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Highly efficient protein separations in high-performance capillary electrophoresis, using hydrophilic coatings on polysiloxane-bonded columns[1]

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Abstract

Three methods for preparing hydrophilic coatings on polysiloxane bonded CElect H-type capillary electrophoresis columns have been shown. The polyalkylsiloxane-bonded phase is the first coating layer on the capillary surface, and nonionic surfactant, hydrophilic polymer, or polymer surfactant, adsorbed onto this first layer through hydrophobic interactions, forms the second coating layer. The resultant capillary surfaces are inert, hydrophilic, and suitable for highly efficient protein separations. The effectiveness and applicability of these capillary surface modification methods were tested for the separations of a variety of proteins over a wide range of buffer pH values under different capillary electrophoretic operation modes.

Keywords: Hydrophilic coatings; Coating methods; Columns; Proteins.

1. Introduction

Rapid development of biotechnology and molecular biology has increased the demand for protein separations. Nowadays, the requirements of a technique used for protein separation generally include high resolution, fast analysis, high sensitivity, small sample consumption, and ease of coupling with structure elucidation techniques. Capillary electrophoresis (CE), with its various modes of operation, has shown great potential to meet these requirements and has proven to be a powerful and versatile separation technique. In CE, a fused-silica capillary column is still the most commonly used separation channel, due to its flexibility, uniform inner diameter, and excellent optical properties. Silanol groups on the inner surface of the fused-silica tubing, however, are ionized in the presence of most of the running buffers used in CE. The negatively charged surface, accompanied by hydrophobic interactions on the surface, creates adsorption problems in CE separation of proteins, which result in tailing peaks and poor reproducibility. Thus, attempts to solve this problem have been emphasized in the last decade, in order to make CE a more suitable technique for protein separation.

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Several approaches have been suggested to overcome the problem of sample adsorption in performing protein separation in CE: (1) use a high or low buffer pH [1,2], a high buffer concentration [3], or a zwitterionic buffer [4]; (2) make dynamic modifications to the capillary surface by using buffer additives such as charged small molecules [5,6] and polymers [7,8], or nonionic polymers [9]; and (3) create a permanent coating on the capillary surface by bonding a small molecule [10,11], polymer [12–27] onto the surface. While some extent of success has been achieved for each of these approaches, it is generally agreed that the permanent modification of the capillary surface provides the most effective way to eliminate adsorption of various proteins, as well as to control the electroosmotic flow which is a consequence of the charged capillary surface.

Covalently bonded capillary surface coatings can be generally classified as hydrophilic or hydrophobic. The interaction of a hydrophilic polymer with a protein molecule is negligible when using common aqueous buffers, thus this kind of coating is ideal for protein separation in CE. Typical hydrophilic coatings include polyethylene glycol [12–15], linear and cross-linked polyacrylamide [16–19], polyvinylpyrrolidone [20], cellulose derivatives [21,22], polyvinyl alcohol (PVA) [23], poly(methyl glutamate) [24], and other hydrogel polymers [25]. In practice, however, hydrophilic coatings are difficult to prepare in a stable, reproducible, and uniform manner. Hydrophobic coatings have been prepared by silane bonding, using HPLC stationary phase preparation methods, or by polysiloxane bonding and cross-linking according to the column preparation methods used in capillary gas chromatography and supercritical fluid chromatography. This latter coating procedure, used in the manufacturing of Supelco's CElect H-type capillary electrophoresis columns produces uniform and stable hydrophobic coatings [26,27]. The polysiloxane coatings can reduce electroosmotic flow, but not eliminate the flow. The reason is that not all silanol groups can be reacted (not 100% yield). The unreacted silanol groups can still be ionized and produce negatively charged inner surface, but they are covered by the polysiloxane film. Although these polysiloxane coatings are very useful for deactivating the capillary surface and reducing electroosmotic flow, hydrophobic interactions between the nonpolar surface surface coatings and protein molecules have stood in the way of achieving efficient separations.

