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Journal of Chromatography A, 717 (1995) 1–13

JOURNAL OF  
CHROMATOGRAPHY A

# Capillary electrophoretic separation of proteins using stable, hydrophilic poly(acryloylaminoethoxyethanol)-coated columns

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## Abstract

Capillary electrophoretic separations of high efficiency and resolution were obtained using poly(acryloylaminoethoxyethanol)-coated capillaries. The polymer was covalently attached to a silica surface previously modified with a  $\alpha$ -methacryloylpropyl functionality. The latter was realized by catalytic hydrosilylation of allyl methacrylate on an SiH-modified fused-silica capillary. The lifetime of the new type of coating used at pH 8.5 was more than twice that of conventional polyacrylamide.

## 1. Introduction

Capillary electrophoresis is becoming an important tool in the separation and characterization of macromolecules with potentially high efficiency and resolution and automation possibilities. However, the efficiency and reproducibility of capillary columns are in practice significantly lower than those predicted by theory [1,2]. Band-broadening effects occur when large macromolecules distribute themselves between the liquid and the wall; weak reversible interactions strongly affect efficiency while moderate adsorption can impair peak shapes, leading to poor reproducibility. Proteins, having more binding sites, tend to interact with surfaces in different ways and, in uncoated capillaries, characterized by a large surface-to-volume ratio, electrostatic interactions between the limited

amount of injected samples and the large surface may play the most important role.

There are several ways to control electro-osmotic flow and adsorption: (a) buffer changes, high or low pH [3–6], high salt concentrations [7,8] and additives [9–13], (b) adsorption of neutral or charged macromolecules on the wall [14,15] and (c) chemically bonded phases. Regarding the last point, capillaries with covalently bonded polymers are generally obtained by organosilanization, a surface modification procedure derived from the “silane coupling” methodology which has been successfully transferred to capillary electrophoresis. In a general reaction scheme, an organosilane reagent yields an Si–R functionality attached to the support through an Si–O–Si–C– (siloxane) linkage. The R group contains an active functionality which permits the covalent attachment of a polymeric coating. Bruin et al. [16] described glycidoxo-derivatized capillaries to which polyethylene glycol (PEG) 600 was anchored in order to decrease the influence of wall adsorption. PEG-600-modified

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capillaries were used to separate alkaline proteins in acidic buffers, but they are not useful in the more interesting intermediate pH range.

Towns et al. [17] reported diol–epoxy coatings of sufficient thickness and hydrophilicity to reduce protein adsorption. These coatings are stable in both acidic and basic conditions and the electroosmotic flow (EOF) is strong enough to carry both cationic and anionic species past the detector. Although these coatings appear attractive, there are some problems related to the synthesis which limit their applicability. A gradual increase in the EOF was observed during their use; this problem is attributed to the formation of aldehyde groups during the  $\text{BF}_3$ -catalyzed cross-linking step. With time, these groups oxidize to a carboxyl group and increase the negative charge on the capillary wall. Another drawback is related to the nature of the catalyst: boron trifluoride in the form of vapor from an etherate solution is passed through the capillary in a stream of nitrogen to cross-link the oxiranes; delivery of the catalyst in the gas phase limits the length of capillary that can be prepared during the cross-linking step. Nashabeh and El Rassi [18] developed coatings having hydroxylated polyether functions bonded to the surface. These types of coatings, referred to as interlocked or fuzzy coatings, were used in the separation of alkaline proteins at pH 6 or 7.

Among the different types of polymeric phases, polyacrylamide-coated columns appear to be able to suppress electroosmosis to a negligible value. Columns with negligible electroosmosis have broad applicability in separations with polymer networks and in isoelectric focusing; migration times and efficiency are higher when electroosmotic flow is completely suppressed. As early as 1985, Hjertén [2] proposed coating the surface with covalently bonded polyacrylamide strings linked to the wall through  $\gamma$ -(methacryloxy)propylsilyl groups. There are, however, some limitations to the stability of this phase with time. In an effort to overcome this problem, Cobb et al. [19] reported a capillary procedure by which a direct Si–C linkage is formed by sequentially reacting silica with thionyl chloride and the Grignard reagent vin-

ylmagnesium bromide. Linear polyacrylamide is attached to the silica via stable Si–C bonds. Sandoval and Pesek [20] proposed an alternative to a Grignard reagent for the formation of Si–C linkages on silica surfaces. They used catalytic hydrosilylation of olefins on a SiH-containing substrate. In addition to a superior hydrolytic stability, olefin hydrosilylation provides the option of preparing  $\gamma$ -methacryloxypropyl-modified surfaces [21,22] suitable for copolymerization with acrylamide and derivatives.

Excellent results in protein separations with regard to efficiency, peak symmetry and reproducibility were obtained by Schmalzing et al. [23] using a synthetic polyvinylmethylsiloxane diol with a cross-linker, the chemical structure of which is unfortunately unknown. They obtained a polymeric film containing vinyl double bonds, incorporated in polyacrylamide chains cross-linked with formaldehyde.

