Journal of Chromatography, 516 (1990) 69–78
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 580

Polyethyleneimine-bonded phases in the separation of proteins by capillary electrophoresis

JOHN K. TOWNS and FRED E. REGNIER*
Department of Chemistry, Purdue University, West Lafayette, IN 47907 (U.S.A.)

ABSTRACT

A hydrophilic, positively charged, durable coating has been developed for capillary electrophoresis of macromolecules. Polyethyleneimine is adsorbed to the inner wall of fused silica capillaries and the adsorbed coating cross-linked into a stable layer. Capillaries of polyethyleneimine-coated silica gave unique separations owing to the reversal of electro-osmotic flow caused by the positively charged coating. The resulting coating was stable from pH 2–12 and could be used over a wide pH range without substantial change in electro-osmotic flow. High-molecular-weight polymers were needed to give thick coatings which mask silanol groups on the wall. Proteins were resolved quickly and efficiently with good recovery using capillaries of 50 cm in length.

INTRODUCTION

Adsorption of positively charged species onto the walls of fused-silica capillaries is a problem in capillary zone electrophoresis. This is particularly true in the case of basic proteins and peptides where adsorption diminishes solute recovery and resolution. Therefore, in order to optimize the separation of basic solutes, adsorption to the capillary wall must be kept to a minimum.

Electro-osmotic flow is another issue that must be considered in fused-silica capillaries. Ionization of surface silanols generates an electrical double layer which migrates toward the cathode when an electric potential is applied to the capillary. Migration of positive ions in this double layer pulls the solution through the capillary, producing the phenomenon known as electro-osmotic flow. The rate of this flow is related to charge density and increases with the ionization of surface silanols from pH 3 to 9. The net velocity of a charged solute in a fused silica capillary is the sum of the rates of both convective and electrophoretic transport. Ideally, the convective component of transport should remain constant while the electrophoretic component is being varied with pH to optimize selectivity in a separation. This would only be possible in capillaries with no charge or constant charge at the surface.

To this end, modification of capillaries by either masking or deactivating surface silanol groups has been performed by physically coating capillary walls with

0021-9673/90/$03.50

methylcellulose[1,2] and by silane derivatization[3–5], respectively. Although these coatings reduce both adsorption and electro-osmotic flow, they do not solve these problems. Residual silanols still diminish the recovery of basic proteins and cause electro-osmotic flow to vary widely with pH. Coating stability is also a problem. Hydrophilic organosilanes can erode from columns in a matter of days[4].

This paper focuses on the preparation of fused-silica capillaries with a positively charged polymer coating. The function of this bonded phase is to enhance the separation and recovery of positively charged species, to stabilize electro-osmotic flow across the pH range from 5 to 11 and to extend the useful life of capillaries.

EXPERIMENTAL

Chemicals

Polyethyleneimine (PEI) 6 (average rel. mol. mass, M_r = 600) was purchased from Polysciences (Warrington, PA., U.S.A.). PEI 18 and 200 were gifts from Dow Chemical (Midland, MI, U.S.A.). Ethyleneglycol diglycidyl ether (EDGE) and triethylamine (TEA) were purchased from Aldrich (Milwaukee, WI, U.S.A.). All other reagents were commercially obtained, reagent grade if available or the purest grade obtainable if not.

Lysozyme (egg white), cytochrome *c* (horse heart), chymotrypsinogen A (bovine pancreas), ribonuclease A (bovine pancreas), and myoglobin (horse heart) were purchased from Sigma (St. Louis, MO, U.S.A.). The neutral markers, mesityl oxide and dextran blue (M_r 2 000 000), were purchased from Aldrich.

Instrumentation

Capillary electrophoresis was performed on an instrument based on an in-house design. All high-voltage components of the system were contained in a Lucite cabinet fitted with a safety interlock that would interrupt the line voltage to the transformer in the power supply when the cabinet door was opened. A Spellman Model FHR 30P 60/EI (Spellman High Voltage Electronics, Plainview, NY, U.S.A.) power supply was used to apply the electric field across the capillary. The power supply output was connected to 22-gauge platinum-wire electrodes immersed in 3-ml buffer reservoirs along with the capillary ends. Polyimine-coated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, U.S.A.) of 50 and 75 μm I.D., 200 μm O.D. were used with the total length varying between 50 and 100 cm and the separation length from 35 to 85 cm. On-line detection was performed with a variable-wavelength UV absorbance detector (Model V4; Isco, Lincoln, NE, U.S.A.). Detection was monitored at 200 nm for the proteins and peptides and 254 nm for the mesityl oxide. The signal from the detector was fed to a Linear 2000 (Linear, Reno, NV, U.S.A.) strip chart recorder.