Towns and Regnier [28] reported a method involving derivatization of the silica surface with octadecylsilane, followed by the deposition of a layer of nonionic surfactant, for protein separations in CE. In this way, a hydrophilic layer was formed on top of a C_{18} layer coated on the capillary surface, through the hydrophobic interaction between the C_{18} bonded layer and the nonpolar portion of the surfactant. Ng et al. [29] reported a similar combination, in which Pluronic polymers were coated onto capillary tubing previously derivatized with silylating agents. Columns produced through these two methods have been shown to provide high-efficiency protein separations. In our opinion, the silane surface pretreatment methods used in these reports can be improved on to provide more stable and reproducible results, by using polyalkylsiloxane bonding and crosslinking to treat the capillary surface. On the other hand, the tailoring of surfaces through the formation of highly ordered molecular arrangements, or so called chemical assemblies, on solid surface have been shown to be very useful for creating desirable surface properties for a variety of applications [30]. All these reported procedures led us to investigate methods of creating organized hydrophilic assemblies on the capillary surface, as well as solving the problem of hydrophobic interaction of nonpolar bonded phases. The present work will show three ways of building chemical assemblies, based on the use of additives dynamically coated onto permanently coated hydrophobic CEelect columns. A variety of applications demonstrate separations of proteins under different buffer systems and separation modes.

2. Experimental

2.1. Instrumental

Capillary electrophoresis was conducted using either an ABI Model 270A-HT capillary electrophoresis system (Foster City, CA, USA) or a Beckman Model P/ACE 2100 capillary electrophoresis system (Fullerton, CA, USA). Untreated and coated fused silica capillary columns were obtained from Supelco (Bellefonte, PA, USA). The coated columns were: (1) CElect-H50, a weakly hydrophobic C_1 phase, 50 μm I.D.; (2) CElect-H150, a moderately hydrophobic C_8 phase, 50 μm I.D.; and (3) CElect-H275, a highly hydrophobic C_{18} phase, 75 μm I.D. Detection was performed by UV absorbance at 214 nm except where noted. Samples were injected either by vacuum or pressure for a defined period of time.

2.2. Reagents and materials

Tris–HCl, sodium dihydrogen phosphate, methylcellulose (4 000 cP), dextran (average M_r 2 000 000), ampholine (pH 3.5 to 9.5), and all standard proteins were purchased from Sigma (St. Louis, MO, USA). Brij-35 was obtained from Fluka (Ronkonkoma, NY, USA). UCON, a copolymer of polyethylene glycol and polypropylene glycol (50 HB 660), was purchased from Analabs (Norwalk, CT, USA). Tetramethylethylenediamine (TEMED) was obtained from Bio-Rad (Richmond, CA, USA). Supelcoat PS2, a proprietary polymer surfactant, was obtained from Supelco (Bellefonte, PA, USA). Deionized water for preparing buffer solutions and rinsing columns was obtained from a Milli-Q water system (Millipore, Milford, MA, USA). Protein solutions were prepared by dissolving the protein in a mixture of the related buffer and deionized water (1:5) at a concentration of 1 to 3 mg/ml. The concentration of the additives and the column rinse procedure will be described in the text.

3. Results and Discussion

When CE is applied to protein separations, a stable, insert, coated column is demanded for reliable and precise results. As mentioned, a hydrophilic coating is required to reduce the interaction between protein and capillary surface. The preparation of stable hydrophilic coatings, however, involves complicated procedures. Polyalkylsiloxane bonded and cross-linked columns have shown such properties as high stability, reduced and less pH-dependent electroosmotic flow, and good column-to-column reproducibility [26]. Protein adsorption due to hydrophobic interaction is the primary limitation restricting these columns from being widely used for protein separations in CE. We have found, however, that these nonpolar phases can be made suitable for protein separation by assembling an additional hydrophilic layer on top of the nonpolar bonded phase.

3.1. Basic protein separation using Brij-35 on a C_{18} bonded column

This is a modified method based on Towns' and Regnier's report [28]. The column was prerinsed for 1 h with a 0.5% Brij-35 in water solution to coat a hydrophilic layer onto the bonded phase. The buffer also contained 0.001% Brij-35, to prevent the adsorbed surfactant layer from washing off the surface. The separation of three basic proteins is shown in Fig. 1. It should be mentioned that basic proteins are readily adsorbed onto the silanol group-enriched bare fused-silica capillary surface at near neutral buffer pH. By using a bare fused-silica column under the same conditions, no peaks were observed. Apparently, the combination of Brij-35 and a nonpolar C_{18} bonded column produced an inert surface suitable for protein separation. In this separation, the stability of this coating is crucial for reproducible protein separations. Thus, the more stable polyalkylsiloxane bonded phase is recommended over the less stable single bonded phase.

