different selectivity in the separations of basic drugs and related compounds. Due to the surface groups and the EOF values in the eCAP™ capillary being significantly higher than those in CHPEI25 dynamic-coated capillary, the latter provides better peak resolution with slightly longer analysis time. The results discovered herein offer an alternative choice of dynamic-coated capillary to separate hydrophilic compounds.

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CHAPTER 6

Overall Conclusions

Polyethyleneimine-based polymers are well-known functional polymers and their applications are very wide. They have not only been utilized for industrial purposes, but also in biomedical and chemistry applications. Recently, core-baed hyperbranched polyethylenimine (CHPEI) has been successfully utilized as a capillary coating to manipulate or suppress the electroosmotic flow (EOF). Due to its abundance of amine surface groups, CHPEI is classified as a polyvalent polymer. The positive surface groups of CHPEI interact with silanol groups of treated bare fused-silica capillary (BFS).

Due to the small size and relative inaccessibility of the capillary surface, characterization of coated capillaries is extremely difficult. CHEPI coated capillaries are characterized by measuring their EOF as a function of buffer pH using a neutral molecule as an EOF marker. Two generations of CHPEI, CHPEI5 and CHPEI25, were selected to immobilize onto BFS surface. Both coated capillaries exhibit anodic EOF in acidic pH and cathodic EOF in neutral and basic pH. In addition, they reveal pH-independent EOF, when the buffer pH is greater than 6.

Different coating methods were also investigated using CHPEI25 as a coating material. It was revealed that the CHPEI coating is not only simple to immobilize onto the capillary surface but the diversified coating methods, static, dynamic and hybrid, could also be carried out by rinsing procedure. The static-coating method can be achieved by flushing a treated BFS with a high concentration of CHPEI solution. Typically, CHPEI concentration ranges from 5-20% (w/v) are optimal for this coating method.

Unlike the static-coating method, the dynamic-coating procedure requires much lower CHPEI concentration in a running buffer. A high concentration of CHPEI in the buffer can cause high current, and as a consequence elevates the background signal. This study reveals that to obtain satisfactory separation quality (e.g. resolution and migration time) an approximate CHPEI25 concentration of 1% (w/v) in the running buffer should be used.

Coated capillaries obtained from static and dynamic CHPEI25-coating methods exhibit EOF reversal in neutral and basic pH ranges. Dynamic-coated capillaries provide only anodic EOF in the pH ranges from 4-9. In contrast, reversal of the EOF in static coated capillaries can be obtained when the buffer pH is greater than 6. The different characteristics of the CHPEI25 coating based on coating method may find usef in analyte selectivity alteration and enhanced peak resolution.

Further study of the hybrid-coating method by using a static-coated capillary and CHPEI containing buffer to separate groups of analytes was also investigated. This study intends to solve problems associated with physical coating depletion and mismatch of the mobility of analyte and background electrolyte. The findings reveal that static-, dynamic- and hybrid-coating methods have shown their benefits in terms of selectivity, migration time repeatability and analysis time.

A comparison of the performance of dynamic amine coated capillaries was carried by comparing CHPEI25- and eCAP^{TM}-coated capillaries. The eCAP^{TM}, amine, dynamic-coated capillary consist of quaternary amine surface groups. This dynamic-coated capillary enhances sample mobility differentiation at higher pH. It has been used effectively for basic proteins and basic drugs separations. Surface groups in CHPEI25

coated capillary mainly consist of primary amines, which provide different selectivity in the separation of basic drugs and related compounds. However, CHPEI25 coated capillary provides better peak resolution with reasonable analysis time.

It is evident that a novel CHPEI material is extremely versatile being utilized as a dynamic-, static- or hybrid-capillary coating. Adsorption of the CHPEI coating onto the silica surface can minimize solute-wall interactions, suppress the EOF, and improve the stability of the EOF and migration-time repeatability. In addition, CHPEI coatings alter the analyte selectivity. The commercial availability of CHPEI coating solutions enhances the utility of surface-modified capillaries and facilities growth in this field, since coated capillaries can be used in all the major modes of CE.

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APPENDIX

Structure of Analytes

Phenol and related compounds	
Phenol 94.11 g/mol pK_a 9.95	
2-Chlorophenol 126.58 g/mol pK_a 8.49	
3-Chlorophenol 126.58 g/mol pK_a 8.85	
4-Nitrophenol 139.22 g/mol pK_a 7.15	

Basic amino acids	
Tyrosine 181.19 g/mol pK_a 2.2, 9.2, 10.5	
Tryptophan 204.23 g/mol pK_a 2.5, 9.4	
Phenylalanine 165.19 g/mol pK_a 2.2, 9.3	

Aniline and its derivatives	
Aniline 93.13 g/mol pK_a 4.63	
3-Chloroaniline 127.57 g/mol pK_a 3.46	
3-Nitroaniline 138.14 g/mol pK_a 2.47	
Organic acids	
Lactic acid 90.08 g/mol pK_a 3.85	

Tartaric acid 150.09 g/mol pK_a 2.98, 4.34	
Succinic acid 118.09 g/mol pK_a 4.16, 5.61	
Citric acid 192.12 g/mol pK_a 3.14, 4.77, 6.39	
B vitamins	
Nicotinamide (Vitamin B_3) 122.12 g/mol	

Pyridoxine (Vitamin B_6) 169.18 g/mol	
Riboflavin (Vitamin B_2) 376.36 g/mol	
Basic drugs and related compounds	
Imidazole 68.08 g/mol pK_a 14.5	
4-Aminobenzoic acid 137.14 g/mol pK_a 2.50	

3, 4, 5-Trimethoxybenzoic acid 212.2 g/mol	
Trimethoprim 290.32 g/mol	

VITA

Cheerapa Boonyakong was born on June 5, 1972 in Hat Yai, Songkhla Province, Thailand. She attended Prince of Songkhla University (Hat Yai, Songkhla) and received her bachelor's degree in 1995 and then she joined Sitthiporn Associates Co., Ltd. as a chemist for two years. After receiving her master's degree in chemical engineering from Prince of Songkhla University in 2000, she joined the National Institute of Metrology (Thailand) (NIMT) and received a scholarship from the Ministry of Science and Technology to pursue her graduate study in chemistry. She then attended Georgia State University (Atlanta, Georgia, USA) and received master degree in chemistry in 2005. She started her doctoral program at University of Missouri (Columbia, Missouri, USA) in August 2005 and graduated with her PhD in chemistry in May 2009 under the direction of Professor Sheryl A. Tucker.