Polyacrylamide coatings when used at a pH higher than 8.5 show an increase in EOF with time, resulting from partial hydrolysis of the amido bonds. N-Mono- and -disubstituted acrylamide are known to be more stable to hydrolysis; the presence of a long, flexible alkyl chain is capable of protecting the amido plane as it can fluctuate in the nearby space and shroud the amido group. In addition, a number of papers have dealt with the problem of limited stability of siloxane linkages [24,25]. In order to overcome both problems, we have proposed a stable coating which combines the superior hydrolytic stability of an N-substituted acrylamide monomer, namely acryloylaminoethoxyethanol [26,27], with the enhanced surface attachment derived from direct Si–C linkages on the silica surface [19–22].

## 2. Experimental

### 2.1. Materials

3-(Morpholino)propanesulfonic acid (MOPS), tris(hydroxymethyl)aminomethane (Tris), N,N-bis(2-hydroxyethyl)glycine (bicine),  $\alpha$ -chymotrypsinogen A,  $\beta$ -lactoglobulin from bovine

milk, trypsin from bovine pancreas, trypsin inhibitor from soybean, myoglobin from horse skeletal muscle, trypsinogen from bovine pancreas and  $\alpha$ -lactalbumin from bovine milk were purchased from Sigma (St. Louis, MO, USA). Lysozyme from hen egg white and ribonuclease A from bovine pancreas were obtained from Boehringer (Mannheim, Germany), spermine and 3-(trimethoxysilyl)propyl methacrylate from Aldrich Chemie (Steinheim, Germany), acrylamide, ammonium peroxodisulfate and N,N,N',N'-tetramethylethylenediamine (TEMED) from Bio-Rad Labs. (Hercules, CA, USA) and methylcellulose of low molecular mass (Methocel Premium Grade) from Dow Chemical (Midland, MI, USA). Water and all the other reagents were of analytical-reagent grade. N-Acryloylaminoethoxyethanol (AAEE) was synthesized as described by Chiari et al. [26].

## 2.2. Methods

CZE separations were performed in a Waters Quanta 4000 capillary electrophoresis system (Millipore, Milford, MA, USA) and in a Spectra-Phoresis 1000 capillary system (Thermo Separation Products, Fremont, CA, USA). Data collection was performed on a PC computer utilizing SW-Phoresis 1000 software. In all the experiments, 75  $\mu\text{m}$  I.D. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) coated by the methods described below were used. Proteins and benzyl alcohol, used as marker of electroosmosis, were detected at 214 nm. Protein stock solutions (10 mg/ml) were prepared in water and diluted to the final concentration just before the injection. Between runs the column was rinsed by hydrodynamic pressure with the separation buffer for 2 min. Electroosmotic flow was measured at 600 V/cm in 25 mM bicine-Tris buffer (pH 8.5). The samples were injected using hydrodynamic pressure for 0.5 s.

## 2.3. Separation conditions

Separation of the alkaline proteins lysozyme (0.5 mg/ml), ribonuclease A (0.6 mg/ml),

trypsinogen (0.5 mg/ml),  $\alpha$ -chymotrypsinogen (0.5 mg/ml) and myoglobin (0.5 mg/ml) were performed at pH 3.0 and 4.4 in 20 mM phosphate buffer at 270 V/cm, 39  $\mu\text{A}$  and in 50 mM acetate-Tris at 540 V/cm, 26  $\mu\text{A}$ , respectively. The same mixture containing trypsin (0.5 mg/ml) instead of myoglobin was analysed at pH 7.0 using 30 mM MOPS buffer and 1.5 mM spermine at 400 V/cm, 29  $\mu\text{A}$ ; the polarity of the power supply was positive (anodic injection).

The acidic proteins trypsin inhibitor,  $\beta$ -lactoglobulin A and B and  $\alpha$ -lactalbumin were resolved using 25 mM bicine-Tris (pH 8.5) buffer. A field of 500 V/cm was applied and a current of 17  $\mu\text{A}$  was produced. The polarity of the power supply was negative (cathodic injection).

## 2.4. Coating procedure

Capillaries coated with linear polyacrylamide bonded through methacryloxypropylsilyl moieties were prepared as described by Hjertén [2] with some modifications: the capillary was first treated with 1 M NaOH for 5 h, then rinsed and flushed with 0.1 M HCl followed by 0.1 M NaOH. After 1 h it was rinsed with water and tetrahydrofuran (THF). Residual water was eliminated by connecting the capillary to a gas chromatographic oven at 120°C for 45 min under nitrogen pressure. A 50% solution of  $\gamma$ -methacryloxypropyltrimethoxysilane in THF was then pulled through the capillary under pressure for 20 min and allowed to stand for 12 h. After this treatment, the capillary was flushed extensively with THF and water and then filled with a 3% acrylamide solution containing the appropriate amount of catalyst (1  $\mu\text{l}$  TEMED and 1  $\mu\text{l}$  of 40% ammonium peroxodisulfate per ml of gelling solution) and degassed under vacuum (20 mmHg) for 40 min. Polymerization was allowed to proceed overnight at room temperature and then the capillary was emptied by means of a syringe.

