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Poly-*N*-hydroxyethylacrylamide as a novel, adsorbed coating for protein separation by capillary electrophoresis

We present the polymer poly-*N*-hydroxyethylacrylamide (PHEA) (trade name, polyDuramide™) as a novel, hydrophilic, adsorbed capillary coating for electrophoretic protein analysis. Preparation of the PHEA coating requires a simple and fast (30 min) protocol that can be easily automated in capillary electrophoresis instruments. Over the pH range of 3–8.4, the PHEA coating is shown to reduce electroosmotic flow (EOF) by about 2 orders of magnitude compared to the bare silica capillary. In a systematic comparative study, the adsorbed PHEA coating exhibited minimal interactions with both acidic and basic proteins, providing efficient protein separations with excellent reproducibility on par with a covalent polyacrylamide coating. Hydrophobic interactions between proteins and a relatively hydrophobic poly-*N,N*-dimethylacrylamide (PDMA) adsorbed coating, on the other hand, adversely affected separation reproducibility and efficiency. Under both acidic and basic buffer conditions, the adsorbed PHEA coating produced an EOF suppression performance comparable to that of covalent polyacrylamide coating and superior to that of adsorbed PDMA coating. The protein separation performance in PHEA-coated capillaries was retained for 275 consecutive protein separation runs at pH 8.4, and for more than 800 runs at pH 4.4. The unique and novel combination of hydrophilicity and adsorptive coating ability of PHEA makes it a suitable wall coating for automated microscale analysis of proteins by capillary array systems.

Keywords: Capillary coating / Capillary electrophoresis / Dynamic coating / Poly-*N*-hydroxyethylacrylamide / Protein separation EL 5366

1 Introduction

With the Human Genome Project approaching completion, the next challenging endeavor for scientists is the analysis of the proteome, or what is known as proteomics. Polyacrylamide slab gel electrophoresis (PAGE) has been the predominant technique for the separation of cell proteins. However, the resolving power of PAGE is often insufficient to separate all of the different proteins in a sample. Other limitations of PAGE are that it is labor-intensive, relatively slow, and not amenable to automation. The past decade has seen miniaturized electrophoresis, such as capillary electrophoresis (CE), explored for rapid protein analysis. Advantages of CE over PAGE include higher resolution, shorter analysis times, lower consumption of chemicals and samples, easier automation, and on-line optical detection. More recently, microchip electrophoresis has been investigated for the purpose of protein analysis due to the inherent high-speed and high-throughput capabilities of this technique [1–3]. Furthermore, microfluidic devices have the potential for multitask manipulation of proteins on one platform that might perform cell lysing, enzymatic digestion, peptide labeling, separation, and detection [4, 5].

For the CE analysis of proteins in free solution, when only diffusion is considered as the factor influencing separation efficiencies and all other factors are neglected, separation efficiencies of millions of theoretical plates are predicted [6, 7]. In practice, the efficiencies typically achieved in protein separations are considerably lower. Protein peaks are often broad and sometimes impossible to detect, especially when proteins are analyzed at pH values below their isoelectric points (p*I*). Low separation efficiencies are due to different types of interactions, including hydrogen-bonding, electrostatic, and/or hydro-

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Abbreviations: APS, ammonium persulfate; **DMA**, *N*,*N*-dimethylacrylamide; **HEA**, *N*-hydroxyethylacrylamide; **LPA**, linear polyacrylamide; **PDMA**, poly-*N*,*N*-dimethylacrylamide; **PHEA**, poly-*N*-hydroxyethylacrylamide (polyDuramide™)

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phobic interactions, between the proteins and the silica wall of the capillary or microchannel, and have been the major obstacles to useful protein separations by CE. Reversible interactions between the analytes and the silica walls result in broadening of peaks, tailing, and decreased reproducibility, while irreversible interactions completely destroy the separation, foul the capillary, and adversely affect sample recovery. Another problem encountered in protein separation by CE is the presence of electroosmotic flow (EOF) created by the charged silanol groups on the wall surface. Acid-base equilibrium on the silica surface is often slow and exhibits hysteresis [8]. Furthermore, changes in the surface due to adsorption of proteins from the running buffer can result in nonuniform axial distribution of the zeta potential, creating complex, nonuniform liquid flow profiles and uncontrollable EOF in silica capillaries, leading to poor reproducibility of separations and band broadening [9].

Several approaches have been proposed in order to minimize protein adsorption and stabilize EOF. One approach is to work at conditions where the silanol groups are fully protonated [10] or fully ionized [11]. These conditions require working at extreme pH, which may denature proteins. Another approach to control protein adsorption and EOF is to add compounds that compete with the analytes for interaction sites on the capillary wall. Although the family of oligoamines has significantly reduced protein-wall interactions at acidic pH, their use at alkaline pH is hindered by the deprotonation of the amino groups, deteriorating their ability to suppress protein adsorption on the silica wall ([12, 13] and references therein).