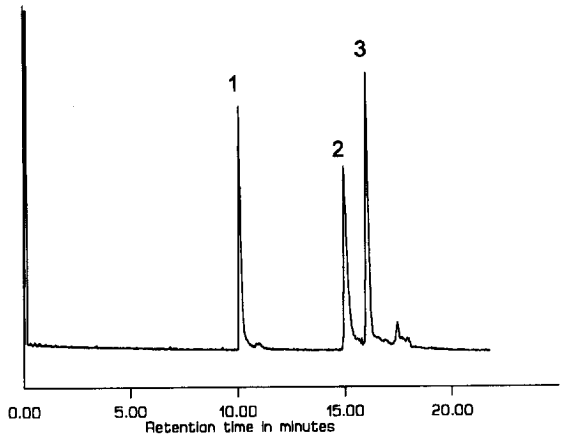

Fig. 1. Basic protein separation, using Brij-35 on a C_{18} bonded column. CElect-H275 bonded column, 10 m*M* sodium phosphate, 0.001% Brij-35, pH 6.0, 30 kV, hydrodynamic injection 2 s, 30°C. Peaks: 1 = lysozyme, 2 = ribonuclease A, 3 = α-chymotrypsinogen A.

3.2. Protein separations using hydrophilic polymer additives on nonpolar bonded phases

Many water soluble polymers or copolymers contain both hydrophobic and hydrophilic regions. Methylcellulose and UCON are typical examples. These kinds of polymers create a hydrophilic coating layer when deposited on a hydrophobic surface. Fig. 2 (top) shows the separation of three basic proteins, using a C_8 column and UCON as the polymer additive. As can be seen, a highly efficient separation is achieved. A bare fused-silica column produced tailing peaks and low efficiency under the same buffer conditions (Fig. 2, bottom). Similar results were obtained when methyl cellulose was used in place of UCON, as shown in Fig. 3. The results shown in Figs. 2 and 3 might be due to adsorption of the polymer additives to the smooth polyalkylsiloxane bonded surface through their hydrophobic portions, leaving their hydrophilic portions exposed to the solutes. Additionally, the polymers are simply dissolved in the buffer solution and they will not interact with protein samples because of their hydrophilic characteristics in the solution.

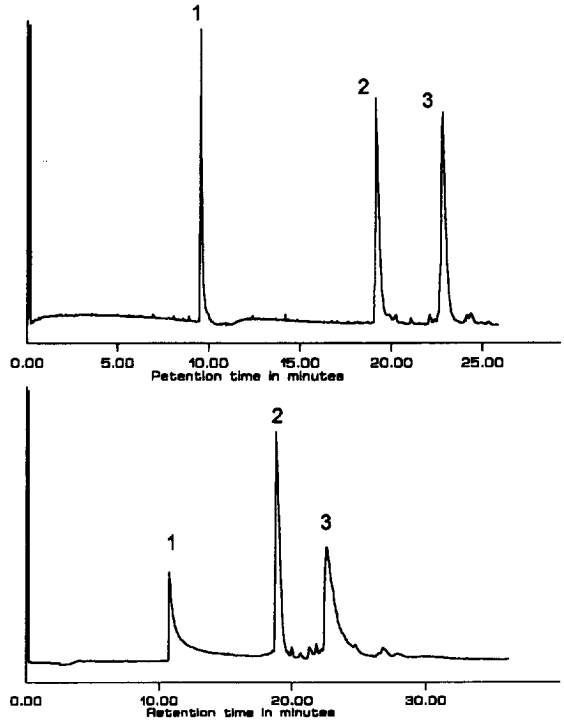

Fig. 2. Basic protein separation, using a bonded column and a bare fused-silica column, with UCON as the polymer additive. CElect-H150 bonded column (top) and CElect-FS50 bare fused-silica column (bottom), 25 m*M* Tris–HCl + 25 m*M* sodium phosphate, pH 6.0, 0.05% UCON, 25 kV, other conditions and peak identifications as in Fig. 1.

The separation of acidic proteins was performed on a C_1 column with added methyl cellulose, as presented in Fig. 4. Since the electroosmotic flow was virtually eliminated by methyl cellulose at concentrations up to 0.1%, the protein sample was injected from cathode to anode. Again, a high-resolution separation was achieved using this method. Although hydrophilic polymers such as PVA and cellulose derivatives have been used to modify the bare fused-silica capillary surface, Gilges and co-workers [9,23] found that only a limited buffer pH range that could be used. It can be seen that the combination of a hydrophilic polymer with a nonpolar bonded phase allows efficient separations of a wide spectrum of protein samples of different p*I* values with the buffer pH near neutral.

