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Short communication

Capillary zone electrophoretic separation of proteins using a column coated with epoxy polymer

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Abstract

Fused-silica capillaries used in capillary zone electrophoresis were covalently coated with epoxy polymer and then cross-linked with ethylenediamine to form a stable layer. This coating was of sufficient thickness and hydrophilicity to reduce both protein adsorption and electroosmotic flow. The electroosmotic flow-rate showed about a twofold decrease but was still sufficient to carry both positive and negative species to the detector. As a consequence, high separation efficiencies were obtained in the pH range 2–10. The coatings were stable after more than 350 injections in 2 months. Further, reproducible separations were achieved from run to run and day to day.

1. Introduction

Capillary zone electrophoresis (CZE) is proving to be of great utility in the separation of small molecules such as inorganic ions, amino acids, peptides and nucleotides. Unfortunately, the high resolving power of capillary electrophoresis decreases seriously in the separation of proteins, mainly because of the adsorption of proteins on the inner wall of silica capillaries, which leads to considerable peak broadening and poor reproducibility.

Several approaches have been employed to eliminate the wall adsorption of proteins, e.g., the use of running electrolytes at high [1] or low pH [2]. Although under such circumstances protein adsorption can be greatly decreased, it may limit the selectivity of the system to a narrow pH range and result in protein denaturation. Addition of additives to the running buffer has been applied. Some additives, such as ethylene glycol [3], zwitterions [4] and amines [5], were reported to suppress protein adsorption effectively. However, high concentrations of additives must be used in the running buffer as competitive ions against protein adsorption, and these will also lead to a decrease in the system selectivities. Further, the use of capillary surface coatings has been advocated. The inner surface of silica capillaries was coated with a polymer monolayer which can shield the silanol groups. Several coatings based on the derivatization of capillaries with simple organosilanes have been developed for the CZE of proteins, e.g., glycol [6], polyethylene glycol (PEG) [7,8], poly(methyl gluta-

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mate) [9], carbohydrate [10] and polyacrylamide [11]. Each has attained some success in reducing protein adsorption. However, these silane derivatives are easily hydrolysed under basic conditions and the column performance deteriorates rapidly.

To solve this problem, Cobb et al. [12] treated the capillary surface with a Grignard regent, followed by reaction of acrylamide to form a linear polymer which exhibited enhanced stability at both high and low pH. However, as both the surface adsorption and electroosmotic flow (EOF) were diminished at the same time, cationic and anionic species were swept in opposite directions. Two runs must be made to analyse a mixture of anionic and cationic compounds. Towns and Regnier [13] reported that nonionic surfactant coatings that were adsorbed on the surface of octadecylsilane-derivatized capillaries can reduce protein adsorption. This approach was successful in reducing protein adsorption while still allowing sufficient electroendosmosis to carry both cationic and anionic species to the detector. However, high concentrations of nonionic surfactant must be used in the buffer solution, which may contaminate the sample collected.

Recently, a hydrophilic cross-linked coating based on the polymerization of a bis(epoxide) on the capillary surface was reported [14]. This coating was of sufficient thickness and hydrophilicity to decrease protein adsorption but still allowed sufficient electroosmotic pumping. A high separation efficiency was achieved between pH 5 and 10. However, the results reported were obtained during a 120-h lifetime and the longer use of coatings was not discussed.

This paper is concerned with the preparation of a stable coating that would decrease protein adsorption on capillaries while still maintaining enough electroosmotic flow to carry both positive and negative species to the detector. Towards this aim, an epoxy polymer was covalently bonded on the capillary surface and then cross-linked with ethylenediamine to produce a stable layer that would extend the pH range used and the capillary lifetime.

2. Experimental

2.1. Apparatus

A capillary electrophoresis system was purchased from the Beijing Institute of New Technology Application (Beijing, China). Fused-silica capillaries (Herbei Yongnian Optical Fibre Factory, Herbei, China) of 50 μm I.D. were used to prepare the capillaries. Capillaries were 66 cm long with a separation length of 44 cm. UV detection at 214 nm was applied.