Coating the inner capillary wall with polymeric materials that are either chemically bonded to or physically adsorbed on the capillary surface has been a popular approach for controlling EOF and protein adsorption. The polymeric coatings sterically mask the silanol groups, thus minimizing their availability for interacting with proteins. Furthermore, the polymeric layer suppresses EOF by increasing the solution viscosity in the electric double layer near the capillary surface, without affecting the bulk solution viscosity [7]. Many polymers have been used as covalent capillary coatings [7, 10, 14–23], which are generally stable over a wide range of pH, offering flexibility in the choice of separation conditions, and do not require regeneration between runs. However, the procedures adopted in preparing covalent coatings are laborious and time-consuming. Furthermore, the coating procedure typically requires an *in situ* polymerization step that is difficult to control, affecting the reproducibility of the quality of the prepared coating. Also, *in situ* polymerization can clog the capillary by producing very viscous polymer solution in the lumen of the capillary that sometimes cannot be flushed out. Thus, in a number of applications including capillary arrays and microchip electrophoresis, the difficulties associated with the production of covalent coatings are considerable and have a high impact on cost.

Adsorbed wall coating is an attractive alternative to covalent coating due to the simplicity and speed of the coating protocol. Most adsorbed coatings are prepared by adsorbing the polymer from a dilute solution onto the inner capillary surface, which obviates the need for organic solvents and viscous solutions. Furthermore, the preparation of an adsorbed coating can be automated and reproduced due to *a priori* knowledge of the coating polymer properties. Gilges *et al.* [24] showed excellent separation of proteins using adsorbed poly(vinyl alcohol) (PVA)-coated capillaries. However, this coating scheme can be used only at pH less than 5. For separations up to pH 9, thermal pretreatment at 140°C was needed to convert PVA to a water-insoluble state and permanently immobilize it on the capillary surface. Only 40 runs were possible at pH 8.5 without loss of efficiency before the performance abruptly decreased. Busch *et al.* [25] reported a method to shield the surface silanols by physically adhering a thin film of cellulose acetate onto the capillary wall, by flushing an acetone solution of cellulose acetate followed by drying with helium gas. A polyethylene oxide (PEO) adsorbed coating can be prepared by flushing the capillary with HCl to fully protonate the wall surface prior to introducing the polymer solution [26]. Regeneration of the coating is needed before each protein separation run. The PEO coating did not efficiently suppress EOF at pH 8.2 [27], limiting its application for acidic protein separations. Verzola *et al.* [28] compared the performance of hydroxypropylmethylcellulose (HPMC), hydroxyethylcellulose (HEC), PVA, and poly-*N,N*-dimethylacrylamide (PDMA) as quenchers of the interaction of myoglobin with the silica wall. The polymers HEC, HPMC, and PVA could inhibit protein adsorption by, at most, 50%, whilst PDMA was much more efficient, inhibiting protein adsorption on the silica surface by 85%. However, the hydrophobic nature of PDMA could provide more adsorption sites for more hydrophobic proteins, rendering it unsuitable for protein separation [15, 29]. In another study, thermally immobilized HEC was used for capillary isoelectric focusing of proteins [30]. For more recent advances in the field of polymeric wall coatings, the reader is referred to the review published by Doherty *et al.* [31].

Adsorbed coatings formed by hydrophilic polymers, such as methylcellulose and PVA, can be easily removed from the capillary wall simply by washing with water [29, 32]. More hydrophobic polymers, such as PDMA [29, 33] and polyvinylpyrrolidone [34], have a higher affinity for the wall surface, forming more stable coatings that cannot be

easily desorbed by an aqueous phase. Water acts as a somewhat "poor" solvent for these polymers, and hydrophobic interactions with the siloxane groups of the skeleton structure of the silica surface may favor polymer adsorption [35]. Doherty *et al.* [36] found that moderately hydrophobic polymers, such as PDMA, adsorb on the capillary surface in a loopy configuration that spans the thickness of the electrical double layer, efficiently suppressing EOF. More hydrophobic polymers, such as poly-*N,N*-diethylacrylamide, form denser but thinner polymer layers, that do not efficiently suppress EOF. Some studies [26, 29, 37] have shown that hydrogen bonding between polymer chains and surface silanols may play a role in the adsorption mechanism and the stability of some polymer coatings. Thus, the hydrophilic-hydrophobic balance of the polymer, its potential for hydrogen bonding with the wall, and the nature of the solvent apparently dictate the adsorption properties of the polymer and the thickness of the coating layer.

An ideal coating for protein separation by CE would combine the ease of production of a dynamic, adsorbed coating with the long-term stability and performance typical of covalently coated capillaries. In a previous paper [38], we have developed poly-*N*-hydroxyethylacrylamide (PHEA) (polyDuramide™) as a hydrophilic, polymeric coating and as a sieving matrix for applications in DNA sequencing by CE. This polymer was also employed as a wall coating in mutation detection applications using linear polyacrylamide (LPA) sieving matrices [39]. PHEA was shown to uniquely combine high hydrophilicity and capillary coating ability, properties that are highly desirable for protein separations by CE. The capillary coating ability suppresses EOF and minimizes electrostatic interactions whilst the polymer hydrophilicity eliminates hydrophobic interactions between the polymeric coating and analyte. Acrylamide-based polymers with structures closely related to that of PHEA have been reported as wall coatings for CE of proteins [40]. In this paper, we investigate the performance of a PHEA adsorbed coating for protein separations by CE. This performance is compared to that of a PDMA adsorbed coating and a polyacrylamide covalent coating. The stability of performance of the PHEA wall coating is evaluated under acidic and basic conditions. Finally, high-speed, high-efficiency protein separation is demonstrated using PHEA as a capillary wall coating.

