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Noncovalent Polycationic Coatings for Capillaries in Capillary Electrophoresis of Proteins

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The adsorption of proteins with net positive charges (p*I* > pH) on the walls of fused-silica capillaries is a common problem in the analysis of proteins by capillary electrophoresis. This paper explores the use of polycationic polymers as noncovalent coatings to limit this problem. The behavior of three sets of proteins was compared using uncoated and coated capillaries: (i) a protein charge ladder obtained by acetylation of lysozyme (EC 3.2.1.17); (ii) a protein charge ladder obtained by acetylation of carbonic anhydrase II (EC 4.2.1.1); (iii) a test panel of proteins with a range of values of molecular weight and p*I*. Four polycationic polymers were examined: polyethylenimine (PEI; MW_{av} = 15 000), Polybrene (MW_{av} = 25 000), poly(methoxyethoxyethyl)ethylenimine (MW_{av} = 64 000), and poly(diallyldimethylammonium chloride) (MW_{av} = 10 000). Detection of proteins with high p*I* was readily achieved using the first three of these polycationic polymer coatings but not with the poly(diallyldimethylammonium chloride). Examination of the stability of these coatings indicates that they are robust: the change in electroosmotic flow was less than 10% for 25 replications of the same separations, using capillaries coated with PEI or Polybrene. This study demonstrates that the charge ladder obtained by acetylation of lysozyme is a good model with which to test the efficiency of polycationic coatings. A study of the electrophoretic mobilities of the members of this charge ladder at pH 8.3 determined the effective charge of lysozyme ($Z_P(0) = +7.6 \pm 0.1$) and established the acidity of the α-ammonium group of lysozyme ($pK_a = 7.8 \pm 0.1$). Results from the test panel of proteins suggest that protein adsorption is mainly driven by electrostatic interactions.

The adsorption of proteins on the walls of hydrated, uncoated fused-silica capillaries is a common problem in the analysis of proteins by capillary electrophoresis.[1−3] The walls of the fused-silica capillaries are negatively charged at physiological pH as a result of ionization of SiOH to SiO^- (pK_a of SiOH ≈ 2−4).[4] A protein having a positive effective charge, or one having a high molecular weight, tends to adsorb on the surface of these capillaries. Minimizing this adsorption is important in extending the utility of capillary electrophoresis to a broader range of proteins. The objective of this work was to test four polycationic polymers as coatings to prevent the adsorption of proteins onto the walls of the capillaries and to demonstrate the use of these coatings in analyses of proteins that could not be examined using uncoated capillaries.

Buffer additives, noncovalent coatings, and covalent coatings have been reported to decrease protein adsorption on the walls of capillaries.[5−19] Covalent coatings are especially useful in protocols that require minimal concentrations of organic materials in the electrophoresis buffer. The covalent coatings add substantially to the cost of the capillaries and their lifetime may be short. Buffer additives and noncovalent coatings that desorb reversibly may be practical to use in studies in which separating the analyte from the buffer is not important: for example, buffer additives are practical in affinity capillary electrophoresis (ACE)[20] but generally not practical in CE/MS.[21,22] Noncovalent additives or coatings have the advantage that manipulation of the coatings is simpler and regeneration of the capillaries is more straightforward than the covalently modified capillaries.

Li and others have described a method to limit adsorption of proteins based on the adsorption of positively charged polymers on the negatively charged inner surface of the fused-silica capillaries.[9−11] The adsorbed polymeric coating reversed or neutralized the fixed negative charge on the surface of the capillary and prevented the adsorption of positively charged proteins (although it may promote adsorption of *negatively* charged proteins). In this study, we evaluated the utility of this method for limiting the adsorption of proteins on capillaries and examined

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the influence of the molecular structure of the polymer on its ability to minimize the adsorption.

We have used electroosmotic flow (EO) (ν_{EO}, cm s^{-1}) and electrophoretic mobility (μ, cm^2 kV^{-1} s^{-1})[23] of test proteins to evaluate the performance of a capillary. The value of ν_{EO} measures an effective fixed charge density on the wall of the capillary. The value of ν_{EO} is a function of zeta potential (ζ), viscosity (η), the permittivity of vacuum (ϵ_o), and dielectric constant (ϵ_r) of the electrophoresis buffer (eq 1).[24] By measuring the change in ν_{EO},

$$\nu_{EO} = \epsilon_o \epsilon_r \zeta E / \eta \quad (1)$$

we can infer the change in ζ and thus infer the stability of the cationic polymer coatings on the surface of the capillaries. Electrophoretic mobility is defined as the steady-state velocity of the analyte per unit field strength. The value of μ is approximately proportional to the effective charge (Z) of the protein and inversely related to its hydrodynamic drag. The hydrodynamic drag is related to the mass (M) (or the molecular volume and shape) of a protein in solution. Recently, we determined the values of C_P and α to be equal to 6.3 cm^2 min^{-1} kV^{-1} $charge^{-1}$ $kDa^{0.48}$ and 0.48, respectively, under the condition of 25 mM tris−192 mM Gly (pH 8.3, T = 37 °C).[25] The electrophoretic mobility (μ) of a protein should be independent of the velocity of electroosmotic flow (eq 2).

$$\mu \approx (C_P / M^{\alpha}) Z_P \quad (2)$$

The value of ν_{EO} is determined experimentally using eq 3,

$$\nu_{EO} = L_d / t_{MO} \quad (3)$$

where L_d (cm) is the length of the capillary from the inlet to the detector and t_{MO} (s) is the migration time of an electrically neutral marker present in the injecting sample (e.g., mesityl oxide). The value of μ is calculated by eq 4, where t_P is the migration time of

$$\mu = \frac{L_d L_t}{V}\left(\frac{1}{t_{MO}} - \frac{1}{t_P}\right) \quad (4)$$

the protein, L_t is the total length of the capillary, and V is the voltage that is applied across the capillary.

We evaluated the performance of coated and uncoated capillaries by examining three sets of proteins: a protein charge ladder[25−28] obtained by acetylation of lysozyme (EC 3.2.1.17); a protein charge ladder obtained by acetylation of carbonic anhydrase II (EC 4.2.1.1); and a test panel of proteins chosen to cover a representative range of values of pI and molecular weight. These three sets cover a substantial range of charges, molecular weights, and properties. Our interest was to determine the utility and generality of the coatings of polycationic polymers to prevent protein adsorption and to understand better the relation between the structure/composition of a protein and its tendency to adsorb. Since polycationic polymers are commercially available and derivatives can be easily synthesized, evidence of substantial differences in behavior would encourage developing different members of the class for specific purposes.

EXPERIMENTAL SECTION

Reagents. All chemicals were analytical grade and were used as received. Acetic anhydride, dioxane, 1-bromo-2-(2-methoxyethoxy)ethane, 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide (Polybrene, MW_{av} = 15 000),[29] polyethylenimine (PEI, MW_{av} = 25 000), and poly(diallyldimethylammonium chloride) (MW_{av} = 10 000) were purchased from Aldrich. Ovalbumin, insulin, glucose-6-phosphate dehydrogenase (EC 1.1.1.49), adolase (EC 4.1.2.13), streptavidin, enolase (EC 4.2.1.11), alcohol dehydrogenase (EC 1.1.1.1), hemoglobin, carbonic anhydrase II (CAII, EC 4.2.1.1), horse heart myoglobin (Myo), cytochrome *c* (Cyt *c*), and α-chymotrypsinogen A (α-Chym) were purchased from Sigma. Lipase (EC 3.1.1.3), carboxypeptidase B (EC 3.4.17.2), diaphorase (EC 1.6.99.1), papain (EC 3.4.22.2), lysozyme (L0, EC 3.2.1.17), and peroxidase (P, EC 1.11.1.7) were purchased from Worthington. Mesityl oxide (MO) was purchased from Eastman Organic Chemical. The electrophoresis buffer was prepared as 25 mM tris−192 mM Gly (pH 8.3).