2.2. Reagents

Protein samples were purchased from Shanghai Biochemical (Shanghai, China), except for α-chymotrypsinogen, which was purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical-reagent grade.

2.3. Electrophoresis

Protein solutions of 1.0 mg/ml were introduced into the capillary by syphoning for a fixed time (3 s) at a fixed height (10 cm). Dimethyl sulphoxide (DMSO) was used as a neutral marker. Between runs, the capillary was flushed with separation buffer for 3 min.

2.4. Preparation of capillary

Pretreatment of capillaries

Capillaries were first treated with 1.0 mol/l NaOH for 30 min followed by 15 min of washing with deionized water. Then the capillaries were flushed with 0.1 mol/l HCl for 10 min. Residual HCl was removed in a gas chromatographic oven at 100°C for 1 h under a nitrogen pressure of 400 kPa.

Preparation of γ-glycidoxypropyltrimethoxysilane (GOX)

The capillary was filled with 5% γ-glycidoxypropyltrimethoxysilane solution in methylene chloride and allowed to react for 3 h. After washing with methylene chloride for 10 min, the

capillary was heated at 100°C for 2 h under a nitrogen pressure of 400 kPa.

Preparation of epoxy polymer coating

A thin film of diglycidyl ether of bisphenol A (DEBA) was deposited on the pretreated capillary by aspirating DEBA in methylene chloride solution and cross-linked with ethylenediamine. Residual reagents were removed in a gas chromatographic oven at room temperature for 2 h under a nitrogen pressure of 100 kPa. Then the temperature was raised to 100°C for 1 h.

3. Results and discussion

Reactions in the epoxy polymer coating process are shown schematically in Fig. 1. It should be noted that the structures do not represent the exact structure of the coating. GOX was firstly