Capillaries activated with  $\gamma$ -methacryloxypropyl bonded to the wall through a direct silicon-carbon linkage were prepared as described by Montes et al. [22]. Briefly, a hydride-modified support was formed by reacting tri-

ethoxysilane (TES) with a silica substrate in the presence of water and hydrochloric acid using dioxane as the solvent. The  $\gamma$ -methacryloxypropyl group residue was linked to the hydride-modified substrate by hydrosilylation of allyl methacrylate in the presence of a Pt catalyst.

Poly(AAEE)-coated capillaries were obtained by filling  $\gamma$ -methacryloxypropyl-activated capillaries with a 6% aqueous solution of AAEE containing the appropriate amount of catalyst (1  $\mu$ l of TEMED and 1  $\mu$ l of 40% ammonium peroxodisulfate per ml of gelling solution) and degassed under vacuum (20 mmHg) for 40 min. Polymerization was allowed to proceed overnight at room temperature and then the capillary was emptied by means of a syringe.

## 2.5. Stability test

A mixture of acidic proteins, trypsin inhibitor,  $\beta$ -lactoglobulin A and B and  $\alpha$ -lactalbumin, each at a concentration of 0.5 mg/ml, was injected into both types of capillaries using 25 mM bicine-Tris (pH 8.5) buffer. The capillaries were washed with buffer for 2 min between the runs, the analyte was renewed before each run while the catholyte was changed every ten runs. A voltage of 500 V/cm was applied and the temperature was kept at 25°C. After every 15 h of use the ends of the capillary, up to 3 mm, were dipped in a 0.5% (w/v) solution of methylcellulose (MC) and after 20 min of contact the capillary was extensively rinsed with water (20

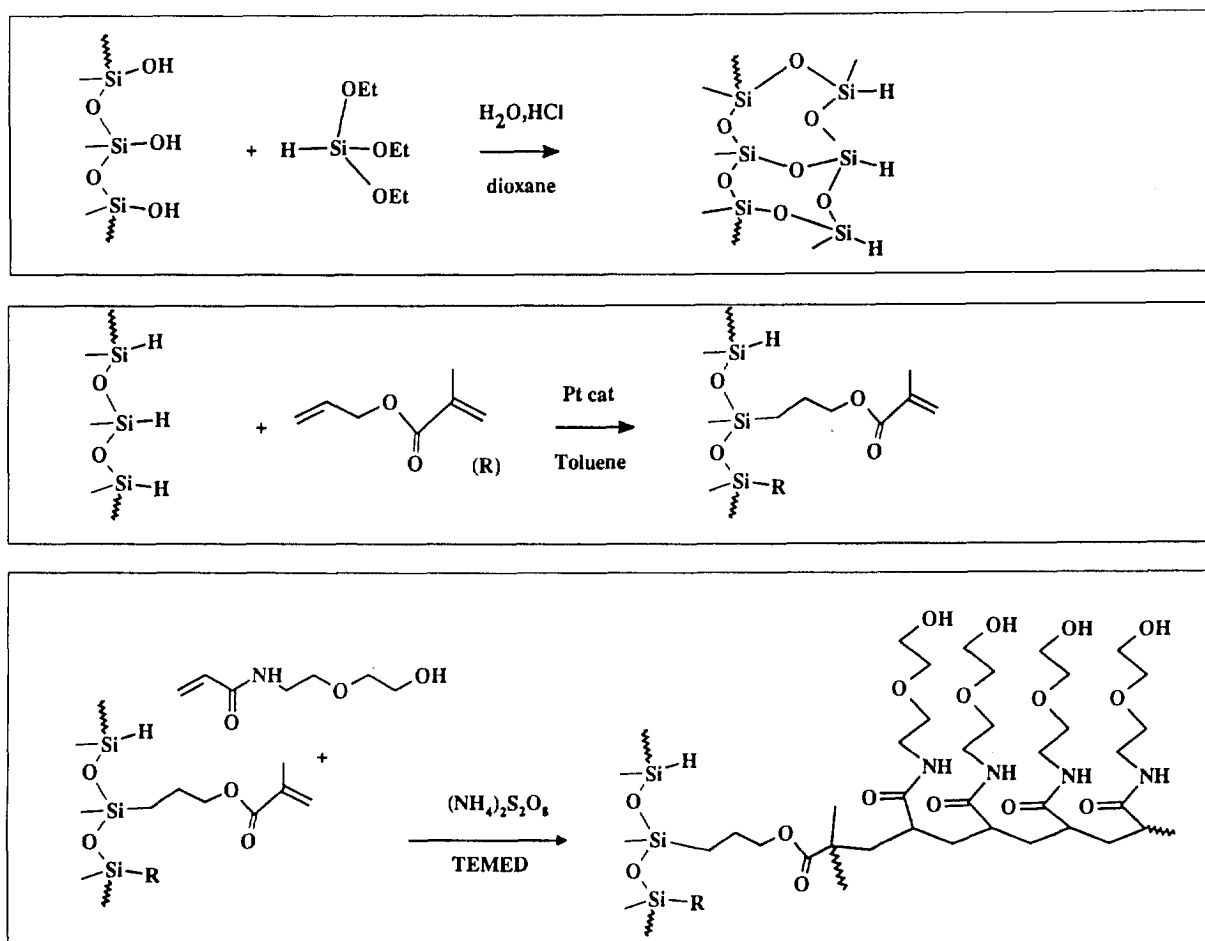


Fig. 1. Schematic representation of the coating procedure.

min) and reconditioned with the alkaline buffer. The EOF was measured at fixed intervals (every 15 h) during the entire test period.