Synthesis of Poly(methoxyethoxyethyl)ethylenimine (Poly-(EG_2)). A DMF solution containing 1-bromo-2-(2-methoxyethoxy)ethane (8.79 g, 48 mmol) was added in drops to a solution of PEI (1 g, 70 μmol) in DMF (20 mL). The mixture was stirred at room temperature for 7 days. The solvent was removed by evaporation under vacuum. The residue was dissolved in water and dialyzed for 7 days. The aqueous solution was evaporated and dried in vacuo: ^{1}H NMR ($CDCl_3$) δ 3.04 (4 H, br), 3.35 (3 H, s), 3.55 (8 H, m). Elemental Anal. Calcd: C, 46.26; H, 8.97; Br, 19.24. Found: C, 45.59; H, 8.78; Br, 17.85. The stoichiometry of this crude material is two EG chains per three nitrogen atoms in a repeating unit of PEI.

Conditions for CE. An Isco Model 3140 capillary electrophoresis system was used in these studies. The capillary tubing (Polymicro Technologies, Inc., Phoenix, AZ) was of uncoated fused silica with an internal diameter of 50 μm, a total length of 72 cm, and a length from inlet to detector of 38 cm. The conditions used for *all* CE experiments were as follows: polarity, negative; voltage, 30 kV; current, 5−6 μA; detection wave length, 214 nm; temperature, 27 ± 2 °C. Samples (8 nL) were introduced into the capillary by vacuum injection. The electrophoresis buffer was prepared as 25 mM tris−192 mM Gly (pH 8.3), unless otherwise specified (see below).

Coating of the Capillary. Each new fused-silica capillary was flushed (velocity 20 cm/min) with 0.1 N NaOH for 15 min and then with deionized water for 15 min. After preconditioning, the capillary was coated with a polymer by flushing the capillary with a 7.5% (w/v) polymer solution, prepared in 25 mM tris−192 mM Gly buffer (pH 8.3), for 15 min. Finally, the capillary was flushed with the electrophoresis buffer (absence of polymer) for 5 min. Recoating of the capillary with polymer was accomplished by using a similar method. The previously coated capillary was flushed with 0.1 N acetic acid (pH 5.0) for 15 min and then with deionized

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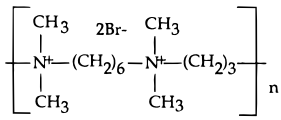
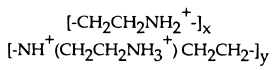
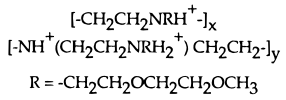
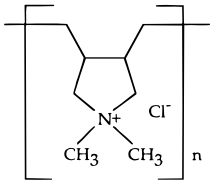
Table 1. Names and Chemical Structures of the Polymers Used as Coatings

compound	structures	MW_{av}	charge density[a]
Polybrene		15 000	0.13
polyethylenimine (PEI)		25 000	0.33[b]
poly((methoxyethoxyethyl)ethylenimine) (poly(EG_2))		64 000[c]	0.13[b]
poly(diallyldimethylammonium chloride)		10 000	0.11

[a] Charge density is calculated as the ratio of the number of positive charges to the total number of non-hydrogen atoms in a repeating unit of the polymer. [b] The calculation assumes that all the amines are present as ammonium ions. [c] The value of molecular weight was calculated based on the molecular formula shown on the left.

water for 15 min. After rinsing, the capillary was recoated with the polymer by flushing the capillary with the 7.5% polymer solution for 15 min. Finally, the capillary was flushed with the electrophoresis buffer (absence of polymer) for 5 min before analysis.

Conditions for Acetylation of Lysozyme. (a) Selective Acylation of ϵ-Amino Groups of Lys. An aqueous solution of lysozyme (0.1 mM, 0.5 mL) was first adjusted to pH 10 (measured by pH paper) by using 0.1 N NaOH, and acetic anhydride (100 mM in dioxane, 4.5 μL) was added. At pH 10, Lys ϵ-amino groups were acetylated more rapidly than the N-terminal α-amino group of Lys on lysozyme. After 5 min at room temperature, 25 μL of a sample was diluted with 65 μL of the electrophoresis buffer (25 mM tris−192 mM Gly, pH 8.3). A stock solution of mesityl oxide (180 mM, 10 μL) was added, and the sample was analyzed by CE.

(b) Selective Acylation of the N-Terminal α-Amino Group of Lys. Lysozyme (4.2 mg) was dissolved in 500 μL of deionized water. An aliquot of the solution of acetic anhydride (100 mM, 4.5 μL) was added to the lysozyme solution. The pH of the solution was maintained at pH 6 (measured by pH paper) by addition of 0.1 N NaOH. At this pH, acylation of the N-terminal α-amino group occurred preferentially over the Lys ϵ-amino groups. After 30 min at room temperature, 25 μL of a sample was diluted with 65 μL of the electrophoresis buffer (25 mM tris−192 mM Gly, pH 8.3). A stock solution of mesityl oxide (180 mM, 10 μL) was added, and the sample was analyzed by CE.

CE of the Charge Ladders of Lysozyme at Different Values of pH. A solution of lysozyme and its six acylated derivatives was prepared at a total concentration of 25 μM in 25 mM tris−192 mM Gly buffer (pH 8.3), and mesityl oxide was added to a final concentration of 18 mM. Several electrophoresis buffers were prepared, at different values of pH (8.7, 7.7, 7.4, 7.1, 6.8, 6.5), by adding 2 N acetic acid or 1 N NaOH, as necessary, to the original tris−Gly buffer. The change of electrophoresis buffer was accomplished by rinsing the capillary with the new CE buffer for 5 min before each CE experiment. Recoating of the capillary between each electrophoresis buffer was not necessary.

RESULTS AND DISCUSSION

Evaluation of Coating Efficiency for Different Polymers. (a) Design and Syntheses of Polymers. We selected four polymers as coatings to prevent adsorption of proteins on the walls of fused-silica capillaries (Table 1). Polybrene, PEI, and poly(diallyldimethylammonium chloride) are commercially available. Polybrene and poly(diallyldimethylammonium chloride) are composed of quaternary amines and have similar charge densities (we define charge density as the ratio of the number of positive charges to the total number of non-hydrogen atoms in a repeating unit of the polymer). PEI has primary, secondary, and tertiary amines; if all of its amines are present in their ammonium forms, its charge density is the highest among the four polymers. Ethylene glycol chains have been demonstrated to prevent adsorption of proteins on a solid surface.[30] We, therefore, synthesized a polymer having ethylene glycol side chains ($CH_2CH_2OCH_2CH_2OCH_3$, EG) by reaction of PEI with 1-bromo-2-(2-methoxyethoxy)ethane. Proton NMR and elemental analysis indicated that two EG chains modified three nitrogen atoms (on average) in a repeating unit of PEI, resulting in a polymer that we have labeled as poly(EG_2). The charge density of the new polymer is similar to Polybrene and poly(diallyldimethylammonium chloride) (Table 1).