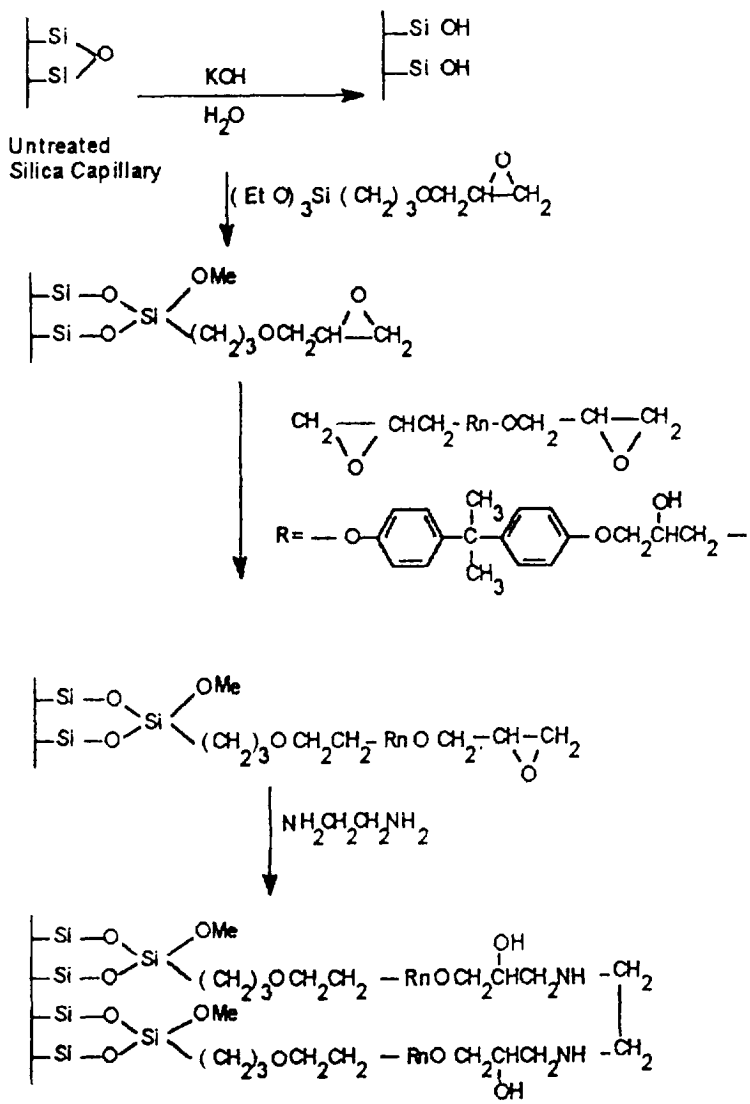

Fig. 1. Typical reaction scheme used in the preparation of epoxy polymer coating.

coupled to the surface silanols and reacted with epoxy polymer (DEBA) to produce a polymer monolayer. Then the surface epoxy groups of DEBA were cross-linked with ethylenediamine to form a stable surface layer. The resulting epoxy polymer contains many ether, hydroxyl and amine groups which may show sufficient hydrophilicity. This cross-linked polymer layer is much more stable than coatings derived from simple organosilanes [6–11]. Towns et al. [14] also used multifunctional oxirane to prepare epoxy polymer coatings, through a five-step process which can effectively reduce protein adsorption at pH 5–10. The present coatings were prepared with only a two-step process and a high-molecular mass epoxy polymer was used to achieve more effective shielding of surface silanols.

3.1. Evaluation of coated capillaries

Masking silanol groups depends on the thickness of the polymer layer deposited on the capillary surface. Therefore, 10%, 50% and 80% epoxy polymer in methylene chloride were used to coat the capillary to determine the optimum conditions. We used four basic proteins to evaluate the coated capillaries. Fig. 2 shows the separation of protein mixtures with coated and uncoated capillaries in 0.05 mol/l phosphate buffer at pH 7.0. Some basic proteins cannot be eluted from the uncoated capillary owing to adsorption on the inner wall (Fig. 2A). However, the four basic proteins can be separated successfully in the coated capillary, which indicates that a substantial decrease in protein adsorption has been achieved.

Table 1 shows the performance of the coated capillaries at the three concentrations examined. With the use of 10% epoxy polymer (coating 1), effective separation can only be achieved at pH 3–5. Above pH 5, the peak shape deteriorated and lysozyme could not be eluted from the capillary. Coating with 50% epoxy polymer (coating 2) showed good efficiency in the pH range 3–7, but the efficiency dropped quickly and both lysozyme and chymotrypsinogen A showed long tailing at pH above 7. This is because the polymer layer deposited on the capillary surface is still not thick enough to mask silanols sufficiently. The use of an 80% epoxy polymer (coating 3) to mask further the surface silanol groups gave good results in terms of efficiency, lifetime and the useful pH range. It should be noted that the efficiency of coating 3 is not as good as that of coating 2 at pH 3–7, probably because epoxy polymer was cross-linked in three directions, and therefore a thicker layer could not be coated as uniformly as a thin layer. However, the EOF of coating 3 is much lower than that of coating 2, which means that more effective shielding of silanols was achieved (Fig. 3). This is very important for a good separation because higher resolution will be achieved at a lower EOF. Further, coating 3 has the longest lifetime and was more stable than others, so it can be used in the pH range 2–11.

3.2. Electroosmotic flow

In CZE, the EOF plays a major role in protein mobility. The protein elution rate is the sum of the EOF and electrophoretic mobility, so decreasing the EOF will improve protein resolution. This is especially true when there is only a small difference among the electrophoretic mobilities of proteins. On the other hand, the EOF is like a pump in liquid chromatography which pumps both positively and negatively charged samples to the detector. Hence it was necessary to maintain a sufficient EOF while decreasing protein adsorption.

The effect of pH on the EOF was examined over the pH range 3–11, using DMSO as a neutral marker. Fig. 3 shows the results obtained for both treated and untreated capillaries. The coated capillaries exhibited a much lower EOF than uncoated capillary because of the effective shielding of surface silanols. However, there was still some increase in EOF over the pH range 3–11, which means that these coatings cannot completely mask all the surface silanols.