2 Materials and methods

2.1 Chemicals

N-Hydroxyethylacrylamide (HEA), trade name Duramide™, was obtained from Cambrex Bio Science Walkersville (Walkersville, MD, USA). Acrylamide, Tris, and ammonium persulfate (APS) were from Amresco (Solon, OH, USA). *N,N*-Dimethylacrylamide (DMA) was from Monomer-Polymer and Dajac Labs (Feasterville, PA, USA). V-50 initiator (2,2'-azobis (2-amidinopropane) dihydrochloride) was from Wako Chemical USA (Richmond, VA, USA). Benzyl alcohol was from Aldrich (Milwaukee, WI, USA). The proteins used in this study, which include cytochrome *c* (horse heart), myoglobin (horse skeletal muscle), ribonuclease A (bovine pancreas), lysozyme (chicken egg white), α-chymotrypsinogen A (bovine pancreas), α-lactalbumin (bovine milk), β-lactoglobulin A and B (bovine milk), and trypsin inhibitor (soybean), were purchased from Sigma (St. Louis, MO, USA) and were used as received. *N,N,N',N'*-tetramethylethylenediamine (TEMED), acetic acid, sodium hydroxide, methanol, and hydrochloric acid were from Fisher Scientific (Pittsburgh, PA, USA).

2.2 Polymer synthesis and characterization

Polymers of HEA and DMA were synthesized by free-radical polymerization in aqueous solution. The initial monomer concentration was 5% w/w and the reaction was thermostatted at 47°C. The solution was deoxygenated by continuous bubbling of nitrogen gas for 2 h. Then, 0.02% w/w V-50 initiator was added and the polymerization was allowed to proceed overnight. The synthesized polymer was then purified by dialysis against deionized water using Spectra/Por cellulose ester dialysis membranes (Spectrum, Gardena, CA, USA), with a molecular mass cutoff of 100 kDa. The purified polymer was then lyophilized and recovered. The weight-average molar mass of the synthesized polymers was determined by gel permeation chromatography (GPC)-multiangle laser light scattering (MALLS). Each polymer sample (100 μL) was injected into the tandem GPC system at a concentration of ~0.5 mg/mL. Each sample was fractionated by passing through a Waters 2690 Alliance Separations Module (Milford, MA, USA) with Shodex (New York, NY, USA) OHpak columns SB-806 HQ, SB-804 HQ, and SB-802.5 HQ connected in series. The flow rate through the columns was 0.35 mL/min and the mobile phase consisted of 100 mM NaCl, 50 mM NaH_2PO_4, and 200 ppm NaN_3. Effluent from the GPC system flows directly into a DAWN DSP Laser Photometer and Optilab DSP Interferometric Refractometer connected in series (both, Wyatt Technology, Santa Barbara, CA, USA), where scattered laser light intensity as a function of angle and refractive index were measured. Tandem GPC-MALLS data were processed using ASTRA software from Wyatt Technology. ASTRA was used to calculate the weight-average molar mass for each analyzed polymer.

2.3 Capillary coating and EOF measurement

Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) used throughout this study had the dimensions of 27 cm total length, 20 cm effective length, and 50 μm ID. To determine the ability of a polymer to suppress EOF as an adsorbed coating, the bare capillary was coated with PDMA or PHEA according to the following protocol: the capillary was washed with 1.0 M HCl for 15 min. The capillary was then flushed with 0.1% w/v polymer solution for 15 min. The preparation of LPA covalently coated capillaries was carried out by flushing each capillary with 1 M HCl for 30 min, then with water for 15 min. Then, the capillary was flushed with 1 M NaOH for 30 min, and rinsed with water for 15 min. A 0.4% v/v 3-methacryloxypropyltrimethoxysilane in 0.4% v/v acetic acid was flushed continuously through the capillary overnight. The capillary was then rinsed with water for 15 min. Coating the capillary was performed by *in situ* polymerization of deoxygenated 4% acrylamide solution, initiated by 1 μL TEMED and 10 μL of 10% APS per mL of acrylamide solution. The polymerization was allowed to proceed overnight. The polymer solution was flushed out with water and the capillary was dried with air. A Beckman P/ACE 5000 (Beckman-Coulter, Fullerton, CA, USA) instrument was used to determine the mobility of EOF in fused-silica capillaries at 25°C. EOF mobility was measured at different pH in following buffers: 25 mM phosphate buffer was used for pH 3 and pH 7, 25 mM acetate buffers were used at pH 4.4 and pH 6, and 25 mM Tris-Bicine buffer was used at pH 8.4. The EOF mobility was measured in the polymer-coated capillaries according to the protocol of Williams and Vigh [41].

2.4 Protein separation by CE

Protein separation by CE was carried out on P/ACE 5000 instrument. Acidic (anionic) protein mixtures, composed of 0.1 mg/mL trypsin inhibitor, β-lactoglobulin A and B, and α-lactalbumin, were injected by 0.5 psi pressure for 2 s at the cathodic end. Separation was carried out in 25 mM Tris-Bicine buffer, pH 8.4, at 500 V/cm and 25°C. Basic (cationic) protein mixtures, composed of 0.1 mg/mL of each protein, were injected by pressure for 2 s at the anodic end, and separated in 25 mM acetate buffer, pH 4.4, at 500 V/cm, unless stated otherwise. All proteins were detected by UV absorbance at 214 nm.