Electrophoresis

Protein solutions of a concentration of 1–5 mg/ml were injected into the capillary by syphoning for a fixed time (1–3 s) at a fixed height (5–8 cm). Mesityl oxide was used as the neutral marker along with dextran blue to determine any sizing differences in the coating. Several buffer solutions were used over the pH range of 3 to 11: 0.01 *M* acetate at pH 3 and 5, 0.01 *M* hydroxylamine–HCl at pH 7, 0.01 *M* diaminopropane at pH 9 and 11. Salt was added to each buffer to give comparable ionic strengths and

currents. During electrophoresis, current through the capillary never exceeded 50 μA with all analyses being run at ambient temperature. No temperature control of the capillary was employed. Between analyses, the capillary was flushed with 1% TFA in propanol, deionized water and the separation buffer for 1 min.

Capillary coating

Capillaries were first treated with 1.0 *M* NaOH for 15 min. followed by 15 min. of washing with deionized water. Residual water was evaporated from the capillaries by connecting the capillaries to a gas chromatography oven at 80°C for 1 h under a nitrogen pressure of 400 kPa. The methanolic solution of polyethyleneimine was then pulled through the capillary by syringe and allowed to sit for 8 h. The methanol–PEI solution was removed from the capillary by pushing nitrogen through the capillary. Nitrogen flow was continued through the capillary for 2 h to evaporate the methanol. In the case of PEI 200, which has water added to reduce the viscosity, the capillary was heated at 80°C for 4 h to remove the 33% water. Next, a 70% solution of EDGE in TEA was pulled into the capillary and allowed to sit for 1 h. This solution was then pushed out with nitrogen. Nitrogen flow through the capillary was continued at 400 kPa for 3 h. The capillary was then heated at 80°C for 30 min.

Picric acid assay

A 2 m × 100 μm I.D. PEI-coated capillary was washed with methylene chloride for 30 min followed by 0.2 *M* picric acid in methylene chloride. Unbound picric acid was removed with an additional methylene chloride wash. Bound picric acid was then released with 5% (v/v) TEA in methylene chloride into a 1-mm cuvette. Washing with the amine solution was continued until the solution eluting from the capillary was colorless. The eluent was then diluted to 0.1 ml and the triethylamine picrate assayed spectrophotometrically. The molar extinction coefficient of TEA picrate in methylene chloride is 14 500 at 358 nm.

RESULTS AND DISCUSSION

Surface derivatization

The synthetic route (Fig. 1) used to prepare positively charged capillaries in these studies was derived from techniques used in liquid chromatography to prepare silica-based anion-exchange packing materials. It has been shown[6–8] that PEI is strongly adsorbed from organic solvents onto a silica surface where it may subsequently be cross-linked with a multifunctional oxirane into a continuous film. This cross-linked layer is held in place by electrostatic adsorption at many sites and cannot be eluted from the silica surface with solvents used in chromatography. Coating thickness is a function of both M_r and concentration of PEI in the coating solution[8]. EDGE was chosen as the cross-linking agent because it was of sufficient length to bridge between adjacent adsorbed PEI molecules and would contribute to the hydrophilicity of the coating. EDGE can react with a polyamine by either an intra- or intermolecular mechanism. The intermolecular reaction serves the purpose of crosslinking the coating into place as shown in Fig 1. In contrast, intramolecular reactions (not shown) can either cross-link within a polyamine molecule or more generally derivatize the polyamine with free oxiranes. These oxiranes are subsequently