(b) Charge Ladder of Lysozyme as a Model System. We used lysozyme as a model for positively charged proteins in this study. Lysozyme is commercially available and inexpensive; its crystal structure is known.[31,32] It has a molecular weight of 14 400 and p*I* of 11.0. Lysozyme has six Lys residues, and other ionizable amino acid residues include 8 Asp, 2 Glu, 11 Arg, 1 His, and 3

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Tyr; its N-terminal amino acid is Lys and its C-terminal amino acid is Leu.[33] Modification of the ϵ-amino groups of Lys by acetic anhydride yielded a series of derivatives of lysozyme that differ in integral units of charge but minimally in hydrodynamic drag (eq 2). CE separates all members of the family of acetylated lysozyme and generates a set of peaks with evenly spaced differences in mobility—a "charge ladder".[28] In previous studies, we have used charge ladders to measure the effective charges of proteins,[28] to estimate values of pK_a for ionizable residues on them,[27] to estimate the influence of charge on protein−ligand interactions,[26] and to measure the molecular weights of proteins in solution.[25]

It has been difficult to examine lysozyme (and many other positively charged proteins) by capillary electrophoresis at neutral pH, since these proteins tend to adsorb to the negatively charged walls of the fused-silica capillaries.[11] Using uncoated capillaries, lysozyme, for example, has a long migration time (over 50 min in a 60-cm capillary at 30 kV), and its peak is severely broadened.[1,34] This study demonstrates that the use of polycationic polymers to modify the surface of the capillaries solves this problem: the charge ladder of lysozyme elutes in 7 min with good resolution and reproducibility using Polybrene-coated capillaries (Figure 1).

(c) Dependence of Coating Efficiency on the Concentration of Polymer in Solution. In choosing the coating conditions necessary for obtaining reproducibility and high resolution, we used the lysozyme charge ladder to monitor the performance of the polycationic polymer coatings. Previous work by Li et al. reported using Polybrene as a buffer additive to coat the capillaries.[9] We first used a 0.05% (w/v) Polybrene solution (25 mM tris−192 mM Gly buffer, pH 8.3) to coat the capillary prior to analysis and then used the electrophoretic buffer (absence of polymer) as a running buffer. We observed that the EO flow was reversed and the charge ladder of lysozyme was successfully detected. After the first run, however, the peaks corresponding to the charge ladder of lysozyme broadened significantly. In order to remedy this effect, it was necessary to coat the capillary after each run. This recoating was time consuming and impractical. We therefore modified the coating procedure by increasing the concentration of the polymer in the coating solution. Using a concentration of Polybrene in the coating solution (25 mM tris−192 mM Gly buffer, pH 8.3) at its solubility limit (7.5% w/v), we were able to detect the lysozyme charge ladder with high resolution and good reproducibility. The stability of the EO flow was excellent, and recoating between runs was not necessary. Thus, we fixed the concentration of polymer as 7.5% (w/v) in the coating solution for the initial coating of the capillary, but not in the running buffer; and we used the same protocol with poly(EG$_2$), PEI, and poly(diallyldimethylammonium chloride).

We did not examine extensively the influence of various factors (e.g., ionic strength, ionic composition, and temperature) on the coating efficiency of the polymers on the surface of the capillary. Rather we compared the coating efficiency of different polymers under identical coating conditions. We tried to rationalize these differences at a physicoorganic level, and the results should help to design new polymers that will maximize the coating efficiency and minimize the adsorption of proteins to the capillary surfaces.

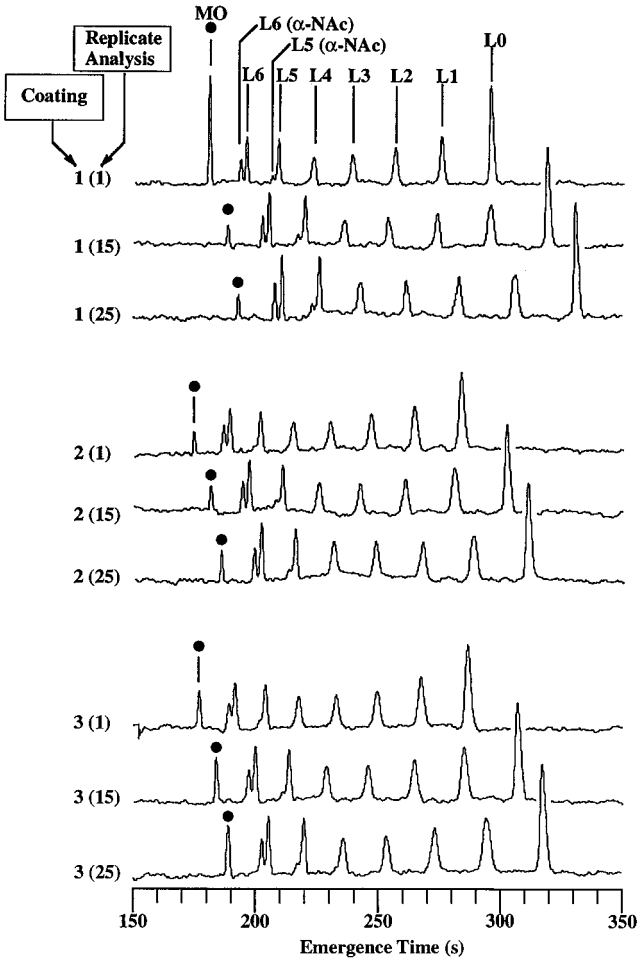

Figure 1. Electropherograms of the lysozyme charge ladder on multiple repeated analyses. The capillary was coated, and then a solution of the members of the lysozyme charge ladder was analyzed repeatedly. The coating solution consisted of 7.5% (w/v) of polymer in 25 mM tris−192 mM Gly buffer (pH 8.3). The running buffer was 25 mM tris−192 mM Gly buffer (pH 8.3) in the absence of polymer. After 25 replicate analyses, the capillary was cleaned and recoated, and replicate analyses were continued. In the notation *X* (*Y*), *X* indicates the number of times the capillary has been coated, and *Y* is the number of repetitions of the analysis after each coating. For example, 1 (15) is the 15th repetition with the original coating. The letter L stands for lysozyme, and the number next to it stands for number of ϵ-amino groups of Lys that are acylated; e.g., L4 is lysozyme with four acylated ϵ-amino groups. L6(α-NAc) is a derivative of lysozyme in which seven amino groups are acylated: six ϵ-amino groups and the N-terminal α-amino group. The peaks for a neutral marker (mesityl oxide, MO) are indicated by filled circles.