The EOF characteristics are different for capillaries coated with different concentrations of epoxy polymer, although they show the same silylation of surface silanols. Coating with 80%

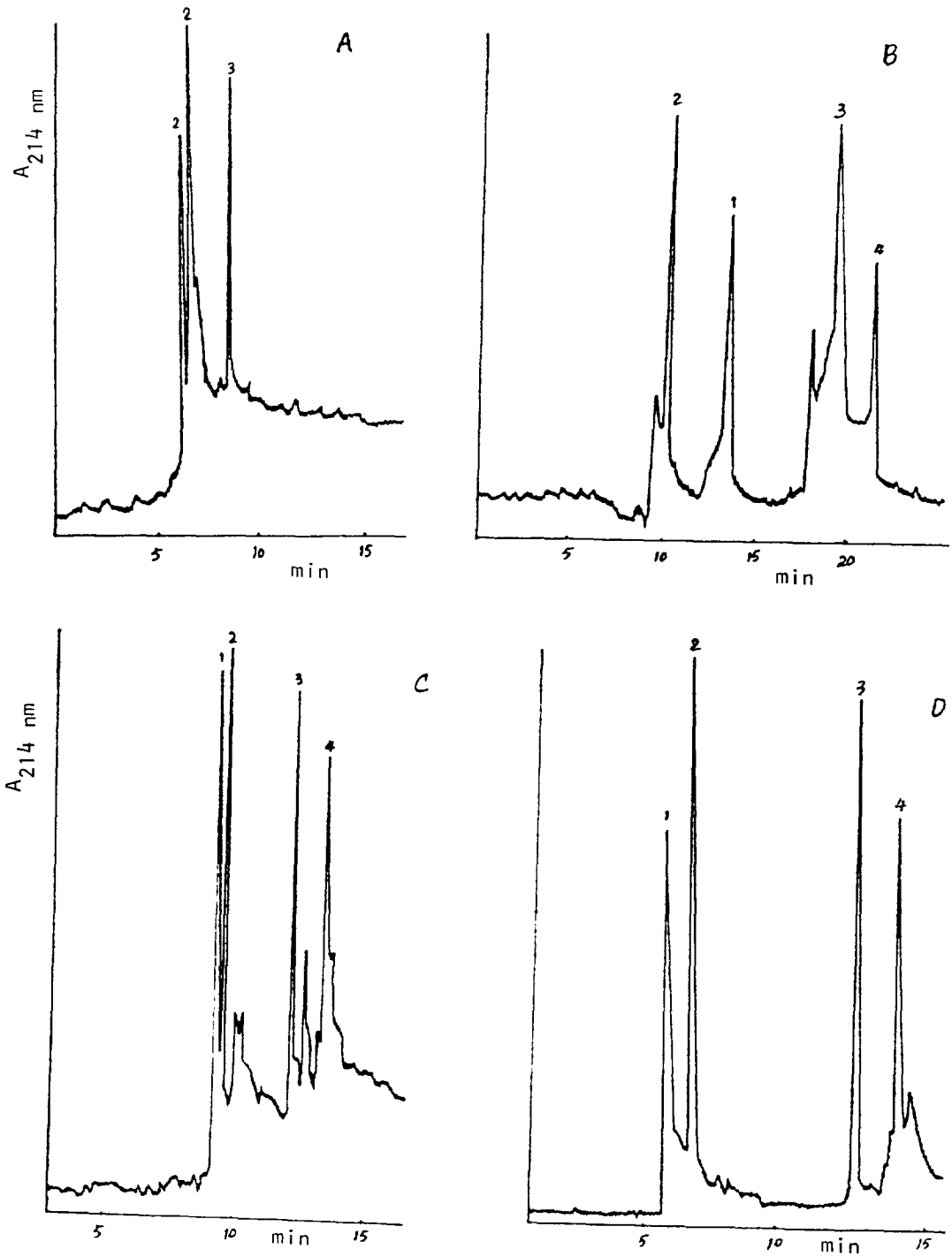

Fig. 2. Capillary electrophoretic separation of a basic protein mixture, using (A) uncoated, (B) epoxy polymer-coated (1), (C) epoxy polymer-coated (2), and (D) epoxy polymer-coated (3) silica capillaries. Electrophoretic conditions: capillaries, 66 cm × 50 μm I.D., 44 cm to detector; 0.05 mol/l phosphate buffer (pH 7 for A, C and D; pH 5 for B); hydrodynamic injection, 5 s at 10-cm height; applied voltage, 19 kV. Peaks: 1 = lysozyme; 2 = cytochrome *c*; 3 = ribonuclease A; 4 = chymotrypsinogen.

Table 1
Separation parameters for epoxy polymer-coated capillaries

Capillary	EOF × 10^{-8} (m^2/V·s)	Plate No.	Useful pH range	Lifetime
Untreated	5.0			
Coating 1	3.3		2–5	20 days
Coating 2	2.1	103 114	2–7	>1 month
Coating 3	1.7	51 210	2–10	>2 months

Electrophoretic condition: capillaries, 66 cm × 50 μm I.D., 44 cm to detector; 0.05 mol/l phosphate buffer (pH 7); applied voltage, 19 kV for protein separation, 24 kV for EOF; lysozyme used to evaluate column efficiency (pH 7); DMSO as neutral marker.

epoxy polymer, the highest concentration used, gives the lowest electroosmotic flow-rate. This is because the higher the concentration of epoxy polymer used, the thicker was the cross-linked layer formed. Hence it can further mask the silanol groups towards the atmospheric binding of electrolyte cations, thus further decreasing the EOF. However, compared with an untreated capillary, the EOF showed a ca. twofold decrease in the pH range 4–7 for all the coated capillaries. This moderate EOF was sufficient to carry both positive and negative species to the detector.