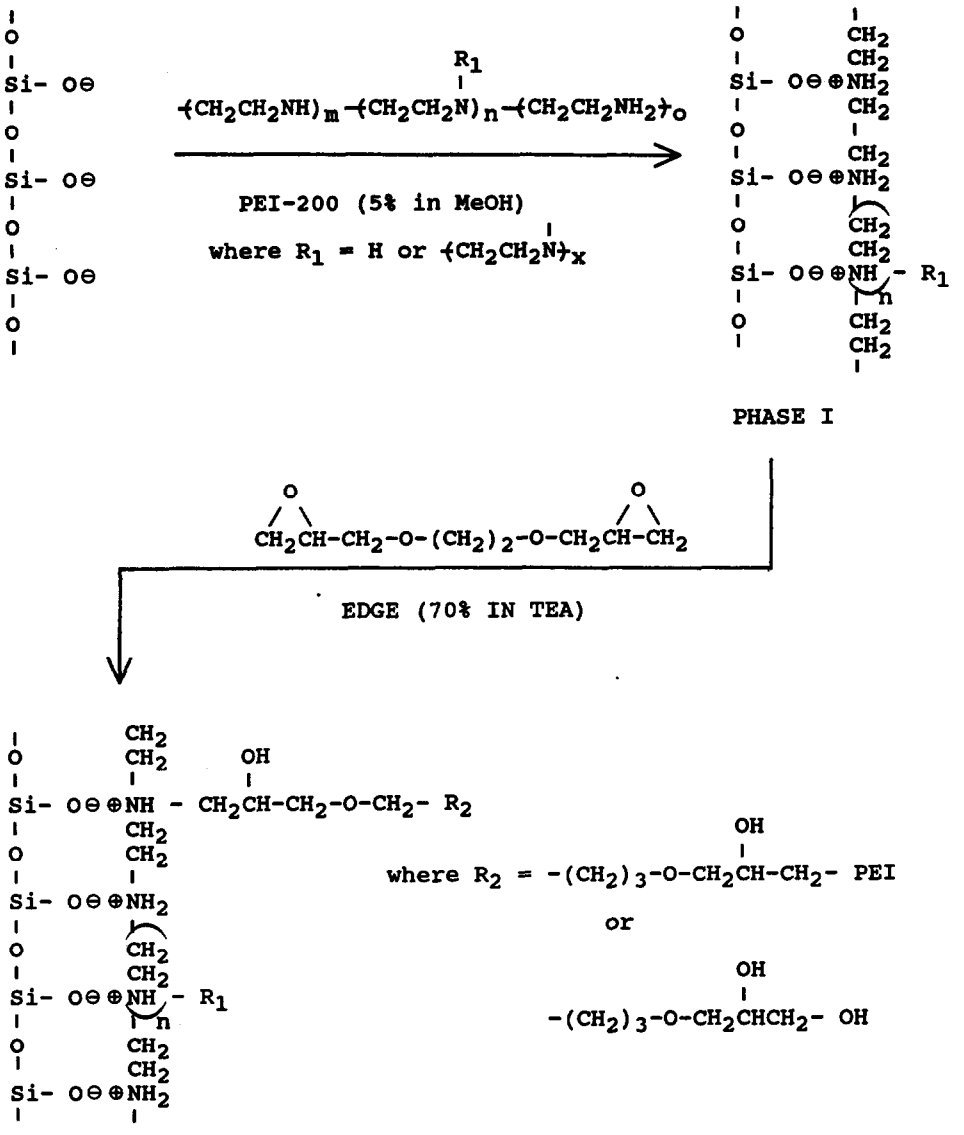

Fig. 1. Synthetic route to an adsorbed PEI-bonded phase. The coating process is two step: (1) adsorption of PEI 200 onto the fused-silica capillary, and (2) cross-linking of the PEI 200 polymer in order to stabilize the coating further.

hydrolyzed to produce a diol-rich coating[8]. Polyethyleneimines are known to be branched polymers with a primary:secondary:tertiary amino group ratio of approximately 1:2:1 (ref. 8). Since the tertiary amino groups are sterically hindered and non-reactive, the primary and secondary amino groups both dominate the electrostatic interaction of the coating with the capillary wall and react with the EDGE.