### 3. Results and discussion

#### 3.1. Surface modification

The overall scheme for preparing linear poly(AAEE)-coated capillaries is illustrated in Fig. 1. The general process requires three steps. First, an intermediate hydride-modified substrate is produced by reaction of triethoxysilane (TES) with the silica substrate in the presence of water and an acid catalyst. Second, the methacryloxypropyl group is covalently linked to the hydride-modified substrate through an allyl double bond by hydrosilylation. In the third step, the methacryl group reacts with AAEE, yielding polymeric linear chains attached at multiple sites to the wall. The surface, after the first two steps, has a methacryloxypropyl functionality that is similar to that obtained after a conventional reaction with a trifunctional organosilane reagent. However, a stronger attachment is produced when a larger number of siloxane linkages are formed on reaction with TES.

By means of  $^{29}\text{Si}$  cross-polarization NMR spectrometry it was demonstrated [22] that about 90% of the Si bearing hydride in the intermediate is fully condensed via formation of three siloxane linkages. In the light of these data, it is clear that in a conventional organosilanization reaction the extent of condensation is lower owing to the steric hindrance of the bulky alkyl groups.

When compared with acrylamide, the acryloylaminoethoxyethanol monomer offers the hydrolytic advantage derived from the N-substitution in acrylamide with the high hydrophilicity associated with a diethylene glycol group. N-Mono- and -disubstituted acrylamides are known to be generally more resistant to alkaline hydrolysis; flexible chains bound to the nitrogen are capable of protecting the amido plane as they can fluctuate in the nearby space and shroud the amido group. However, when alkyl chains are

linked to the nitrogen they should bear OH groups in order to lessen the hydrophobicity of the substituent. In the AAEE monomer the hydroxyl group is distant enough from the amido group to prevent the formation of five-, six- and seven-membered rings closing on to the amido linkage. Such rings favor hydrolysis of the amido bond via a mechanism of N,O-acyl transfer [28].

#### 3.2. Stability test at basic pH

An investigation of the lifetime of the polyacrylamide- and poly(AAEE)-coated capillaries was performed by monitoring EOF variations, efficiency and migration time reproducibilities. Fig. 2 shows the separation of a mixture of four acidic proteins achieved at pH 8.5. This mixture was injected into both types of capillaries until the EOF reached a value incompatible with an efficient separation. The relative standard deviations of migration times of proteins were very high in the last series of 20 injections performed in polyacrylamide-coated capillaries, and the plate counts were ten times lower than the initial value.

When performing long-term stability tests at alkaline pH, one of the problems is the adsorption of proteins at the capillary inlet. In most of the capillaries that we have examined, after about 20 h of use at pH 8.5, the electroosmotic mobility was of the order of  $3 \cdot 10^{-9} \text{ m}^2/\text{V} \cdot \text{s}$ . The EOF became negligible again after the removal of a few millimeters from the capillary ends.

Protein-wall interactions have been the subject of a number of previous studies [29,30] because this phenomenon leads to considerable peak broadening and asymmetry and causes migration time irreproducibility. Towns and Regnier [31] demonstrated that the adsorption of positively charged species which occurs preferentially at the capillary inlet produces a non-uniform axial distribution of the zeta potential within the column. Although proteins with an isoelectric point significantly lower than the pH of the running buffer were employed in this study in order to minimize electrostatic interactions with the wall surface, there are other