3 Results and discussion

3.1 Suppression of EOF

In this study, we investigate the application of the hydrophilic polymer, PHEA, as an adsorbed capillary coating for protein separation by CE, and compare the separation performance with that of polyacrylamide covalent coating and PDMA adsorbed coating. The weight-average molar masses of the PHEA and PDMA used throughout this study are 5.2 and 4.4 MDa, respectively. We found that the efficiency of suppression of EOF by a PHEA coating depends strongly on the capillary pretreatment protocol prior to the coating step. Four different capillaries were subjected to four different pretreatment protocols, composed of the following flushing steps: (i) 1 M NaOH, then water, (ii) 0.1 M HCl, then water, (iii) 0.1 M HCl, or (iv) 1 M HCl. Each flushing step was for 15 min. Then, each capillary was flushed with 0.1% w/v PHEA solution for 15 min and the EOF was measured afterwards in 25 mM Tris-Bicine buffer, pH 8.4. No attempt was made to optimize the duration of each capillary washing step to decrease the coating preparation time. Table 1 summarizes the residual EOF in the capillaries subjected to the different pretreatment protocols. The best EOF suppression performance was obtained by protocol (iv), that is the 1 M HCl pretreatment just before introducing the polymer solution. Highly acidic pretreatment is needed to protonate the silanol groups on the silica surface, increasing their availability for hydrogen bonding with the carbonyl, amide or hydroxyl groups in PHEA. Flushing the capillary with 1 M HCl was more efficient than with 0.1 M HCl, regarding protonating the surface. Based on these observations, it can be concluded that hydrogen bonding between PHEA and wall surface plays a critical role in the formation of a stable adsorbed coating for EOF suppression.

Table 1. Effect of capillary pretreatment on EOF suppression by PHEA adsorbed coating

Pretreatment	EOF[a)] ($\times 10^{10}$ m^2/Vs)
(i) 1 M NaOH, water	689.5±24.8
(ii) 0.1 M HCl, water	10.35±1.12
(iii) 0.1 M HCl	9.71±0.75
(iv) 1 M HCl	1.76±0.09

a) Reported values represent the average ± standard deviation of three runs.

Figure 1 shows the effect of pH on EOF mobility in PHEA-coated capillaries and compares it with what is observed for an uncoated capillary. When the same capillary was used at different pH, the EOF mobility increased significantly as the pH was increased from 3 to 8.4. In contrast, when PHEA-coated capillaries were each used at a given pH, the PHEA coating is shown to be more stable over the range of pH commonly used for protein separations. The PHEA coating resulted in about two orders of magnitude reduction of EOF compared to the uncoated

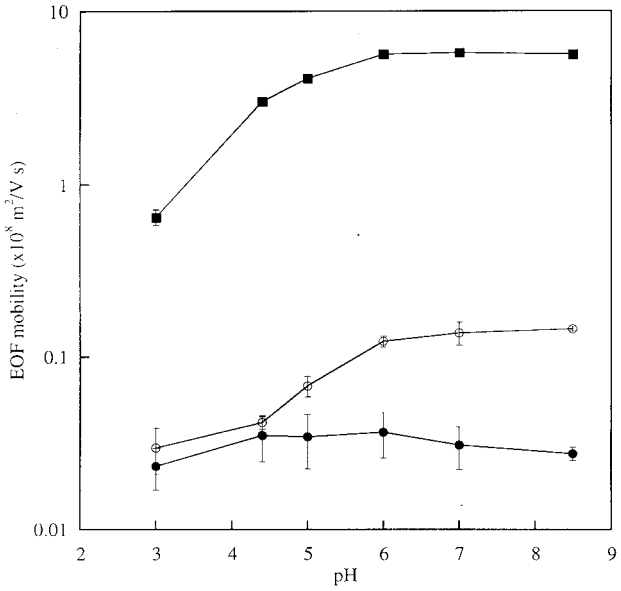

Figure 1. Effect of changing pH on EOF mobility measured (■) in a bare fused-silica capillary, and (○) in a single PHEA-coated capillary (error bars correspond to standard deviation of three EOF measurements). The data points shown as (●) represent the EOF mobility measured in different PHEA-coated capillaries at different pH, where each capillary was used to measure the EOF mobility at one pH value (error bars correspond to standard deviation of three EOF measurements in each capillary).

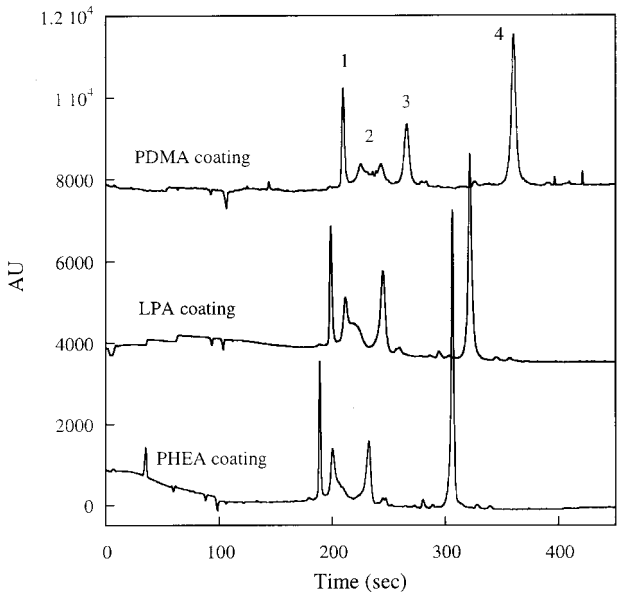

Figure 2. Acidic protein separation in adsorbed PHEA-, adsorbed PDMA-, and covalent LPA-coated capillaries. Separation conditions: 25 mM Tris-Bicine buffer, pH 8.4; 500 V/cm; 10 μA; 25°C; 20 cm separation distance. Sample: 0.1 mg/mL protein. Injection: 0.5 psi, 2 s. Peak identification: 1, trypsin inhibitor; 2, β-lactoglobulin A; 3, β-lactoglobulin B; 4, α-lactalbumin. Peaks identified by spiking.

capillary. The excellent EOF suppression capability of adsorbed PHEA provides flexibility in the choice of the operating pH for a given separation.