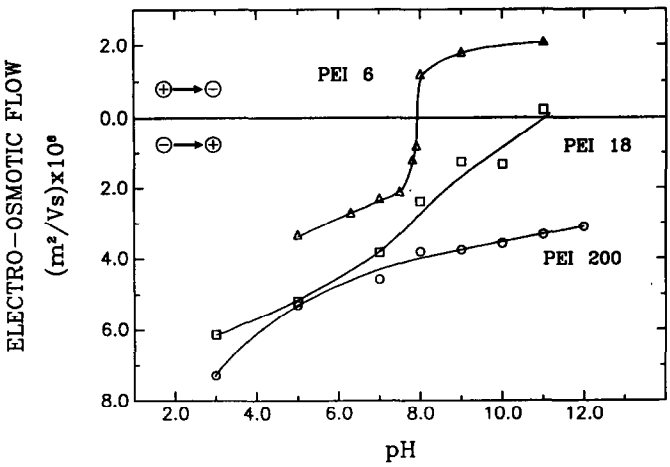

Fig. 2. Plot of electro-osmotic flow as a function of pH for three sizes of polyethyleneimine: PEI 6 (M_r 600); PEI 18 (M_r 1800); PEI 200 (M_r 20 000).

Influence of polymer size on electro-osmotic flow

The influence of polyamine molecular mass on electro-osmotic flow over the pH range from 3 to 11 is shown in Fig. 2. There is a strong correlation between the electro-osmotic flow-rate, direction of flow and polymer size. For low-molecular-mass polymer (PEI 6), the electro-osmotic flow moves from the negative to the positive electrode at low pH, indicating a positively charged wall. However, as the pH is increased, there is a sharp reversal in direction of the flow between pH 7.9 and 8.0. Flow reversal is the result of a switch in the net charge on the wall from positive to negative within that very small pH range. This is unusual in that the change occurs abruptly over a small pH range and the magnitude of the change (as expressed in flow-rate) is large on either side of this small range. These observations may be explained in the following way. It is known that the ionization of primary, secondary and tertiary amines in PEI is almost linearly related to pH between pH 4 and 10, *i.e.* PEI is not totally ionized until pH is dropped to 4 or less[6]. This is the result of the very high charge density in the polymer and electronic interaction between adjacent amine groups. In contrast, ionization of surface silanols begins at pH 3 and is not complete until the solution pH is raised to greater than 8. Again the broad titration curve is due to high charge density and electronic effects occurring at the surface. Below pH 7.9, positively charged amine groups dominate at the surface of the capillary causing the zeta potential to be positive. Above pH 8.0, surface silanol groups are more abundant than amine groups and the negative electrical potential of the silica surface projects through the polyamine coating into the solution and establishes a positively charged double layer. Between pH 7.9 and 8.0, the surface is isoelectric and electro-osmotic flow is zero. Although surface charge density is changing substantially on either side of this isoelectric point, electro-osmotic flow is almost constant. This shows that electro-osmotic flow is only weakly coupled to charge density.

As the molecular mass of the polymer is increased to 1800, electro-osmotic flow at low pH is faster than with PEI 6. This is due either to an increase in the positive

charge that a larger polymer would possess, or to a more efficient masking of the negative groups on the fused silica. However, this coating apparently does not completely mask all surface silanols. An increase in pH still results in a significant decrease in electro-osmotic flow. This decrease continues until at pH 11 the negative silanol groups once again predominate and push the electro-osmotic flow in the opposite direction (positive to negative electrode). When an even larger molecular mass polymer is coated onto the capillary (PEI 200), the electro-osmotic flow is further increased. However as the pH is increased, the electro-osmotic flow stays relatively constant, decreasing only slightly from pH 3 to 8 and almost staying constant from pH 8 to 12. This makes it possible to run a PEI 200-coated capillary between pH 3 and 12 without a significant change in electro-osmotic flow. Over this range, pH conditions can be employed to give the best selectivity without adversely effecting the analysis time. Uncoated fused silica capillaries are very different in contrast. Silanol ionization begins at pH 3 and continues to increase through pH 8. This means that electro-osmotic flow is extremely pH dependent[10,11].