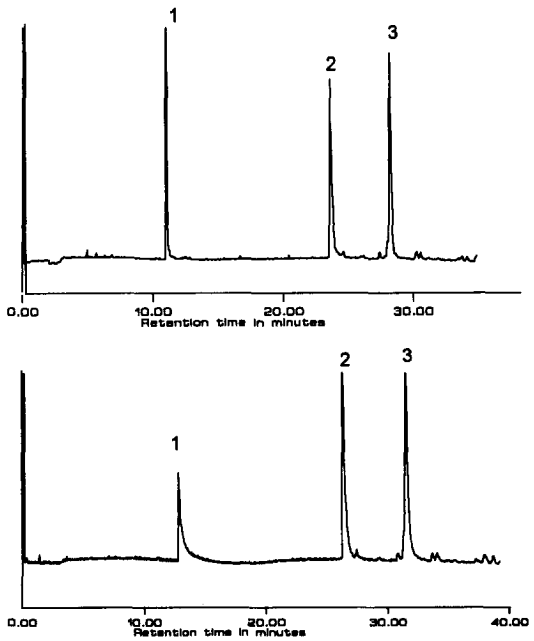

Fig. 3. Basic protein separation, using a bonded column and a bare fused-silica column, with methylcellulose as the polymer additive. CE conditions are the same as in Fig. 2, except that 0.05% methylcellulose was added to the buffer.

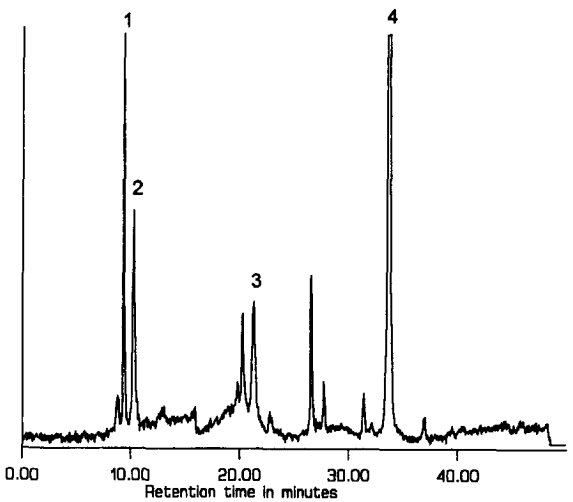

Fig. 4. Acidic protein separation, using a bonded column with methylcellulose as the polymer additive. CElect-H50 bonded column, 25 m*M* sodium phosphate, pH 8.0, 0.1% methylcellulose, −25 kV, hydrodynamic injection 2 s, 25°C. Peaks: 1 = trypsin inhibitor, 2 = β-lactoglobulin B, 3 = carbonic anhydrase, 4 = transferrin.

3.3. Protein separations using polymer surfactants on nonpolar bonded columns

We have investigated polymer surfactants that would interact strongly enough with the nonpolar bonded phase to allow the use of simple buffers during protein analyses. Thus, a stable hydrophilic layer can be formed on the nonpolar bonded phase through a single rinse of the column. Fig. 5 shows the electropherogram of three basic proteins, using Supelcoat PS2, a polymer surfactant, on a C_1 column. In this separation, the column was rinsed with 0.1% Supelcoat PS2 in deionized water for 20 min, then with the separation buffer for 10 min before the sample was injected. Although the polymer surfactant was not added to the buffer, evidently, the surface maintained a hydrophilic layer which was stable to buffer rinse. Before each analysis, the column was rinsed with the polymer surfactant solution, thus creating a fresh hydrophilic coating layer on the capillary surface.

3.4. Comparison of the methods

Three coating methods and their effectiveness have been demonstrated. The results are tabulated in Table 1. The most significant difference

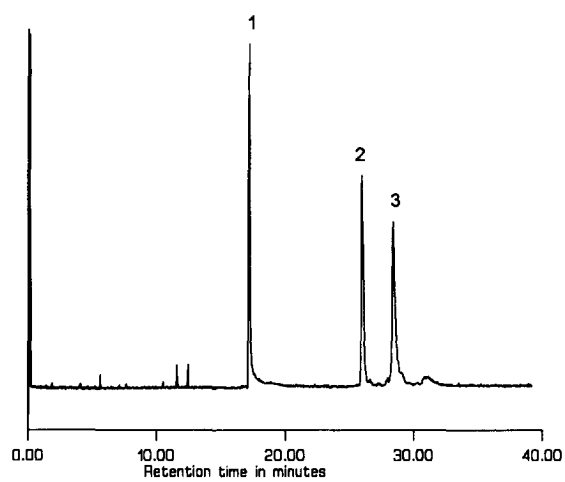

Fig. 5. Separation of basic proteins, using Supelcoat SP2 on a C_1 bonded column. CElect-H50 bonded column, other conditions as in Fig. 2.