3.2 Protein-polymer coating interactions

Proteins can interact reversibly or irreversibly with a polymer-coated silica surface in several ways, including electrostatic interactions with unmasked, ionized silanol groups, hydrogen bonding with surface-bound donor or acceptor moieties, and hydrophobic interactions between protein surface-bound nonpolar patches and polymer hydrophobic regions. To investigate the effect of interactions between protein analytes and polymer coatings, the separation of model proteins in capillaries coated with different acrylamide-based polymers, including PHEA, PDMA, and LPA, was systematically evaluated. An acidic protein test mixture containing 0.1 mg/mL trypsin inhibitor, β-lactoglobulins A and B, and α-lactalbumin was separated in capillaries coated with these three different polymers in 25 mM Tris-Bicine buffer, pH 8.4. At this pH, the Si–OH groups are largely deprotonated, and the EOF is maximized by the use of low ionic strength buffer. In addition, at basic pH, low-p*I* proteins become negatively charged and thus are repelled by the negatively charged silanols. This reduces electrostatic interactions, allowing a better evaluation of other kinds of interaction with the surface. Figure 2 compares the protein separation electropherograms, which show that the β-lactoglobulin A peak was split into two small peaks in a PDMA-coated capillary, and badly tailed in an LPA-coated capillary, whilst the best peak shape for that and other proteins was obtained in a PHEA-coated capillary. Table 2 compares migration time reproducibility and peak efficiency of the acidic proteins separated in the three polymer-coated capillaries. For the proteins studied, the migration time increases while the peak efficiency decreases with increasing polymer hydrophobicity, which increases in the order PDMA > LPA > PHEA [38]. Both PHEA and LPA coatings demonstrated excellent reproducibility of migration times of the three proteins, with relative standard deviation (RSD) values less than 0.65%. On the contrary, migration time reproducibility was poorer in PDMA-coated capillaries, with RSD values about one order of magnitude greater than in PHEA- or LPA-coated capillaries. Chiari *et al.* [17] and Gelfi *et al.* [15] reported a similar dependence of migration time reproducibility on polymer hydrophilicity.

Table 2. Migration time reproducibility (n = 50) and peak efficiency of acidic proteins separated in polymer-coated capillaries

Protein	PHEA-adsorbed coating			PDMA-adsorbed coating			LPA covalent coating		
	t (min)	RSD (%)	N_p ($\times 10^{-5}$ m^{-1})	t (min)	RSD (%)	N_p ($\times 10^{-5}$ m^{-1})	t (min)	RSD (%)	N_p ($\times 10^{-5}$ m^{-1})
Trypsin inhibitor	3.13	0.25	3.08	3.48	2.69	2.15	3.32	0.18	2.94
β-Lactoglobulin B	3.85	0.35	1.05	4.40	3.49	0.90	4.10	0.29	0.98
α-Lactalbumin	5.06	0.47	4.68	5.95	5.95	2.46	5.40	0.64	3.55

Comparing protein peak efficiencies produced by the different capillary coatings investigated in this study, the highest peak efficiencies were obtained in the PHEA-coated capillary indicating minimal interactions between proteins and the polymer coating. The slower migration times and lower efficiencies obtained in LPA-coated capillaries are most likely due to weak interactions, primarily hydrogen bonding between proteins and LPA [42, 43]. Reversible hydrogen bonding interactions between proteins and hydrophilic polymer coatings temporarily retain the proteins at the capillary surface, resulting in retardation of the analyte and in band broadening, similar to what happens in chromatographic separations. When a PDMA coating is used, hydrophobic interactions between hydrophobic protein patches and hydrophobic regions of the polymer are stronger. These interactions retain the proteins at the surface for longer times, resulting in increased retardation and decreased efficiencies. When the analyte-polymer interactions are strong enough, as in the case of β-lactoglobulin A separated in a PDMA-coated capillary, a significant portion of the protein sample is adsorbed to the capillary wall, giving a sample electropherogram that has two peaks. The first is due to the sample remaining in solution, and the second caused by the desorption of analyte. It is initiated by a drop in solution concentration immediately after the passage of the first peak [44]. Splitting of the β-lactoglobulin A peak into two peaks has been observed by Chiari *et al.* [29]; this protein is generally known to exhibit low peak efficiency in CE due to its relatively strong interactions with the surface.