Estimation of coating thickness

Polymer thickness influences the electro-osmotic flow by masking the deprotonated silanol groups on the capillary wall. The thicker the coating, the greater the masking of negative charges on the wall and the greater the electro-osmotic flow. To determine the thickness of the organic layer, a modification of the picric acid assay for ion-pairing capacity was employed[12]. Assuming a density of 0.9 g/ml for ethyleneimine and that approximately one third of the total nitrogen bound in the organic coating is at the surface and accessible to picric acid[7], a rough estimation of the coating thickness can be made from the picric-acid ion-pairing capacity. Table I shows surface nitrogen amounts and coating thickness estimates as a function of polymer molecular mass for 1, 5, and 25% PEI 200 in the methanol coating solution. As expected, the larger the molecular mass of polymer, the thicker the coating. In the case of PEI 200, a coating thickness estimated to be 30 Å results in a significant masking of the negative capillary wall charges.

TABLE I

SURFACE NITROGEN CONCENTRATION AND ESTIMATION OF COATING THICKNESS FOR POLYETHYLENEIMINE CAPILLARIES

Polymer size	*PET in methanol (%)*	*Concentration of surface nitrogens (μmoles/m²)*	*Estimation of coating thickness[a] (Å)*
PEI 200	25	46.8	70
(M_r = 20000)	5	16.2	24
	1	11.8	17
PEI 18	5	8.7	13
(M_r = 1800)			
PEI 6	5	6.7	10
(M_r = 600)			

[a] Assuming 33% of bulk nitrogens are surface nitrogens[7] and an ethyleneimine density of 0.9 g/ml.

The migration reproducibility was studied using the PEI-200 coating on 50 cm × 75 μm capillaries. It was found that the run to run reproducibility was 0.9% relative standard deviation (R.S.D.) ($n=6$), while the day to day reproducibility was 2.4% R.S.D. ($n=5$). A section to section reproducibility of 3.1% R.S.D. ($n=6$) was determined by cutting a three-meter-long coated capillary into six segments. The column-to-column migration reproducibility was examined over a three-month period and found to be 4.7% R.S.D. ($n=14$).

The stability of the coating was tested using a 50 cm × 75 μm coated capillary over an eight-day period. The total electrophoresis running time was 100 h or approximately 12 h a day for 8 days. After the neutral marker migration time increased slightly (12 s) over the first three hours, the migration times stayed steady for the next 6 days to the 84-h mark. The migration time then increased a total of 5 s to the 100-h mark where upon the experiment was terminated.

Influence of polymer concentration on electro-osmotic flow

The concentration of PEI 200 in the methanol coating solution was increased from 1 to 25% in order to determine the optimum polymer concentration that would maximize electro-osmotic flow over a wide pH range. The results are plotted in Fig. 3. As expected, electro-osmotic flow decreases for all five concentrations as the pH is increased from 3 to 11. This is the result of a gradual deprotonation of PEI between pH