Table 1
Comparison of the three methods for coating hydrophilic layer on nonpolar bonded phase for protein separations

Method	Surface coverage	Additive in the buffer	Efficiency (plates/m)[a]	%R.S.D. ($n = 8$) of migration time[a]
1	+	Yes	130 000	1.38
2	++	Yes	390 000	2.33
3	+++	No	340 000	2.47

[a] Representing the average of values for lysozyme, ribonuclease A, and α-chymotrypsinogen A.

is the way the additive is coated onto the nonpolar bonded phases. When using Brij-35, the column must be rinsed for at least one hour before it is filled with the buffer, which also contains a small amount of the surfactant. For UCON and methylcellulose, a pre-rinse is not necessary and the buffer rinse alone is enough, but these polymers must be included in the buffer. For polymer surfactant, a 20-min pre-rinse of the column with a surfactant water solution is sufficient, and it is not necessary to add the surfactant to the buffer. The experimental results indicate that methods 2 and 3 give about the same separation efficiencies, which are higher than those of method 1. In practice, the choice of which method to use depends on the nature of the protein sample, the separation requirements, and the CE operation mode. Furthermore, we noted that different bonded phases are best matched with different additives for protein separations in CE: e.g. Brij-35 on the C_{18} phase, UCON and methylcellulose on the C_8 and C_1 phases, and polymer surfactant on the C_1 phase.

3.5. Applications

Fig. 6 shows the separation of proteins in human serum. Several recent reports [31,32] dealt with serum protein separations by CE, and untreated fused-silica columns and high pH buffers were used. Methylcellulose dynamically coated onto a C_1 bonded column provides an inert surface for serum proteins, allowing high-resolution separations. CE has been shown to be a very powerful tool in carbohydrate and glycoprotein separations [33,34]. Fig. 7 shows the

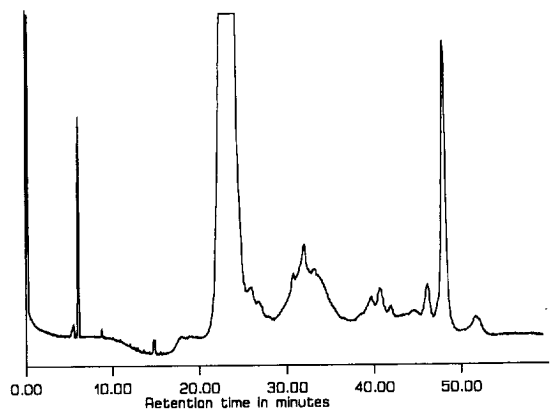

Fig. 6. Separation of human serum proteins. CElect-H50 bonded column, 25 m*M* phosphate, pH 8.0, 0.1% methylcellulose, −25 kV, hydrodynamic injection 6 s.

separation of ribonuclease B glycoforms with five to nine mannose units. By using a polymer surfactant on a C_1 bonded column, a high-resolution separation of the glycoforms was achieved. Fig. 8 gives the result of bovine al-

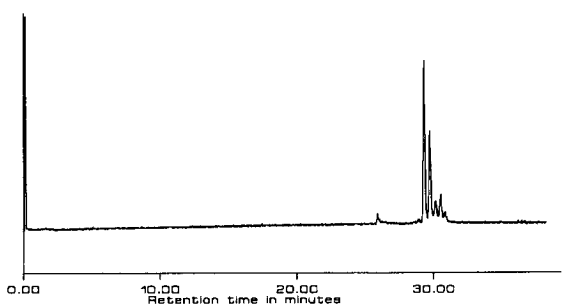

Fig. 7. Separation of ribonuclease B glycoforms, using Supelcoat SP2 on a C_1 bonded column. CElect-H50 bonded column, 75 m*M* Tris-HCl, pH 4.7, 20 kV, hydrodynamic injection 2 s.