(d) Velocity of the EO Flow (ν_{EO}): A Measure of the Stability of the Coatings. The change in ν_{EO} as a function of the number of repetitions, which reflects the change in ζ, can be used to infer the stability of the cationic polymeric coatings on the surface of the capillaries. In a representative series of experiments (e.g., as in Figure 1), after the initial coating of a polymer, we carried out 25 consecutive separations of the lysozyme charge ladder in tris−Gly buffer (pH 8.3) and monitored migration times and mobilities of the neutral marker and charge ladder of lysozyme. We then rinsed the capillary using 0.1 N acetic acid (pH 5), recoated with the polymer, and repeated the replicate analyses. We used the percentage change in the rate of EO flow, (% $\Delta\nu_{EO} = 100\Delta\nu_{EO}/\nu_{EO}$%) to represent the stability of the coatings on the surface of the capillaries. With the

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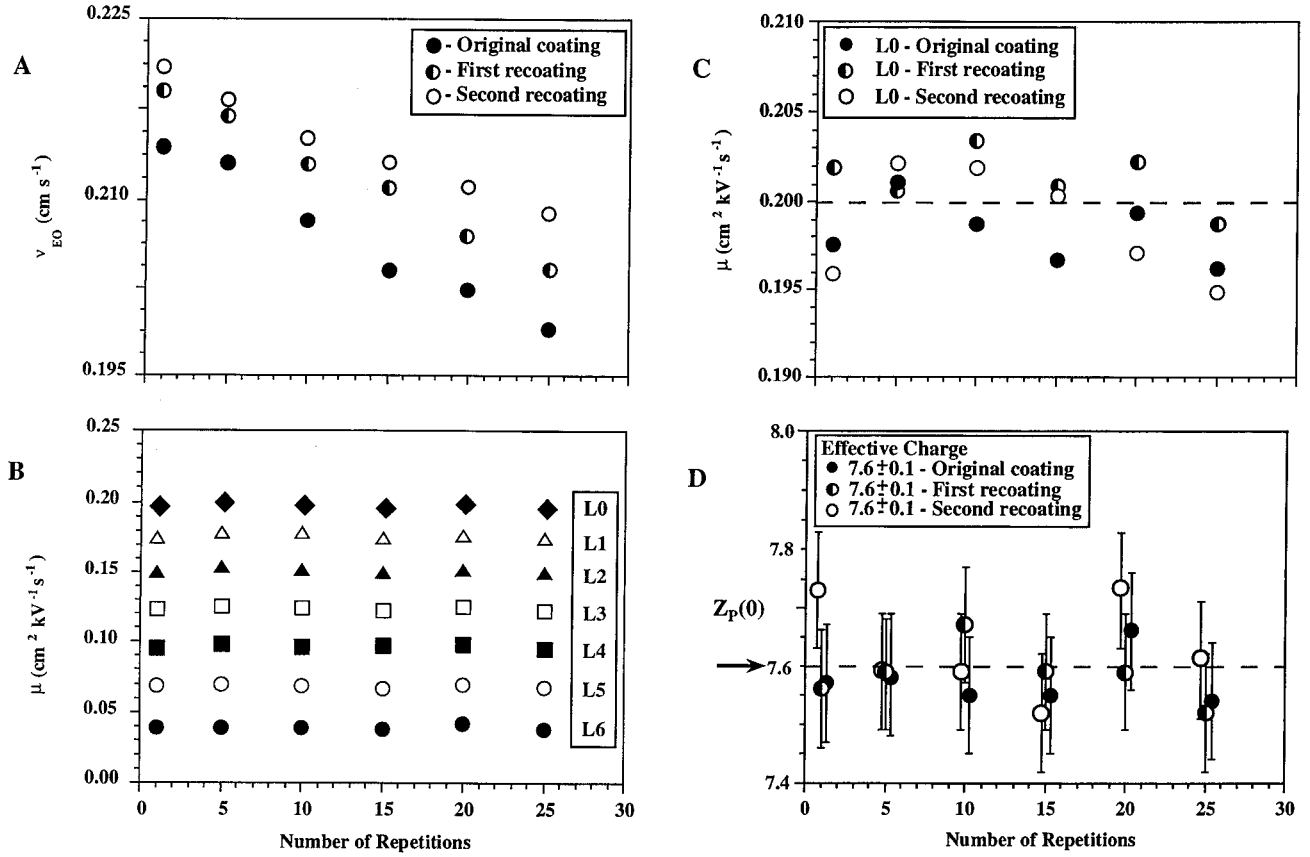


Figure 2. (A) Plot of ν_{EO} as a function of the number of repetitions for three successive applications of the Polybrene-coated capillary. (B) Plot of electrophoretic mobility of lysozyme charge ladder as a function of the number of repetitions for one successive application of the polycationic coating to the capillary. (C) Plot of electrophoretic mobility of native lysozyme as a function of the number of repetitions for three successive applications of the Polybrene-coated capillary. (D) Plot of the measured values of the effective charge of native lysozyme as a function of the number of repetitions in a series of experiments. The points are all at multiples of 5 but are displaced slightly horizontally for clarity. The bars in the figure represent the uncertainties of the measurement.

Table 2. Comparison of Performance of Coatings by the Four Polycationic Polymers[a]

polymer	ν_{EO} (cm s^{-1})	stability[b] (% $\Delta\nu_{EO}$)	μ (cm^2 s^{-1} kV^{-1}) (L0)	μ (cm^2 s^{-1} kV^{-1}) (L6)
Polybrene	0.22	9	0.20	0.040
PEI	0.19	9	0.23	0.048
poly(EG_2)[c]	0.15	7	0.20	0.042
poly(diallyldimethyl-ammonium chloride)	0	0	0	0

[a] All experiments were carried out by coating the capillary with a solution of 7.5% (w/v) polymer in 25 mM tris−192 mM Gly buffer (pH 8.3). The running buffer was 25 mM tris−192 mM Gly buffer (pH 8.3) in the absence of polymer. [b] % $\Delta\nu_{EO} = 100\ \Delta\nu_{EO}/\nu_{EO}$% and is the percentage change in the rate of EO flow during the course of the first 25 repetitions. [c] % $\Delta\nu_{EO}$ is calculated by comparing replicate analyses 5 and 25.

Polybrene-coated capillaries, the value of ν_{EO} did not change significantly (% $\Delta\nu_{EO}$ < 10%) for 25 replications (Figure 2A, Table 2) within the replicate analyses. Recoating produced capillaries characterized by very similar values of ν_{EO} (<2% change compared to those of the same repetition number in the previously coated capillaries) (Figure 2A). We carried out similar analyses for other two polymers, and their results are reported in Table 2.

(e) Dependence of Electrophoretic Mobilities of Proteins on the Number of Repetitions. The value of μ of a protein in solution is related to the effective charge (Z) of the protein and its hydrodynamic drag (eq 2) and should be independent of the polymer coatings on the surface of the capillary. Examination of the electrophoretic mobilities of the proteins as a function of the number of repetition can provide information about the possible interactions between the proteins and polymers on the surface of the capillary wall. Results from the Polybrene-coated capillary showed that although minor drifting of the EO flow occurred (<10%, Figure 2A), the electrophoretic mobility for each families of charge ladder of lysozyme appeared to be reproducible (<2%, Figure 2B,C). We observed similar reproducibility for peak shape and peak area for all of the members of the charge ladder of lysozyme over many repetitions (Figure 1). Furthermore, the electrophoretic mobilities of lysozyme and its derivatives were the same for analyses using Polybrene, PEI, and poly(EG_2) coatings (Table 2). These results indicate that the charge ladder of lysozyme mainly migrated in solution, and their interactions with the positively charged surface of the capillary were minimal.