Separation of alkaline proteins at acidic pH is known to be a sensitive test for shielding of silanol charges by polymer coatings. Irreversible protein adsorption on the capillary surface due to hydrophobic and/or electrostatic interactions between proteins and a wall coating usually results in low sample recovery and a loss of detection sensitivity. A basic protein mixture, composed of 0.1 mg/mL cytochrome *c*, ribonuclease A, and myoglobin, was used as a test mixture. Figure 3 shows the effect of pH on the separation of three basic proteins in a PHEA-coated capillary. The peaks due to ribonuclease A and myoglobin could only be baseline-resolved at pH 4.4. Protein separation at pH 6 was not successful in resolving and detecting all protein peaks. With increasing pH, migration times increase due to the decrease of the net positive charge on the proteins. This increases the availability of the hydrophilic groups on the protein surface for hydrogen bonding with donor and acceptor groups on the PHEA coating, resulting in a loss of peak efficiency and resolution.

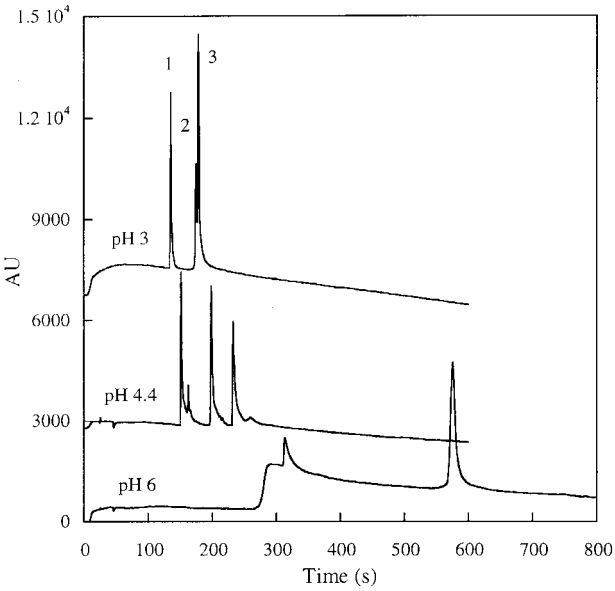

Figure 3. Effect of pH on the separation of basic proteins in PHEA-coated capillaries. Separation conditions: 500 V/cm; 25°C; 20 cm separation distance. Sample: 0.1 mg/mL protein. Injection: 0.5 psi, 2 s. Peak identification: 1, cytochrome *c*; 2, ribonuclease A; 3, myoglobin. Peaks identified by spiking.

At pH 3, the majority of silanol groups will be protonated, and thus, the capillary surface will be practically uncharged [10]. Under these conditions, basic protein separations become possible in a bare silica capillary due to the elimination of electrostatic interactions with the capillary surface. The peaks due to ribonuclease A

Table 3. Migration time reproducibility (n = 20) and peak efficiency for basic proteins separated in PHEA-coated and in uncoated capillaries

Protein	PHEA-adsorbed coating			Bare fused-silica capillary		
	t (min)	RSD (%)	N_p ($\times 10^{-5}$ m^{-1})	t (min)	RSD (%)	N_p ($\times 10^{-5}$ m^{-1})
Trypsin inhibitor	2.28	0.17	2.14	2.54	2.75	1.44
β-Lactoglobulin B	2.92	0.18	1.03	N/A	N/A	N/A
α-Lactalbumin	2.99	0.22	2.23	3.48	3.93	0.63

N/A, not available

and myoglobin could not be resolved and eluted as a single peak (data not shown). Table 3 shows that protein migration was faster, more reproducible, and more efficient in a PHEA-coated capillary than in a bare silica capillary. The comparison highlights the role played by the PHEA coating in improving separation efficiency and resolution by minimizing interactions between proteins and the bare capillary surface.

To investigate the effect of the polymer coating on protein separation, the basic protein test mixture was separated in PHEA-, LPA- and PDMA-coated capillaries. The separations were carried out at pH 4.4, a solution condition which maximizes the electrostatic interactions between proteins and silanol charges [45]. During the experiments with all three coatings under these conditions, it was noticed that there was a gradual, run-to-run improvement in the elution profile with respect to peak intensity, detection sensitivity and peak shape. Generally, the first few runs failed to produce peaks. Then, signals were detected, but the peaks were badly tailed. After few more runs, the peaks become more uniform, sharper and more reproducible. The attainment of steady-state, high-performance separation was fastest in PHEA-coated capillaries and slowest in PDMA-coated capillaries (data not shown). Irreversible protein adsorption could be due to an inhomogeneous coating of the capillary, which could leave some areas of the fused silica uncovered, causing protein adsorption. Another reason could be that the coating layer had a small thickness, permitting the electric charges of the silica surface to adsorb the proteins on top of the polymeric layer. Even though a polymer coating can efficiently suppress EOF, analytes can still be attracted to the surface charges, which will have an influence on the overall separation performance [46]. In addition to electrostatic interactions, it is likely that basic proteins adsorb irreversibly on the PDMA coating by hydrophobic interactions.