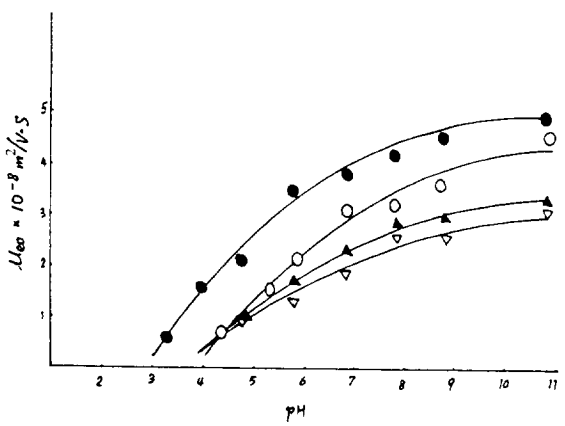

Fig. 3. Dependence of EOF on pH for (●) uncoated, (○) epoxy polymer-coated (1), (▲) epoxy polymer-coated (2) and (△) epoxy polymer-coated (3) silica capillaries.

3.3. Effect of pH on separation

According to the literature [6–11], many coatings based on derivatization with simple organosilanes have been found to be easily hydrolysed above pH 7, because of the unstable Si–O–Si–C bonds. In this work, we also prepared capillaries by silanol derivatization, but the polymer monolayer was further cross-linked with ethylenediamine to form a stable layer. This coating was found to be stable over a wide pH range (2–11). Fig. 4 shows the separation of proteins in coated capillary 3 over the pH range 2–10. Sharp peaks and good efficiencies were obtained at all the pH values used. Therefore, the resolution can be optimized very easily by finding the optimum pH for the specific separation of a protein mixture. Table 2 shows plate numbers for four basic proteins at different pH. It was found that excellent efficiencies and separation were achieved in the pH range 3–8. Below pH 3, although the efficiency is very high, protein denaturation will occur. At pH 10, lysozyme showed slight tailing, which indicated that there were still some unshielding silanols. Chymotrypsinogen A and ribonuclease A, which have a negative charge, should exhibit a slight increase in efficiency; however, the efficiency of ribonuclease A dropped considerably because of its denaturation. Hence both acidic and basic conditions, which may result in protein denaturation, cannot give good separation and efficiencies.