The above experiments also demonstrate the use of neutral markers as internal standards to correct for changes in EO flow in the CE analysis. In such cases, the absolute mobility of an analyte in solution depends on a variety of external factors that change EO flow (e.g., coating quality, solution condition, etc.). The relative mobility of the analyte to an internal standard (either a neutral marker or other charged markers) will be independent of these external influences. One direct application of the internal standard is in affinity capillary electrophoresis,[20] where use of high concentrations of binding ligands in solution often changes the

value of EO flow and, thus, the absolute mobility of the protein−ligand complex. The calculation of the relative mobility of the complex (e.g., its electrophoretic mobility) is necessary for the CE analysis.[20]

We used the value of effective charge ($Z_P(0)$; see discussion below) as another measure of the consistency of the data. The high reproducibility of $Z_P(0)$ (<3%) over a number of repetitions supports further the inference that minor drifting in ν_{EO} in capillaries coated with Polybrene does not influence the measurement of the effective charge of lysozyme by CE (Figure 2D).

(f) Comparison of Coating Efficiency for Different Polymers. When poly(diallyldimethylammonium chloride) was used as a coating, we could detect neither the neutral marker nor the charge ladder of lysozyme. We speculate that the inability of this polymer to coat the capillary is due to the restricted conformations of its backbone and consequently diminished Coulomb interactions between the polymer and the walls of the capillary.

Unlike Polybrene and polyethylenimine coatings, EO flow of poly(EG_2) coating during the first five repetitions increased. We believe that a multilayer structure formed along the surface of the capillary after the initial coating. During multiple repetitions of analyses when the poly(EG_2) was absent in the electrophoresis buffer, the current continued to increase until the surface coating stabilized; this desorption and restructuring gave rise to the equilibration of the polymer on the capillary. After the fifth repetition, the value of ν_{EO} did not change significantly (% $\Delta\nu_{EO}$ < 10% for repetitions 5 through 25, Table 2) within the replicate analyses, signifying the equilibration of the polymer coating on the capillary.

The stabilized value of ν_{EO} of poly(EG_2) coating is, however, smaller than that observed with Polybrene or PEI coating (Figure 3, Table 2). There are at least two possible reasons for this decreased value of ν_{EO} for poly(EG_2) coatings: (i) a decrease in ζ as a result of the screening of surface charges by the flexible EG chain on the polymer, or the smaller charge density of poly(EG_2) than that of PEI; (ii) an increase in η at the interface of solution and poly(EG_2) coatings relative to that of the interface of Polybrene or PEI coatings. We did not distinguish between these possibilities.

Polybrene, PEI, and poly(EG_2) coatings gave similar values of % $\Delta\nu_{EO}$ (Table 2). The small values of % $\Delta\nu_{EO}$ for various polymer coatings demonstrate that these coatings are stable and convenient for CE analysis. Since Polybrene and PEI coatings do not require preequilibration, and they gave faster EO flow and shorter analysis time than poly(EG_2) coatings, it is more convenient to use these two coatings to analyze proteins (see below).

Application of Polybrene Coatings To Characterize the Effective Charge of Lysozyme and pK_a of the N-Terminal α-Ammonium Group of Lys of Lysozyme. (a) Measurement of Effective Charge of Lysozyme. The effective charge $Z_P(0)$ of a protein can be determined by analysis of the electrophoretic mobilities of the members of a charge ladder.[27,28] The electrophoretic mobility (μ_n) of a member of the charge ladder correlates linearly with its charge, $Z_P(n)$, thus the number of modifying acetyl groups on a protein (n; eq 5). A plot of μ_n vs the values of n

$$\mu_n \approx \frac{C_P}{M^\alpha} Z_P(n) = \frac{C_P}{M^\alpha} Z_P(0) - \frac{C_P}{M^\alpha} n \qquad (5)$$

gives $Z_P(0)$ as the x-axis intercept. Least-squares analysis of the mobilities from the lysozyme charge ladder gave a value of $Z_P(0) = 7.6 \pm 0.1$ at pH 8.4 (Figure 2D). This value is consistent with a value of 7.4 estimated from protein titration curves in a previous study.[35]

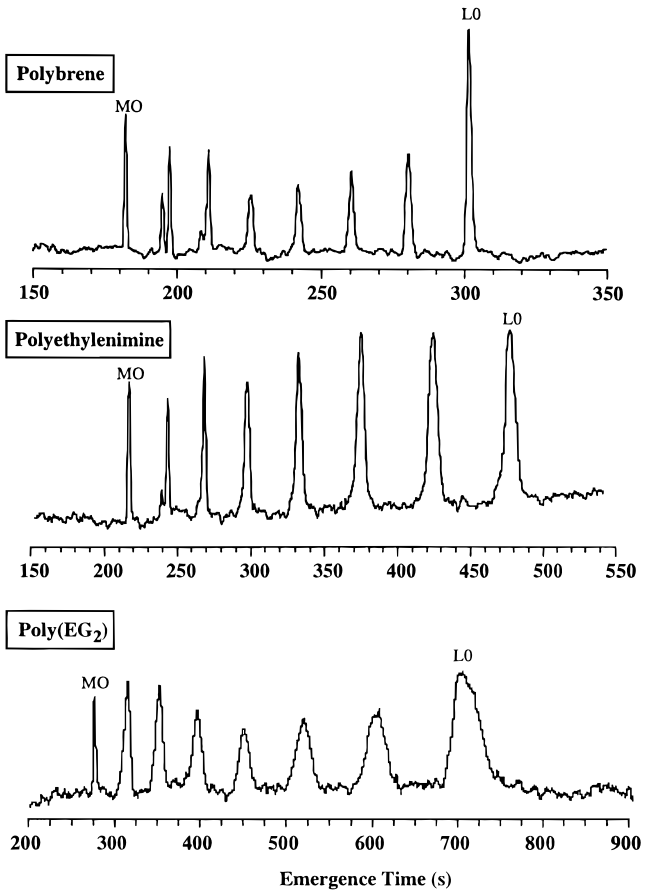


Figure 3. Electropherograms of the lysozyme charge ladder using Polybrene, PEI, and poly(EG_2) polymer coatings. Note that the time scale for each electropherogram is different. Different preparations of the lysozyme charge ladder were used; differences in intensities are not significant.

(b) Selective Acylation of N-Terminal α-Amino Group of Lys and ϵ-Amino Groups of Lys in Lysozyme. We described previously the selective acylation of the α-amino group at the N-terminus of insulin at low pH, and the selective acylation of Lys ϵ-amino groups at high pH.[27] When acylation of lysozyme is carried out at pH 10−11, it is primarily on the lysine ϵ-amino groups, and the acylation yields a charge ladder with members separated by integral increments of charge (Figure 4A). The N-terminal amino acid of lysozyme is Lys. At values of pH between 5 and 7, the α-amino group of the N-terminal amino acid—which has a lower value of pK_a and has a larger probability existing as neutral nucleophilic state than do the ϵ-amino groups—acylates selectively. We acylated an aqueous solution of lysozyme at pH 6 with acetic anhydride; this reaction gave a simple electropherogram in which native lysozyme (L0), and lysozyme acetylated on one ϵ-amino and α-amino group (L1 and L1(α-NAc)) were the only species to appear (Figure 4B). This solution was then made basic (pH 10), and additional acetic anhydride was added. The resulting charge ladder involved acylation of both the ϵ-amino groups of Lys and the N-terminal α-amino group (Figure 4C).

(35) Tanford, C.; Roxby, R. *Biochemistry* **1972**, *11*, 2192−2198.