The steady-state performance for protein separation in the three "preconditioned" polymer-coated capillaries was compared. In PDMA-coated capillaries the baseline does not return to zero (data not shown) due to irreversible protein adsorption at the detection window [9, 44]. Table 4 summarizes the average migration times, RSD and peak efficiencies of basic proteins separated in the three polymer-coated capillaries. Excellent reproducibility of migration times was obtained in PHEA- and LPA-coated capillaries, whilst the variability of migration times was much higher in PDMA-coated capillaries. Cifuentes *et al.* [47] derived a simple expression for the quantification of protein interaction with the capillary wall:

$$\frac{1}{t} = \frac{\mu_p}{l}E - \frac{\mu_p Q_c}{l\varepsilon x^2} \quad (1)$$

Table 4. Migration time reproducibility (n = 50) and peak efficiency of basic proteins separated in polymer-coated capillaries

Protein	PHEA-adsorbed coating			PDMA-adsorbed coating			LPA covalent coating		
	t (min)	RSD (%)	N_p ($\times 10^{-5}$ m^{-1})	t (min)	RSD (%)	N_p ($\times 10^{-5}$ m^{-1})	t (min)	RSD (%)	N_p ($\times 10^{-5}$ m^{-1})
Cytochrome *c*	2.49	0.33	2.13	2.52	2.13	1.65	2.47	0.31	2.01
Ribonuclease A	3.27	0.37	2.01	3.28	3.68	1.72	3.19	0.43	2.31
Myoglobin	3.76	0.62	2.38	3.81	4.27	1.51	3.66	0.41	2.66

Table 5. Interaction strengths of model proteins with different polymer-coated capillaries, estimated from the slope and intercept of Eq. (1)

Protein	p*I*	Slope ($\times 10^5$)			Intercept ($\times 10^5$)		
		PHEA	PDMA	LPA	PHEA	PDMA	LPA
Cytochrome *c*	10.6	1.34	1.48	1.32	−2.37	−15.57	−2.65
Ribonuclease A	9.3	1.04	1.18	1.03	−2.02	−14.40	−2.51
Myoglobin	7.0	0.90	1.05	0.89	−1.92	−14.23	−1.83

where t is the protein migration time, μ_p is the protein electrophoretic mobility, E is the electric field strength, l is the separation distance, Q_c is the electric surface charge, ε is the dielectric constant of the buffer, and x is the average distance between capillary surface and protein. A plot of $1/t$ *vs.* E gives a straight line for each protein, where the slope is a function of the protein electrophoretic mobility, and the intercept is a measure of the degree of electrostatic interactions between the capillary wall and the analyte. The higher the absolute value of the intercept, the stronger are the electrostatic interactions with the wall. The intercept is a function of both the analyte mobility and analyte surface adsorption. Thus, the value of the intercept depends on the buffer ionic strength, pH, and polymer coating, making it difficult to compare data reported in the literature taken with different buffer systems [20, 21, 47]. To our knowledge, here we report the first study that compares the parameters of Eq. (1) as a function of hydrophilicity of polymer coating, using the same buffer and pH, to investigate the effect of the wall coating on protein-wall interactions. Table 5 summarizes the values of the slope and intercept for the three model proteins investigated. As noted in previous reports [20, 21], for a given polymer coating, there is a strong correlation between the protein p*I* value and the intercept value. The higher the p*I*, the greater the charge on the protein at given pH, and the stronger the electrostatic interactions with the surface charges, and hence, the lower its peak efficiency. Furthermore, the intercept values in PHEA- and LPA-coated capillaries are comparable, and are about one order of magnitude smaller than those in PDMA-coated capillaries. This is not surprising considering the relatively high residual EOF mobility in PDMA-coated capillaries, given in Table 6, which indicates that surface charges are only partially shielded by the PDMA coating. For each protein, the peak efficiency correlates with the absolute value of the intercept in the three polymer-coated capillaries, indicating that interactions are most significant in PDMA-coated capillaries, thus resulting in the smallest peak efficiencies. The PHEA coating has minimal interactions with the proteins, giving the highest peak efficiencies.

Table 6. Comparison of EOF in different polymer-coated capillaries at different pH, initially and after 50 consecutive protein separation runs

Coating	EOF at pH 4.4 ($\times 10^{10}$ m^2/Vs)		EOF at pH 8.4 ($\times 10^{10}$ m^2/Vs)	
	Initial	Final	Initial	Final
PHEA	5.34	6.97	3.68	7.34
PDMA	4.93	17.38	6.47	16.87
LPA	4.76	6.30	3.69	7.83

Although the protein peak efficiencies reported in this study are significantly lower than the efficiencies reported for some coatings, such as PVA [24] and cellulose acetate [25], they match efficiencies reported by many other adsorbed or covalent coatings [14, 16, 17, 20, 22, 45, 48–52]. However, a fair comparison of the protein separation performance of different coatings cannot be done unless all other variables of the electrophoresis system, including detection system, injection conditions, protein samples quality, capillaries and buffers, are fixed. In this study, we have shown that, under the same conditions, the adsorbed PHEA coating can provide protein separation performance, at least, as good as that of covalent polyacrylamide coating, which is commonly used in commercial electrophoresis instruments. Furthermore, the ease of preparation of PHEA adsorbed coating is another advantage for this coating.

3.3 Stability of the PHEA coating

The stability of the PHEA coating under acidic and basic separation conditions was evaluated. Figure 4 and Table 7 show the change in protein migration times and peak efficiencies, respectively, in consecutive protein separation runs at basic pH. Excellent reproducibility was obtained for up to 270 runs, after which there was a dramatic change in migration times and drop in peak efficiency. At basic pH, ionization of silanol groups decreases the hydrogen bonding sites on the capillary surface, thus