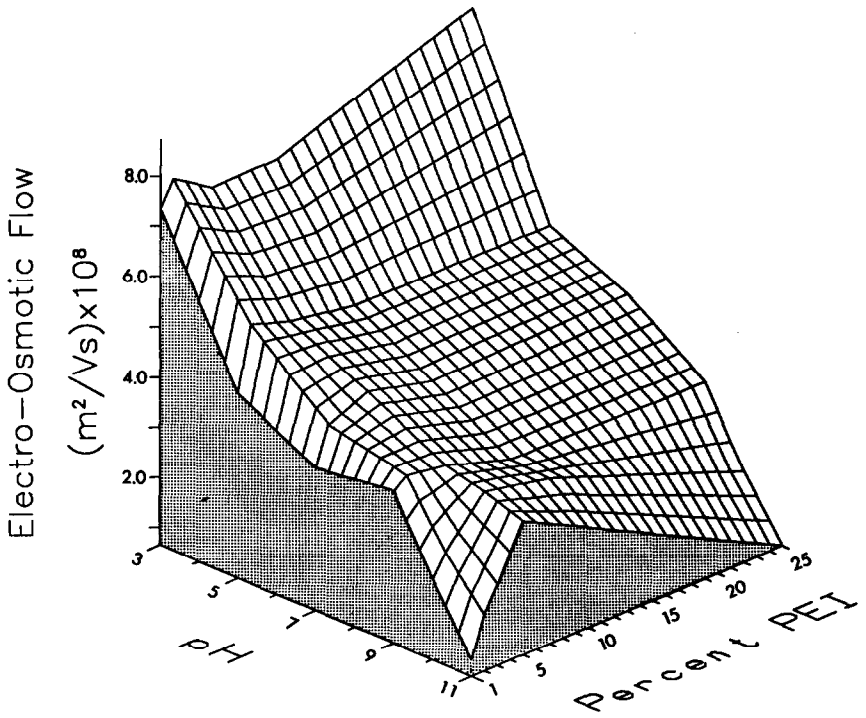

Fig. 3. Three-dimensional plot of electro-osmotic flow as a function of polymer concentration in methanol (1, 2, 5, 10 and 25%) for a given pH.

4 and 11. The fact that substantial electro-osmotic flow occurs at pH 11 indicates that the polyamine coating is still charged. This is probably due to quaternization of the amine during the cross-linking reaction[8]. It is to be expected that an increase of polymer concentration from 1 to 25% would result in a larger suppression of charge from the silica capillary and an increase in electro-osmotic flow. This is not strictly the case. Electro-osmotic flow is maximum with 5% PEI in all cases. This effect is largest at pH 11. The increase in electro-osmotic flow from 1 to 5% can be attributed to an increase in the coating thickness which is estimated to increase from 17 to 24 Å over this concentration range. This results in a more complete suppression of the negative charge on the capillary column. However, the opposite is observed as the PEI concentration is increased further; then the electro-osmotic flow decreases. Coating thickness is estimated to increase from 24 to 70 Å over this range. A satisfactory explanation for this phenomenon has not been developed.

Applications

Electrophoresis has been a valuable analytical technique for large, biological molecules such as proteins. This is why major emphasis is being placed on developing capillary electrophoresis for the separation of proteins. It has been demonstrated above that surface charges on the PEI (200)/EDGE coating do not change significantly over the pH range 3–12, *i.e.* fluctuation in electro-osmotic flow is minimal over this range. This uncoupling of electro-osmotic flow and pH allows pH manipulation of selectivity[9,13]. In the separation of amphoteric compounds such as peptides and proteins, net charge and electrophoretic mobility can vary significantly with pH[14,15]. Fig. 4 shows how variations in pH from 3 to 11 can optimize the separation of five proteins. At low and high pH, two sets of proteins (lysozyme/cytochrome *c* and chymotrypsinogen A/ribonuclease A) have similar migration rates and are therefore not well resolved. At pH 7, the migration rates are far enough apart so that these

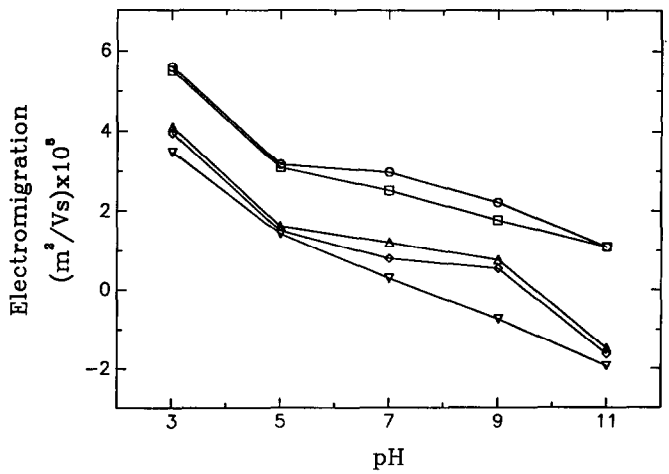

Fig. 4. Plot of electromigration for five proteins as a function of pH. ○ = Hen-egg lysozyme, p*I* 11.1; □ = horse-heart cytochrome *c*, p*I* 9.4; △ = bovine chymotrypsinogen A, p*I* 8.8; ◇ = bovine ribonuclease A, p*I* 8.7; ▽ = horse-heart myoglobin, p*I* = 7.3.