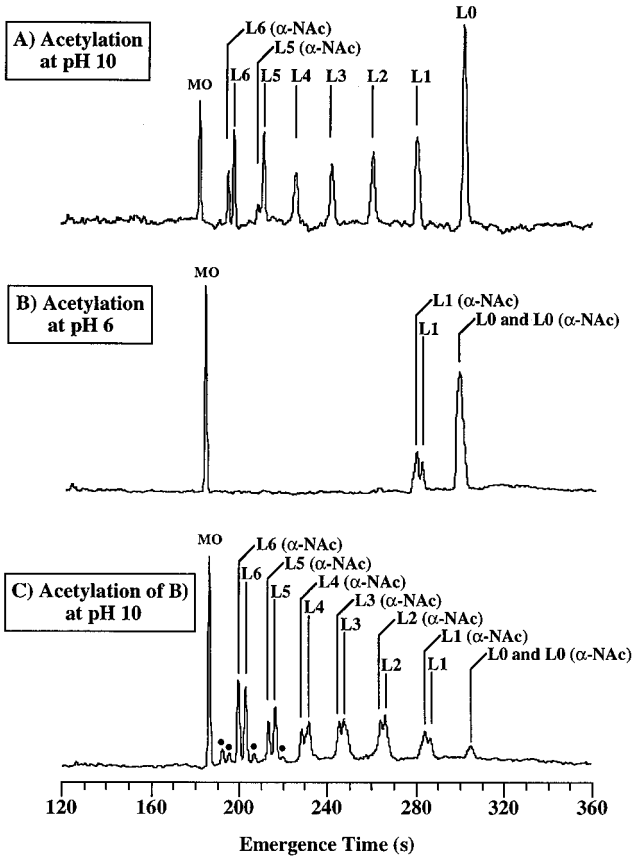


Figure 4. Electropherograms of charge ladders of lysozyme under selective acylation procedures: (A) Charge ladder from acetylation of lysozyme at pH 10. Under these conditions, the ϵ-amino groups of Lys can be selectively acetylated. (B) Charge ladder from acetylation of lysozyme at pH 6. At this pH, the α-amino group of the N-terminal Lys can be selectively acetylated, resulting a mixtures of L0, L0(α-NAc), L1, and L1(α-NAc). (C) Charge ladder from further acetylation of the sample shown in (B) at pH 10. The acetylation resulted in a charge ladder of lysozyme in which both the ϵ-amino groups and α-amino group were modified. The peaks labeled with ● in (C) are probably due to minor modification of other ionizable resides on lysozyme (e.g., His or Tyr residues). The pH of the electrophoresis buffer was 8.3 (25 mM tris−192 mM Gly). Separation of L0 and L0(α-NAc) was not obtained under these experimental conditions.

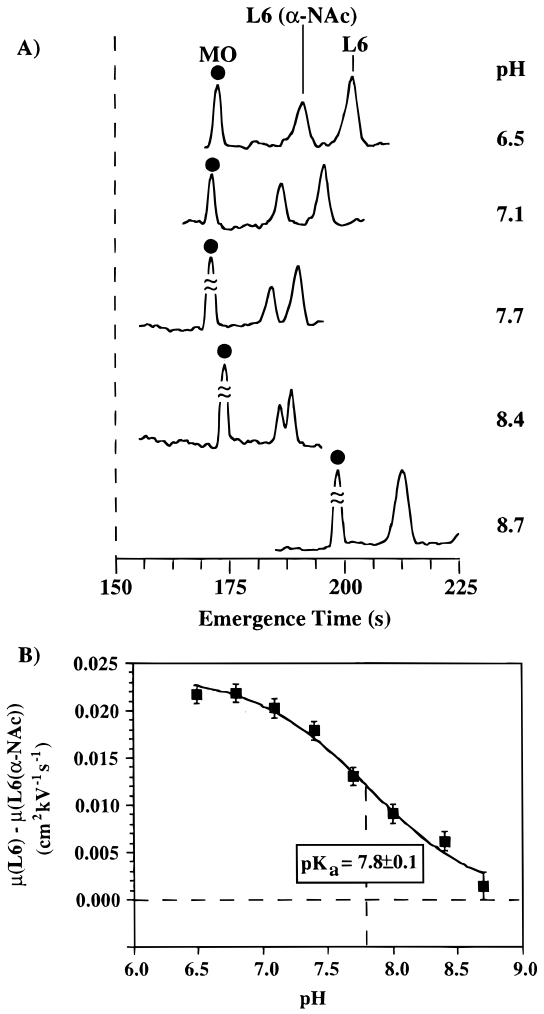


Figure 5. (A) Electropherograms of L6 and L6(α-NAc) at several values of pH in the electrophoresis buffer (25 mM tris−192 mM Gly). L6 is a derivative of lysozyme with six acylated ϵ-amino groups; L6(α-NAc) is a derivative of lysozyme in which seven amino groups are acylated: six ϵ-amino groups and the N-terminal α-amino group. The time scale applies to all experiments. The peaks for the neutral marker (MO) are indicated by filled circles. (B) Dependence of $\Delta\mu$ ($\Delta\mu = \mu$ (L6) $- \mu$ (L6(α-NAc)) on the pH of the electrophoresis buffer (25 mM tris−192 mM Gly). The least-squares fit of the data to eq 6 yields a value of p$K_a = 7.8 \pm 0.1$ for the α-ammonium group of the N-terminal Lys of lysozyme.

(c) Determination of the pK_a of the α-Ammonium Group of the N-Terminal Lys of Lysozyme. We examined the dependence of the difference in electrophoretic mobility of the native protein relative to a derivative of the protein with the ionizable residue of interest masked ($\Delta\mu = \mu$(L6) $- \mu$(L6(α-NAc)) on the pH of the buffer (eq 6; C is a constant) to determine this

$$\Delta\mu = \mu_{(L6)} - \mu_{(L6(\alpha-NHAc))} = C/(1 + 10^{pH-pK_a}) \qquad (6)$$

value of pK_a.[27] The value, μ(L6), is the electrophoretic mobility of lysozyme with all of its Lys ϵ-amino groups acylated (L6). The value, μ(L6(α-NAc)), is the electrophoretic mobility of lysozyme when all ϵ-amino groups of lysine *and* the N-terminal α-amino group (L6(α-NAc)) are acylated. Figure 5A shows electropherograms from which values of $\Delta\mu$ between μ(L6) and μ(L6(α-NAc)) were plotted as a function of pH. These data were fit to eq 6 using the pK_a of the α-ammonium group of L6 as an adjustable parameter: the least-squares best-fit value was p$K_a = 7.8 \pm 0.1$ (Figure 5B). The N-terminal amino acid of lysozyme is Lys; this α-ammonium group is expected to have p$K_a \approx 7.7$ according to literature.[36]

Implication for the Mechanism of Protein Adsorption. (a) Comparison of Coated and Uncoated Capillaries Using Charge Ladders of Carbonic Anhydrase and Lysozyme. We selected the charge ladders of carbonic anhydrase II (CAII)[28] and lysozyme as models to test the efficiency of coated and uncoated capillaries (Figure 6). The charge ladders of carbonic anhydrase and lysozyme produce a wide range of charged derivatives with protein derivatives having charges ranging from −20 (fully acylated carbonic anhydrase) to +7.6 (native lysozyme). The charge ladder of lysozyme was successfully detected using a Polybrene-coated capillary but not an uncoated capillary; that of CA was detected using an uncoated capillary but not a Polybrene-

(36) Rickard, E. C.; Strohl, M. M.; Nielsen, R. G. *Anal. Biochem.* **1991**, *197*, 197−207.