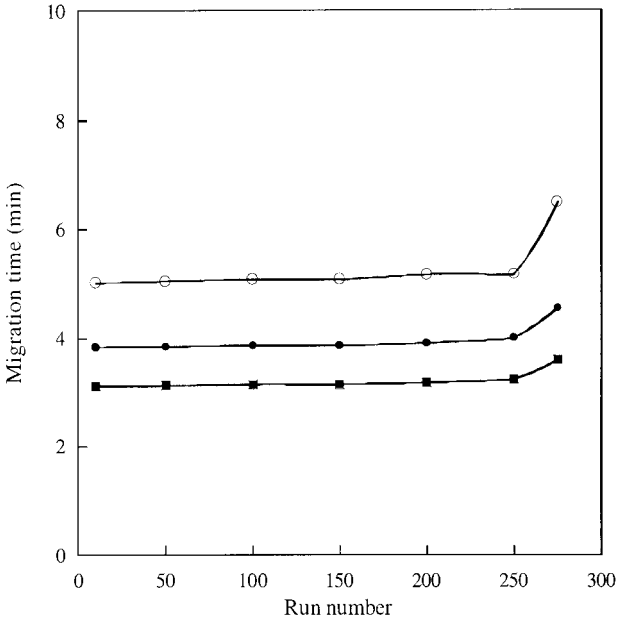

Figure 4. Change of migration time of (○) trypsin inhibitor, (●) β-lactoglobulin B, and (■) α-lactalbumin with run number in PHEA-coated capillary. Conditions: same as in Fig. 2.

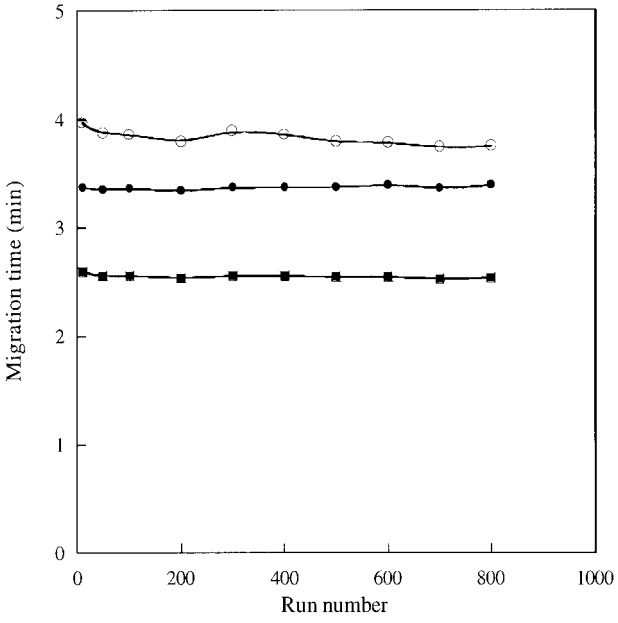

Figure 5. Change of migration time of (○) cytochrome *c*, (●) ribonuclease A, and (■) myoglobin with run number in PHEA-coated capillary at pH 4.4. Other conditions are same as in Fig. 3.

Table 7. Change of acidic protein efficiency in consecutive separation runs in a PHEA-coated capillary

Run number	Peak efficiency ($\times 10^{-5}$ m^{-1})		
	Trypsin inhibitor	β-Lacto-globulin B	α-Lactal-bumin
10	3.14	0.77	5.62
50	2.40	0.85	3.51
100	2.00	0.98	2.82
150	2.02	1.05	2.82
200	2.01	1.00	2.88
250	1.77	1.09	2.71
275	0.80	0.68	0.56

destabilizing the PHEA coating. The addition of 0.1% PHEA to the running buffer significantly improved the coating stability at basic pH [38]. Several attempts to regenerate the coating and regain the high-efficiency separations were unsuccessful, indicating that the coating is difficult to remove from the wall. At acidic pH, the PHEA coating was much more stable and its performance was reproducible for more than 800 consecutive runs. The changes in protein migration times, peak efficiencies, and EOF are shown in Fig. 5 and Table 8. The high-efficiency separation and stability of the adsorbed PHEA layer prove the high quality of this coating for protein analysis by CE.

Table 8. Change of EOF and basic protein peak efficiency in consecutive separation runs in a PHEA-coated capillary

Run	EOF	Peak efficiency ($\times 10^{-5}$ m^{-1})		
	($\times 10^{10}$ m^2/Vs)	Cyto-chrome *c*	Ribo-nuclease A	Myo-globin
10	1.42	1.36	1.76	0.96
50	2.81	2.00	1.82	2.16
100	3.62	2.17	1.97	2.55
200	5.01	2.50	2.21	2.68
300	7.07	2.60	2.73	3.25
400	3.65	2.44	1.96	3.02
500	0.94	2.66	2.21	3.87
600	0.97	2.66	1.69	3.19
700	2.77	2.95	1.97	3.61
800	6.27	2.82	2.04	3.25

4 Concluding remarks

In this study, the application of PHEA as an adsorbed coating for silica microchannels has been demonstrated. Preparation of the PHEA coating is fast and simple, allowing the incorporation of the procedure in automated CE instruments and microfluidic chips. PHEA offers a unique combination of hydrophilicity and adsorbed capillary-coating ability, which are important for improving separation efficiency and reducing capillary cost. Protein sepa-

ration in PHEA-coated capillaries was accomplished with excellent reproducibility and moderate-to-good efficiency, due to minimal interactions between proteins and the polymer coating. Furthermore, the long-term stability of the PHEA coating in consecutive protein separation runs has demonstrated the suitability of PHEA coating for high-throughput electrophoretic protein separations. This work demonstrates the excellent potential of PHEA as a microchannel wall coating for protein and other biomolecule analysis.

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