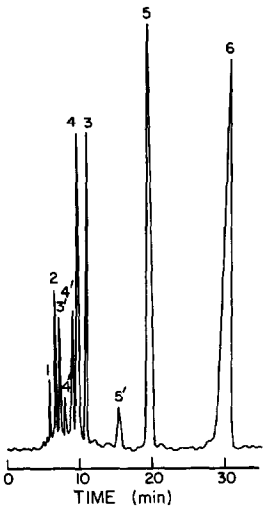

Fig. 5. Capillary electrophoretic separation of mode proteins on a PEI-200–EDGE coated capillary. Conditions: capillary length, 50 cm; separation length, 35 cm; I.D., 75 μm; buffer, 0.02 *M* hydroxylamine–HCl at pH 7.0; separation potential, 12.5 kV. Peaks: 1 = Mesityl oxide (neutral marker); 2 = horse-heart myoglobin; 3 = bovine ribonuclease A; 4 = bovine chymotrypsinogen A; 5 = horse heart cytochrome *c*; 6 = hen-egg lysozyme. Peaks 3′, 4′ and 5′ are impurities in 3, 4 and 5, respectively.

proteins can be well separated. This is demonstrated in Fig. 5 which shows the separation of the five proteins using a PEI(200)–EDGE capillary of 50 cm in length. The coating reversed the electro-osmotic flow so the polarity of the power supply must be switched from that used on uncoated fused-silica capillaries. The reversed flow results in lysozyme (p*I* 11.1) arriving at the detector last as compared to using uncoated capillaries where it would be the first to arrive. The p*I* values for the proteins range from 7.3 for myoglobin to 11.1 for lysozyme. Thus by using a buffer at pH 7, the coulombic repulsion of proteins from the positively charged capillary surface overcomes any solute–wall adsorption.

CONCLUSION

Adsorbed polyamine coatings on fused-silica capillaries may be cross-linked to give a thick, reproducible positive layer. This coating has the advantage that it is stable from pH 2 to 12 and does not adsorb positively charged proteins. Thick coatings of approximately 30 Å are needed to effectively mask the underlying negative silanol groups.

The most notable advantage of this coating lies in its ability to retain a relatively constant positive surface charge over a wide pH range. This makes it possible to vary the pH to optimize selectivity without significantly affecting the rate of electro-osmotic flow.

ACKNOWLEDGEMENTS

The authors thank James R. Gord for his help in the graphical presentation of the data. Funds for this research were provided by Pfizer, Inc. and a research grant from the National Institute of Health (GM 25431).

REFERENCES

1 S.Hjerten, *J. Chromatogr. Rev.*, 9 (1967) 122.
2 B. J. Herren, S. G. Shafer, J. V. Alstine, J. M. Harris and R. S. Snyder, *J. Colloid Interface Sci.*, 115 (1987) 46.
3 J. W. Jorgenson and K. D. Lukacs, *Anal. Chem.*, 53(1981) 1298.
4 J. W. Jorgenson and K. D. Lukacs, *Science (Washington, D.C.)*, 222 (1983) 266.
5 S. H. Chang, K. M. Gooding and F. E. Regnier, *J. Chromatogr.*, 120 (1976) 321.
6 A. J. Alpert and F. E. Regnier, *J. Chromatogr.*, 185(1979) 375.
7 G. Vanecek and F. E. Regnier, *Anal. Biochem.*, 121 (1982) 156.
8 R. M. Chicz, Z. Shi and F. E. Regnier, *J. Chromatogr.*, 359(1986) 121.
9 V. Pretorius, B. J. Hopkins and J. D. Schicke, *J. Chromatogr.*, 264(1974) 385.
10 K. A. Cobb, J. Liu and M. Novotny, presented at *40th Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy, Atlanta, GA, March 6–10, 1989,* Abstract 1422.
11 R. M. McCormick, *Anal. Chem.*, 60 (1988) 2322.
12 W. S. Hancock, J. E. Battersby and D. R. K. Harding, *Anal. Biochem.*, 69 (1975) 497.
13 J. W. Jorgenson and K. D. Lukacs, *Clin. Chem.*, 27 1981) 1551.
14 S. Hjerten and M. D. Zhu, *J. Chromatogr.*, 346 (1985) 265.
15 R. J. Wieme, in E. Heftman (Editor), *Chromatography—A Laboratory Handbook of Chromatograpic and Electrophoretic Methods,* Van Nostrand-Reinhold, New York, 3rd ed, 1975, Ch. 10.