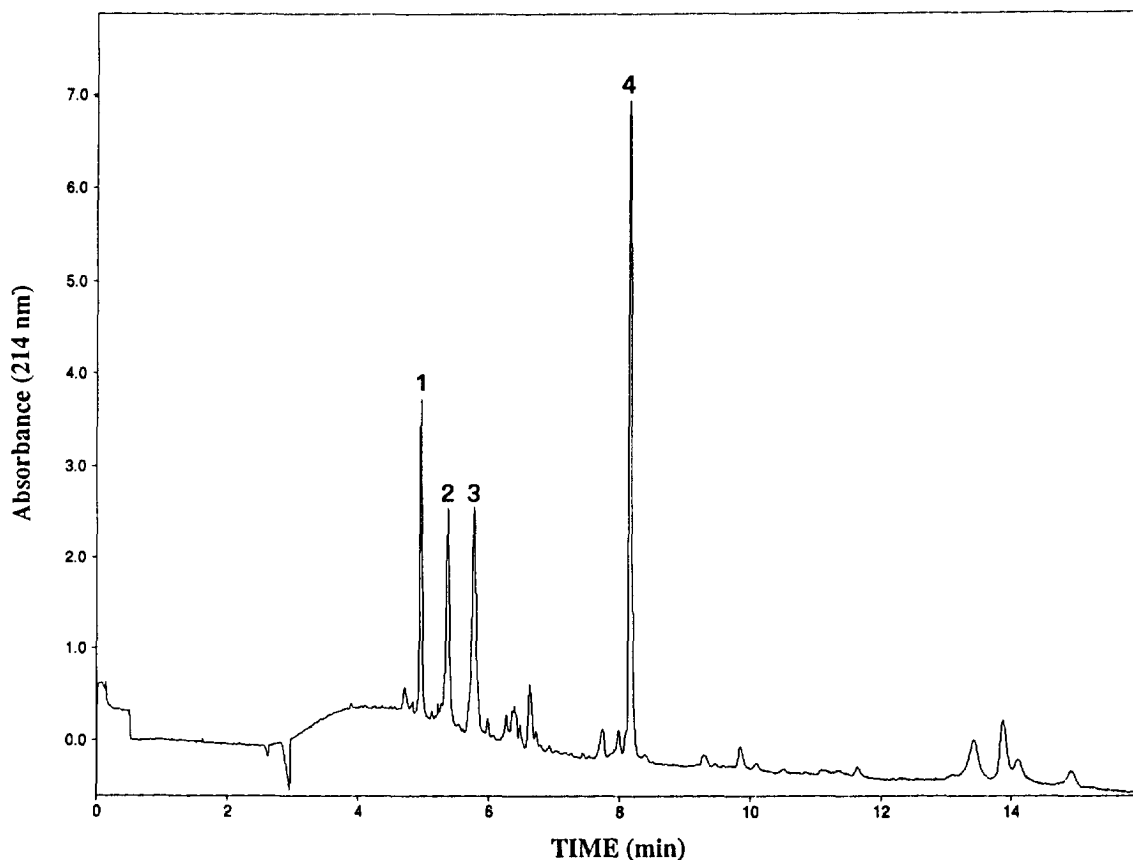


Fig. 2. Typical electropherogram of acidic proteins obtained in a poly(AAEE)-coated capillary (40 cm total length, 32 cm inlet to window) at 25°C, using 500 V/cm (17  $\mu$ A), UV detection at 214 nm and 25 mM bicine-Tris (pH 8.5). Samples: 1 = trypsin inhibitor; 2 =  $\beta$ -lactoglobulin A; 3 =  $\beta$ -lactoglobulin B; 4 =  $\alpha$ -lactalbumin. Reversed polarity, cathodic injection.

possible adsorption modes with the capillary such as hydrogen bonding and hydrophobic interactions which are known to play an important role in the process. In addition, degradation of the coating may occur preferentially at the capillary ends as a result of a possible heterogeneity of the electric field intensity, which increases in the vicinity of sharp corners. During electrophoresis, in particular when high voltage and an alkaline pH are employed, erosion of the coating may take place, thus exposing regions of uncoated silica surfaces in the proximity of the capillary inlet that could be responsible for poor peak shapes and generation of electroosmosis.

A simple solution to both problems, protein adsorption and degradation of the coating at the

capillary ends, is represented by dynamic coating of the ends with methylcellulose. Methylcellulose coatings have been used successfully in both capillary zone electrophoresis and isoelectric focusing and their use in combination with hydrophilic coatings has been reported [32,33]. In a dynamic coating, in order to prevent leakage of the polymer from the wall, a small amount of polymer, typically ranging from 0.01 to 0.4%, is added to the running buffer. In our case, the treatment with MC was limited to the ends of the capillary, since the aim of our investigations was stability of the polymeric coating covalently linked to the wall. Both types of capillaries, one coated conventionally with polyacrylamide and the other coated with poly(AAEE), both as described under Experimental, were subjected to

Table 1  
Reproducibility of migration times

Protein	Poly(AAEE)		Polyacrylamide	
	Average (min)	R.S.D. (%)	Average (min)	R.S.D. (%)
Trypsin inhibitor from soybean	5.12 <sup>a</sup>	0.55	5.68 <sup>a</sup>	1.10
	4.95 <sup>b</sup>	0.46	5.54 <sup>c</sup>	3.60
$\beta$ -Lactoglobulin A	5.42	0.78	6.26	1.86
	5.38	1.27	6.00	4.71
$\beta$ -Lactoglobulin B	5.80	0.86	6.87	2.27
	5.85	1.9	6.52	5.00
$\alpha$ -lactalbumin	8.37	0.78	9.51	1.51
	8.24	0.75	9.43	6.27

25 mM bicine-Tris (pH 8.5) buffer; 500 V/cm.

<sup>a</sup>  $n = 40$  (runs 1-40).

<sup>b</sup>  $n = 40$  (runs 260-300).

<sup>c</sup>  $n = 20$  (runs 130-150).

the MC treatment after every 15 h of use. In both cases this treatment improved the capillary performance. The dynamic coating of the ends physically shields any charges on the wall deriving either from adsorbed proteins or from exposed silanols. Poly(AAEE)-coated capillaries, owing to their higher stability, could be used in the separation of acidic proteins at pH 8.5 for 100 h, with 300 runs of 20 min each, whereas polyacrylamide-coated capillaries after 40 h (120 injections) of use showed strong variations in the migration times and a dramatic decrease in efficiency. In polyacrylamide-coated capillaries the treatment with MC restored the initial characteristics, but this effect was temporary and was lost after a few runs (typically less than ten).