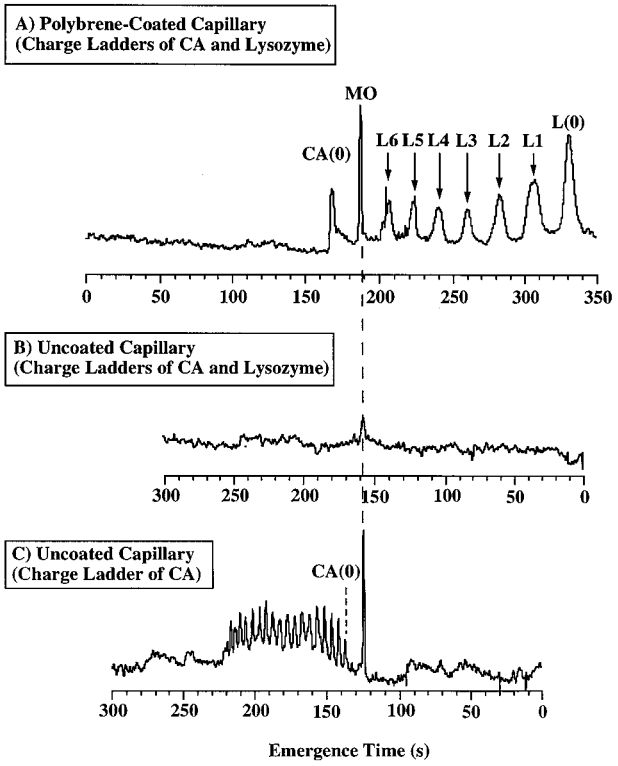


Figure 6. Electropherograms obtained using a Polybrene-coated capillaries and an uncoated capillary: (A) Charge ladders of lysozyme and CAII using a Polybrene-coated capillary. (B) Charge ladders of lysozyme and CAII using an uncoated capillary. (C) Charge ladder of CAII using an uncoated capillary. The time scales of (B) and (C) are reversed, and the neutral markers (MO) in (A−C) are aligned. Native CAII and lysozyme are labeled CA(0) and L(0), respectively.

coated capillary nor a capillary coated with the other positively charged polymers examined here. The charge ladder of CAII was, however, not detected in the presence of the charge ladder of lysozyme using an uncoated capillary. As a comparison, the charge ladder of lysozyme was detected in the presence of CAII charge ladder using Polybrene-coated capillary, where electroosmotic flow (ν_{EO}) did not change significantly (% $\Delta\nu_{EO}$ < 10% for five repetitions) and the electrophoretic mobilities of the charge ladder of lysozyme remained unchanged. These results demonstrate that Polybrene-coated capillary is able to analyze positively charged proteins that uncoated capillaries fail to detect, and further, it is more tolerant to proteins carrying opposite charges relative to the charges on its surface than uncoated capillaries.

(b) Dependence of Protein Adsorption on the Values of p*I* and Molecular Weight of Proteins. We selected a panel of proteins (Table 3) that vary in values of p*I* (4.7−11.0) and molecular weight (6000−161 000) in order to survey the generality of the procedure using polymers as coatings. These proteins cover a wide range of physical properties (e.g., effective charge and charge distribution, shape, or hydrodynamic drag, etc.); all proteins are commercially available. We surveyed their propensity to adsorb to the Polybrene-coated capillaries by examining the dependence of their electrophoretic mobilities, peak areas, and peak shapes on the number of repetitions. We define a protein as not adsorbing to the capillaries if these parameters changed by less than 5% for 10 consecutive repetitions of the electrophoretic separation.

Figure 7 summarizes the tendencies of these proteins to adsorption as a function of their values of pI and molecular weight.

Table 3. p*I*, MW, and Subunit Information for a Test Panel of Proteins[a]

ID	compd (abbrev)	p*I*	MW[b] ($\times10^{-3}$)	sub-units
1	ovalbumin	4.7	45	1
2	lipase	5.0	48	1
3	insulin	5.3	6	1
4	carboxypeptidase	6.0	35	1
5	glucose-6-phosphate dehydrogenase	6.0	128	2
6	adolase	6.1	161	4
7	streptavidin	6.4	60	4
8	enolase	6.7	93	2
9	alcohol dehydrogenase (ADH)	6.8	80	1
10	hemoglobin	6.8	65	4
11	diaphorase	8.5	24	1
12	papain	8.8	21	1
13	carbonic anhydrase II (CA)	5.9	30	1
14	myoglobin (Myo)	7.2	18	1
15	peroxidase (P)	7.2	40	1
16	α-chymotrypsinogen A (α-Chym A)	8.8	25	1
17	cytochrome *c* (Cyt *c*)	9.3	13	1
18	lysozyme (L0)	11.0	14	1

[a] Information was obtained from Sigma and Worthington catalogs. [b] Total molecular weight including all the subunits.

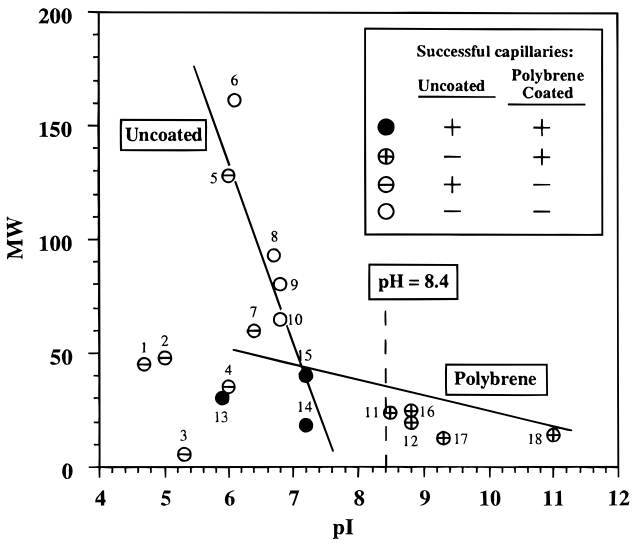
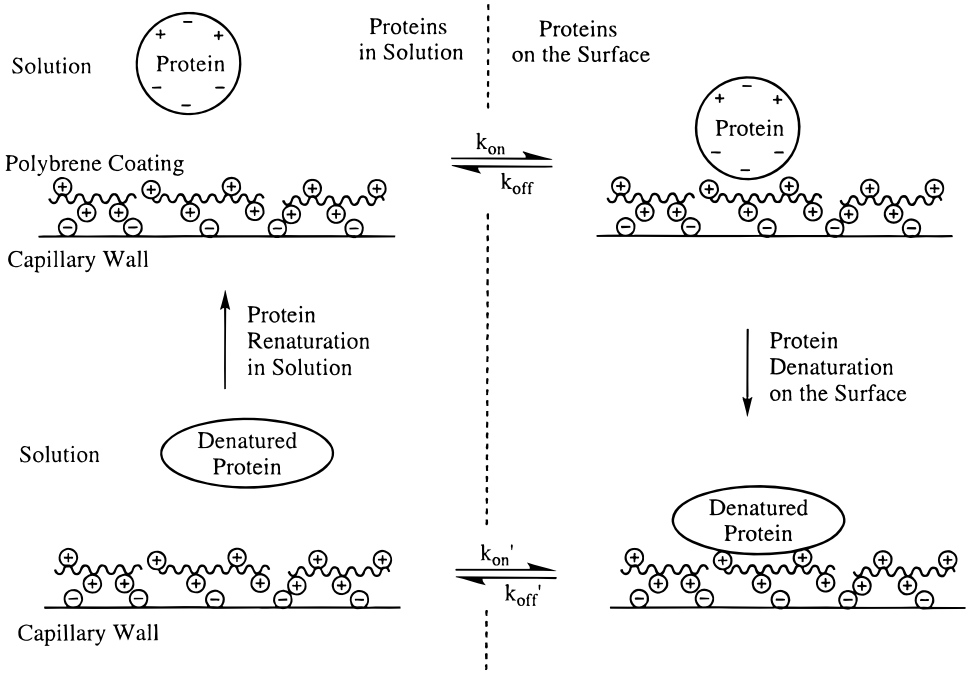