3.4. Reproducibility and stability of the coatings

These studies were carried out on capillaries with coatings 2 and 3. Table 3 summarizes the run-to-run, day-to-day and month-to-month migration reproducibility expressed in terms of relative standard deviation (R.S.D.) with four basic proteins at pH 5.0. It should be noted that the month-to-month reproducibility reflects results obtained when the capillary was further used over the pH range 3–10 for 1 month. Both coatings exhibit good run-to-run and day-to-day reproducibility over their useful pH range. However, after the capillary had been further used

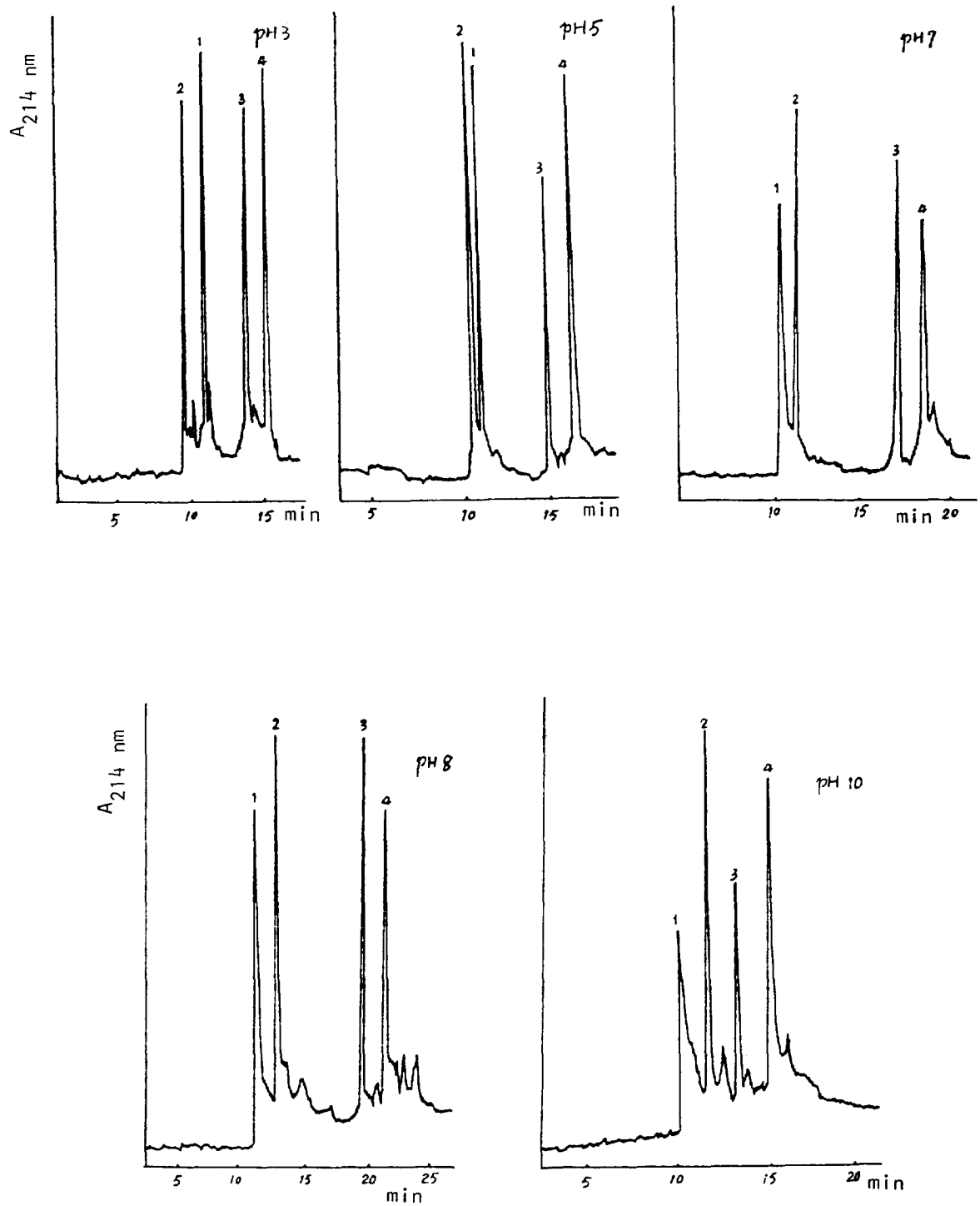

Fig. 4. Separation of basic proteins over the pH range 3–10 on epoxy polymer-coated (3) capillary. Peaks: 1 = lysozyme; 2 = cytochrome *c*; 3 = ribonuclease A; 4 = chymotrypsinogen.

Table 2
Efficiency (plate number) for four basic proteins at various pH values

Protein	pH 2.9	pH 5.0	pH 7.0	pH 8.2	pH 9.5	pH 5 (after 2 months)
Lysozyme	115 070	81 450	51 210	50 850		58 730
Cytochrome *c*	119 170	95 690	104 430	86 560		92 380
Chymotrypsinogen A	87 720	51 060	59 010	58 520	65 410	45 690
Ribonuclease A	167 250	196 990	227 580	100 400	67 030	91 240

Electrophoretic conditions: capillary with coating 3, 66 cm (44 cm to detector); 0.05 mol/l phosphate buffer; applied voltage, 19 kV.

over a wide pH range (2–11), it can be seen that coating 3 is more reproducible than coating 2 simply because the former is thicker and more stable than the latter.

Both coatings 2 and 3 are fairly stable over their usable pH ranges (Table 1). It should be noted that although slight protein adsorption on the coated capillaries was observed under basic conditions, e.g., lysozyme showed slight tailing on coating 3 at pH 10 (Fig. 3), the coatings were still stable and did not hydrolyse under the conditions used. After