Table 1 shows the reproducibility of migration times for consecutive runs at pH 8.5 in both types of capillaries. The average retention times of the first series of 40 injections are compared with the last series of 40 injections; in poly(AAEE)-coated capillaries a total number of 300 injections (corresponding to 100 h of use) were performed, whereas in polyacrylamide-coated capillaries only 150 injections were carried out. In this case the first 40 injections were compared with the last 20, since the profile of the 150th run, which is the 15th run after the MC treatment, was not interpretable. The average migration times are lower in both cases in the last series of injections, probably as a consequence of the MC treatment. The efficiency (see Tables 2

Table 2  
Efficiency for four basic proteins run at different pH values

Protein	Efficiency (plates/m)			Protein pI <sup>a</sup>
	pH 3.0	pH 4.4	pH 7.0	
Lysozyme (egg white)	356 000	614 000	316 000	11.0
Ribonuclease A	156 000	605 000	353 000	9.3
Trypsinogen	361 000	415 000	201 000	8.7
$\alpha$ -Chymotrypsinogen A	429 000	445 000	149 000	9.5

Separation conditions as in Figs. 4, 5 and 6.

<sup>a</sup> pI values from Refs. [11] and [19].

Table 3  
Efficiency for four acidic proteins run at pH 8.5

Protein	Efficiency (plates/m)	$pI^a$
Trypsin inhibitor from soybean	370 000	4.5
$\beta$ -Lactoglobulin A	187 000	5.13
$\beta$ -Lactoglobulin B	131 000	5.23
$\alpha$ -Lactalbumin	585 000	4.8

Separation conditions as in Fig. 2.

<sup>a</sup>  $pI$  values from Refs. [11] and [19].

and 3) and the reproducibility of migration times between run numbers 1 and 300 in poly(AAEE)-coated capillaries (Fig. 3a) confirm the increased quality of the new phase, combining stable

attachment to the silica with a stable polymeric layer. In contrast, over a period of 150 h the conventional polyacrylamide-coated capillaries were seriously damaged (Fig. 3b). Even though the MC treatment restores the initial performance (see lane 2 in Fig. 3B, representing the ninth run after the treatment), the effects of such a treatment are quickly lost (lane 3) and in the last series of injections the R.S.D. is very high.

Another parameter which was evaluated as a quality criterion was electroosmosis. The EOF was determined with a buffer at high pH and low ionic strength to maximize silanol ionization. Notwithstanding this, the EOF was found to be reduced to a negligible value in columns coated with both linear poly(AAEE) and linear polyacrylamide. The initial EOF, calculated using an