Figure 7. Dependence of protein adsorption on the values of p*I* and molecular weight for the test panel of proteins. The numbers on the table indicate the protein number given in Table 3. The lines are aids to the eye in organizing the proteins into sets that can be detected using uncoated capillaries, using Polybrene-coated capillaries, using either type of capillary, or not at all.

We compared the results from Polybrene-coated capillaries with those from uncoated capillaries. We draw three conclusions from these results: (i) protein adsorption correlates with p*I*. The proteins (MW < 50 000) with low values of p*I* (<8) are easily analyzed using uncoated capillaries but not with Polybrene-coated capillaries; proteins with high values of p*I* (>8) are easily detected using Polybrene-coated capillaries but not with uncoated capillaries. (ii) Molecular weight also seems to influence the tendency of a protein to adsorb to the capillaries. Proteins having MW > 50 000 clearly show a higher tendency to stick to the capillaries than those having smaller values of molecular weight. (iii) The polycationic polymer coating seems to be more tolerant to the charge of a protein than an uncoated capillary (that is, negatively charged carbonic anhydrase, myoglobin, and peroxidase could be detected using a capillary coated with a polycationic polymer,

Scheme 1. Mechanism of Protein Adsorption Mediated by Electrostatic Interactions[a]



[a] The surface of the capillary is composed of Polybrene that interacts noncovalently with the negatively charged surface of the bare silica capillaries. A net negatively charged protein in solution is attracted to the Polybrene surface by long-range Coulomb interactions. Once the proteins is adsorbed on the surface, it may undergo conformational changes to maximize the electrostatic interactions. The current study, however, cannot distinguish these two states of proteins on the surfaces. The denatured protein, once desorbed into solution, may renature itself to reduce the exposure the hydrophobic surface areas.

while proteins having positive charges were not detected on an uncoated capillary).

(c) Influence of Electrostatic Interactions on the Adsorption of Proteins. We previously observed adsorption of proteins on hydrophobic surfaces[30] and biospecific surfaces.[37] Protein adsorption on hydrophobic surfaces is mediated through nonspecific hydrophobic interactions between the hydrophobic surface of the protein and the adsorbing surface, and denaturation of proteins has been hypothesized following initial adhesion. Increasing the proportion of ethylene glycol among the hydrophobic surface effectively reduces the adsorption of proteins.[30] We have prepared a biospecific surface by attaching benzenesulfonamides to a gold surface through ethylene glycol linkers and discovered that only its binding protein, carbonic anhydrase, adsorbed to such surfaces. This experiment clearly demonstrates that carbonic anhydrase retained its native conformation on the biospecific surface.

The results from the current study emphasize the influence of electrostatic interactions on the protein adsorption (Scheme 1). Uncoated and Polybrene-coated capillaries provided highly negatively charged and positively charged surfaces, respectively. We found that proteins of opposite charges readily adsorbed to these surfaces (Figure 7). A silanol surface appeared to be more sensitive to the charge of proteins than the Polybrene-coated surface.

Scheme 1 illustrates the possible mechanism of adsorption of proteins on the charged surface. The adsorption may first occur through long-range electrostatic interactions between proteins and the charged groups on the surface of the capillary. The magnitude of this interaction depends on the effective charge of the protein and the electrostatic potential (ζ) on the surfaces. Once the protein contacts the adsorbing surface, depending on the charge distribution on the protein, the charged groups on the surface may induce the protein to collapse to maximize the electrostatic interactions. In this process, the protein may undergo conformational changes and denaturation. The denatured protein on the surface can be desorbed into solution and the protein may renature itself to reduce the exposure of its hydrophobic surfaces. Our study here, however, cannot distinguish whether the proteins on the surface are native or denatured.

The origin of higher tendency to adsorption for larger proteins is less straightforward to rationalize than that with charge and may be due to the larger contacting surface areas possible between the proteins having higher molecular weights and the walls of capillary, due to inhomogeneous distribution of charges on larger proteins, or both. The tendency of protein adsorption perhaps depends on several factors: effective charge of proteins, charge density, charge distribution on proteins, and electrostatic properties of the adsorbing surfaces. Molecular modeling studies should be useful to understand these correlations.

CONCLUSIONS

Adsorption of proteins to the surface of the fused-silica capillaries is a major problem in applying CE in protein biochemistry. Here we have developed the method originally reported by Li and others[9−11] based on the use of noncovalent coatings of cationic polymers on the walls of fused-silica capillaries. Among the four polymers we examined, Polybrene and polyethylenimine coatings are effective in preventing the adsorption of positively charged proteins. Examination of a panel of proteins (having values of p*I* from 4.7 to 11 and values of molecular weight from 6000 to 161 000) using both uncoated and Polybrene-coated capillaries indicates that protein adsorption correlates qualitatively

(37) Mrksich, M.; Grunwell, J. R.; Whitesides, G. M. *J. Am. Chem. Soc.* **1995**, *117*, 12009−12010.

with values of p*I* and molecular weight. Proteins having molecular weights less than 50 000 can be readily analyzed using either uncoated or coated capillaries, depending on the values of p*I* of these proteins. Proteins having molecular weights above 50 000 have a stronger tendency to adsorb both to uncoated and coated capillaries and still present a challenge for CE analysis.

We have observed the significant influence of electrostatic interactions on the adsorption of proteins by CE. Uncoated and Polybrene-coated capillaries provided highly negatively and positively charged surfaces, respectively. Proteins of opposite charges stuck to these surfaces. We cannot distinguish whether the proteins keep their native conformations on the surface, however. Protein adsorption onto the surface of uncoated capillaries appears to be more sensitive to the charges on proteins than that with Polybrene-coated capillaries.

These polycationic coatings will be specially effective in analyzing proteins with high values of p*I* for which analyses are limited by adsorption on uncoated fused-silica capillaries. In this study, we demonstrate that the lysozyme charge ladder is a good model system to test the efficiency of these polycationic coatings. In the process, we characterized the effective charge of lysozyme to be +7.6 at pH 8.3, and the pK_a of the α-ammonium group of its N-terminal Lys to be 7.8.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Electropherograms of the charge ladder of lysozyme on poly(EG_2) coated capillaries (2 pages). Ordering information is given on any current masthead page.

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