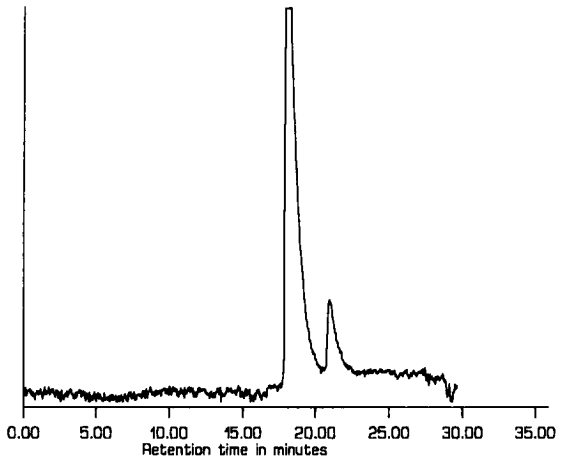

Fig. 8. Separation of bovine albumin monomer (major peak) and dimer CElect-H50 bonded column, 0.1 *M* sodium phosphate, pH 2.7, 0.05% methylcellulose, 3.2% dextran, 30 kV, hydrodynamic injection 8 s.

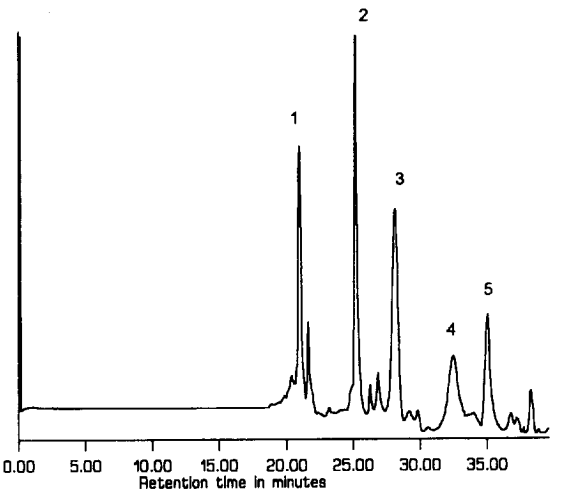

Fig. 9. CIEF of protein mixture using a C_8 column and UCON as a polymer additive. CElect-H150 bonded column (45 cm effective, 60 cm total), anode: 10 m*M* phosphate, cathode: 20 m*M* NaOH, protein mixture in 5% ampholine 3.5 to 9.5, 0.5% UCON, 10 μl TEMED/ml sample, 25 kV, hydrodynamic injection 1 min, UV absorbance detection at 280 nm. Peaks: 1 = cytochrome *c* (p*I* = 9.6), 2 = α-chymotrypsinogen A (p*I* = 8.8), 3 = myoglobin (p*I* = 7.2), 4 = carbonic anhydrase (p*I* = 5.9), 5 = β-lactoglobulin A (p*I* = 5.3).

bumin monomer (major peak) and dimer separation by capillary gel electrophoresis (CGE). The entangled polymer solution filled into the separation column provides a molecular sieving effect which allows the separation of proteins based on size. In this separation system, native bovine albumin was used as the sample, dextran was the sieving polymer, and methylcellulose was used to modify the capillary surface.

Fig. 9 shows another mode of CE, e.g. capillary isoelectric focusing (CIEF). In earlier studies, CIEF was performed on coated columns with no electroosmotic flow; the focused protein zones were eluted and detected by salt mobilization [35,36], vacuum [37], or pressure [38]. Mazzeo and Krull [39] developed a method for CIEF in which focusing and elution occurred in the same step. The EOF was reduced, but not totally eliminated, by adding methylcellulose to the sample/ampholyte mixture. In their later work [40], they showed that improved results could be obtained by using a CElect-H1 (C_8) bonded column, as the coated column gave less pH-dependent electroosmotic flow. Our studies suggested that the coating of methylcellulose on the nonpolar phase also plays an important role. Referring to the work of Mazzeo and Krull, we used UCON instead of methylcelluose for CIEF separations, because UCON and methycellulose behave in the same manner, as we had shown previously.

4. Conclusion

We have demonstrated that hydrophilic coatings formed by combining dynamically coated nonionic surfactants or hydrophilic polymers with permanently bonded polyalkylsiloxane CElect H-type columns produce highly efficient protein separations under a variety of CE conditions. Important features of this method include (1) the assembled hydrophilic capillary surface provides an alternative for difficult-to-make permanently coated hydrophilic columns, (2) high-performance protein separations in HPCE can be achieved without buffer pH limitation, and (3) rinsing the capillary surface with additive solution before each analysis freshes the surface and enhances the reproducibility of the

separations. The polymer surfactant Supelcoat PS2 can be used to rinse the column without the subsequent need to add the surfactant to the buffer. The present work also shows that the bonded columns give superior performance, versus bare fused-silica columns in forming a hydrophilic capillary surface for protein separations.

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