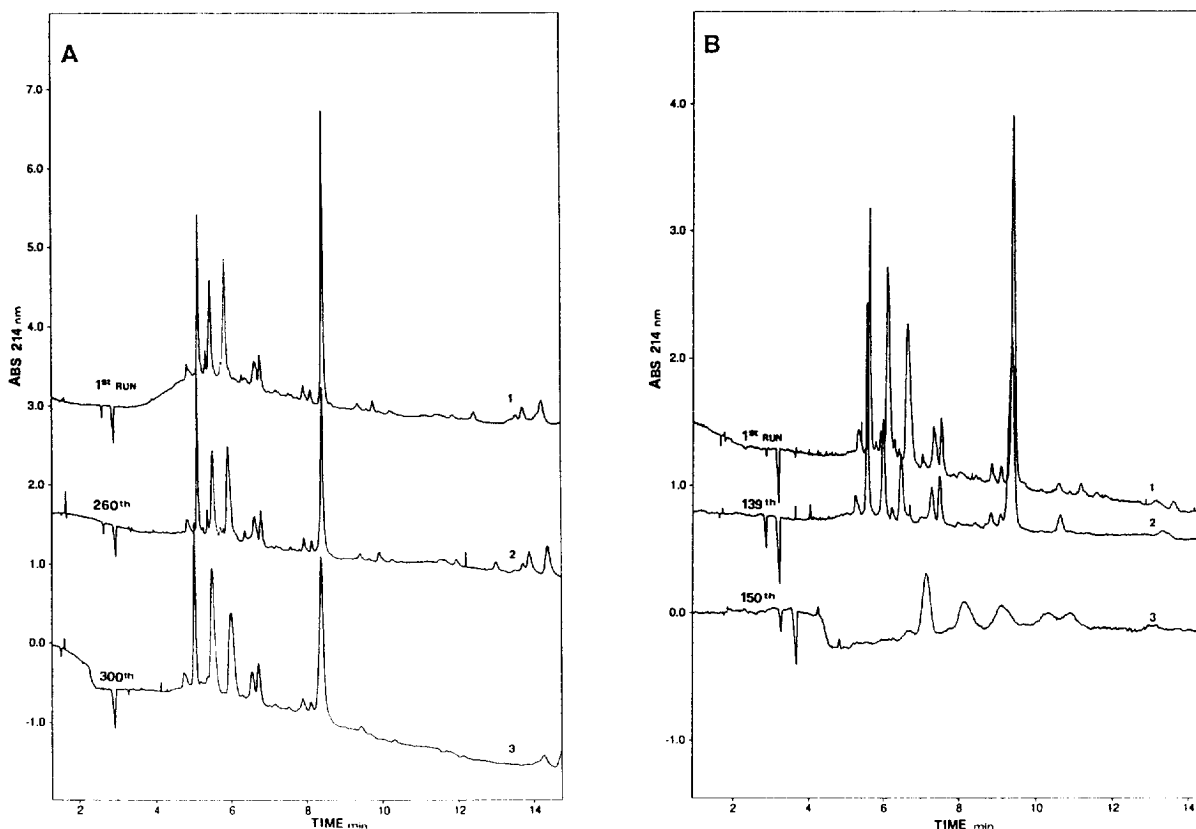


Fig. 3. (A) Protein separations in poly(AAEE)-coated capillary, 1st, 260th and 300th runs. Conditions as in Fig. 2. Samples, from the left: 1 = trypsin inhibitor; 2 =  $\beta$ -lactoglobulin A; 3 =  $\beta$ -lactoglobulin B; 4 =  $\alpha$ -lactalbumin. (B) Protein separations in polyacrylamide-coated capillary, 1st, 139th and 150th runs (44 cm total length, 36 cm inlet to window), conditions as in Fig. 2. Samples, from the left: 1 = trypsin inhibitor; 2 =  $\beta$ -lactoglobulin A; 3 =  $\beta$ -lactoglobulin B; 4 =  $\alpha$ -lactalbumin.



equation proposed by Jorgenson and Lukacs [1], was lower than  $4 \cdot 10^{-10} \text{ m}^2/\text{V}\cdot\text{s}$ . Such a reduction was one of goals of this work as it influences other parameters characterizing the quality of a coating such as efficiency and transit time reproducibility. In evaluating such parameters we found that the performance of coated capillaries is acceptable as long as the EOF is lower than  $1.2 \cdot 10^{-9} \text{ m}^2/\text{V}\cdot\text{s}$ . When the EOF reached such a limiting value the plate counts became at least ten times lower than the initial values and the R.S.D. of the migration times higher. This means that a low EOF can be associated with high efficiency and migration time reproducibility. The EOF variations as a function of hours of continuous use are summarized in Fig. 4. The electroosmotic mobility was measured every 15 h after the removal of 3 mm from the capillary ends in order to avoid the presence of degraded ends influencing the determination. With polyacrylamide coatings the EOF increased gradually with time and after 80 h it was found to be  $3 \cdot 10^{-9} \text{ m}^2/\text{V}\cdot\text{s}$ , whereas with poly(AAEE) coatings, the EOF increased to  $0.8 \cdot 10^{-9} \text{ m}^2/\text{V}\cdot\text{s}$  and after 100 h of use the plate count was still acceptable. The lifetime of the new of coating was more than twice that of conventional polyacrylamide.

### 3.3. Protein separations

The goals of this work were to produce capillaries with a stable hydrophilic coating capable of suppressing the EOF to a negligible value, and to investigate the variation of migration reproducibility and efficiency caused by an EOF increase after prolonged use at pH 8.5 in the presence of an applied potential. Acceptable reproducibilities may be difficult to achieve without accurate control of the capillary temperature during CE separations. The instrument employed in the protein separations utilized a Peltier thermoelectric device to control the capillary temperature. When polyacrylamide-coated capillaries are used for the separation of proteins, a significant difference is observed between the number of theoretical plates effectively obtained and the values predicted by the theory, probably owing to insufficient masking of the silanol groups. Some workers have hypothesized that hydrophobic interactions [19] or hydrogen bonding [34] cause a reduction in efficiency. The high plate numbers that we achieved in the separation of basic and acidic proteins using poly(AAEE)-coated capillaries indicate that virtually no interactions occurred between proteins and the silica surface (Tables 2

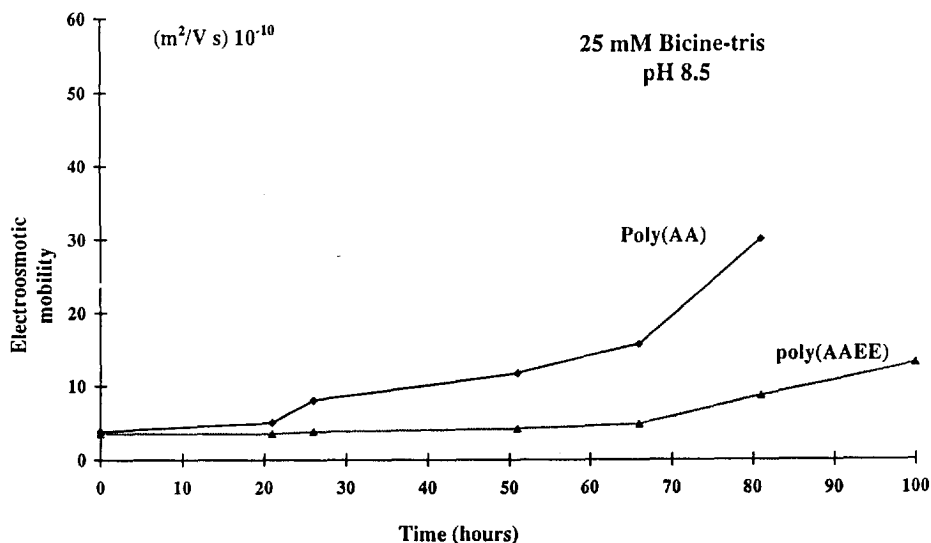


Fig. 4. Variation of electroosmosis as a function of hours of use in 25 mM bicine-Tris buffer (pH 8.5). EOF was measured using benzyl alcohol as a neutral marker in bicine-Tris buffer (25 mM, pH 8.5), 600 V/cm, 23  $\mu\text{A}$ .