capillary 3 had been further used for several days over the pH range 9–11, we again used four basic proteins to evaluate its efficiency at pH 5. Table 2 shows the performance parameters of coating 3 that was used after 2 months over a wide pH range (2–11). Sharp peaks were still obtained and there was only a slight decrease in performance in comparison with the plate number obtained earlier. This indicated that coating 3 is fairly stable at basic pH. The slight adsorption problem probably occurred because the coating still does not have enough thickness to mask all the silanols.

4. Conclusions

Capillaries covalently coated with epoxy polymer can produce a hydrophilic cross-linked layer. Compared with other approaches, this coating, which involves only a two-step process, is easy to prepare. It was found that epoxy polymer coatings can greatly reduce protein adsorption and electroosmotic flow. However, it still allows sufficient electroosmotic pumping to carry both positive and negative samples to the detector. High efficiency, symmetrical peaks and reproducible separations can be achieved over a

Table 3
Reproducibility of migration times of proteins on epoxy polymer-coated capillaries

Protein	R.S.D. (%)[a]					
	Coating 2			Coating 3		
	Run-to-run	Day-to-day	Month-to-month	Run-to-run	Day-to-day	Month-to-month
Lysozyme	1.4	2.0	21.2	1.2	2.0	10.6
Cytochrome *c*	0.8	1.1	24.8	1.4	2.3	11.5
Chymotrypsinogen A	2.1	2.9	28.7	1.8	2.4	15.3
Ribonuclease A	2.5	3.2	31.5	2.7	3.6	18.8

Capillaries, 66 cm × 50 μm I.D., 44 cm to detector; 0.05 mol/l phosphate buffer (pH 5); applied voltage, 19 kV.
[a] $n = 6$.

wise pH range of 2–10. Although slight protein adsorption still existed above pH 10, these coatings are fairly stable over the pH range 2–11 and can be used for more than 2 months.

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