and 3). The coating procedure adopted here provides efficient shielding of the surface, thus precluding proteins from interacting with silanols. In addition, the hydrophilicity of the polymer reduces hydrophobic interactions. Alkaline proteins, being positively charged at most operative pH values, interact strongly with the wall. These proteins are often used to test the quality of a coating since in uncoated or inefficiently coated capillaries they are irreversibly adsorbed by the wall, leading to inefficient and irreproducible separations. In many cases no peaks are visible at the detector end. The features of this coating allow pH variations to optimize selectivity without a decrease in efficiency. A possible strategy to improve the

separation of basic proteins would be to work at acidic pH. In this way, silanol ionization is reduced and protein charges are maximized.

Fig. 5 shows the separation of five alkaline proteins performed at pH 3.0 in a poly(AAEE)-coated capillary. The peak shapes demonstrate that no interaction occurred between proteins and the surface but the resolution is poor. The lack of resolution is a typical drawback of the use of extreme pH. In Fig. 6, the same mixture was analyzed at pH 4.4. All peaks were resolved and the higher number of theoretical plates obtained in the separation (see Table 2) again indicates that no interactions with the wall were observed.

At extreme pH many proteins are significantly denatured and in their denatured state irrevers-

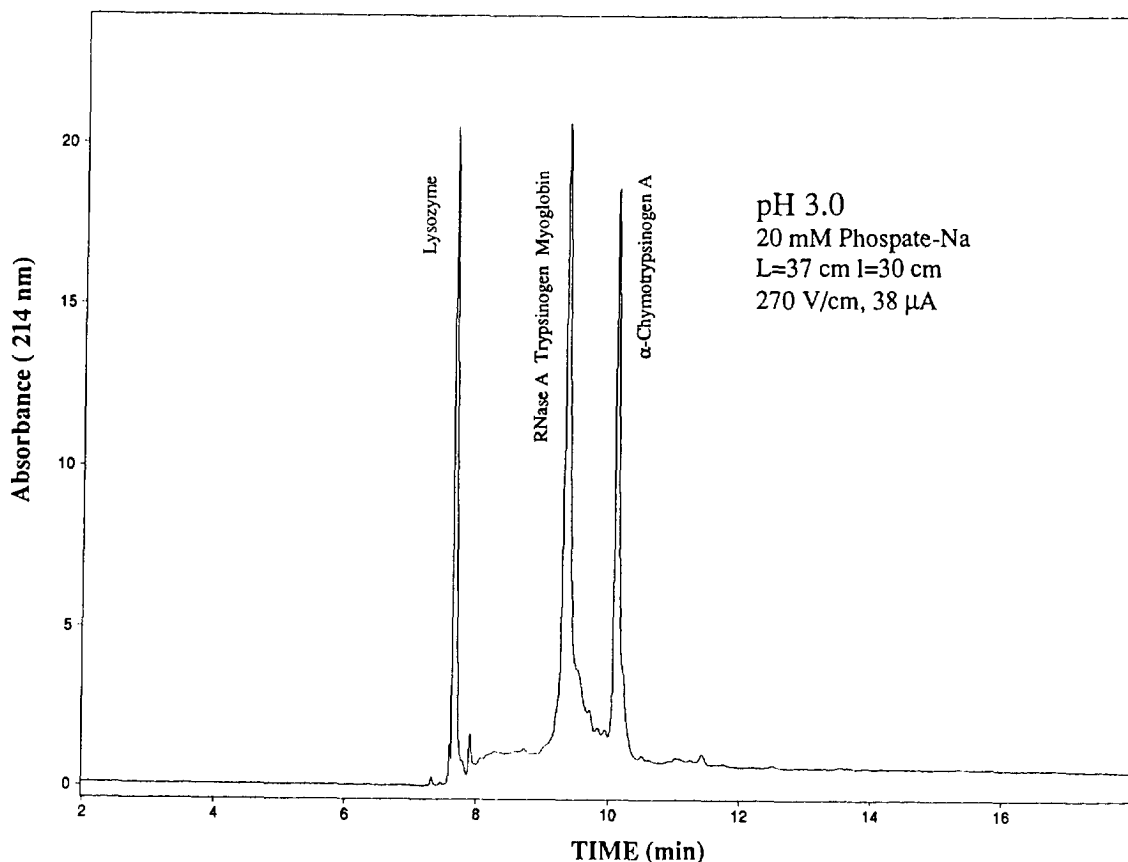


Fig. 5. Electropherogram of alkaline proteins obtained in a poly(AAEE)-coated capillary (36 cm total length, 28 cm inlet to window) at 25°C, using 270 V/cm (39  $\mu$ A) applied field, UV detection at 214 nm and 20 mM sodium phosphate (pH 3.0). Samples: 1 = lysozyme; 2 = ribonuclease A, trypsinogen, myoglobin; 3 =  $\alpha$ -chymotrypsinogen A. Anodic injection.

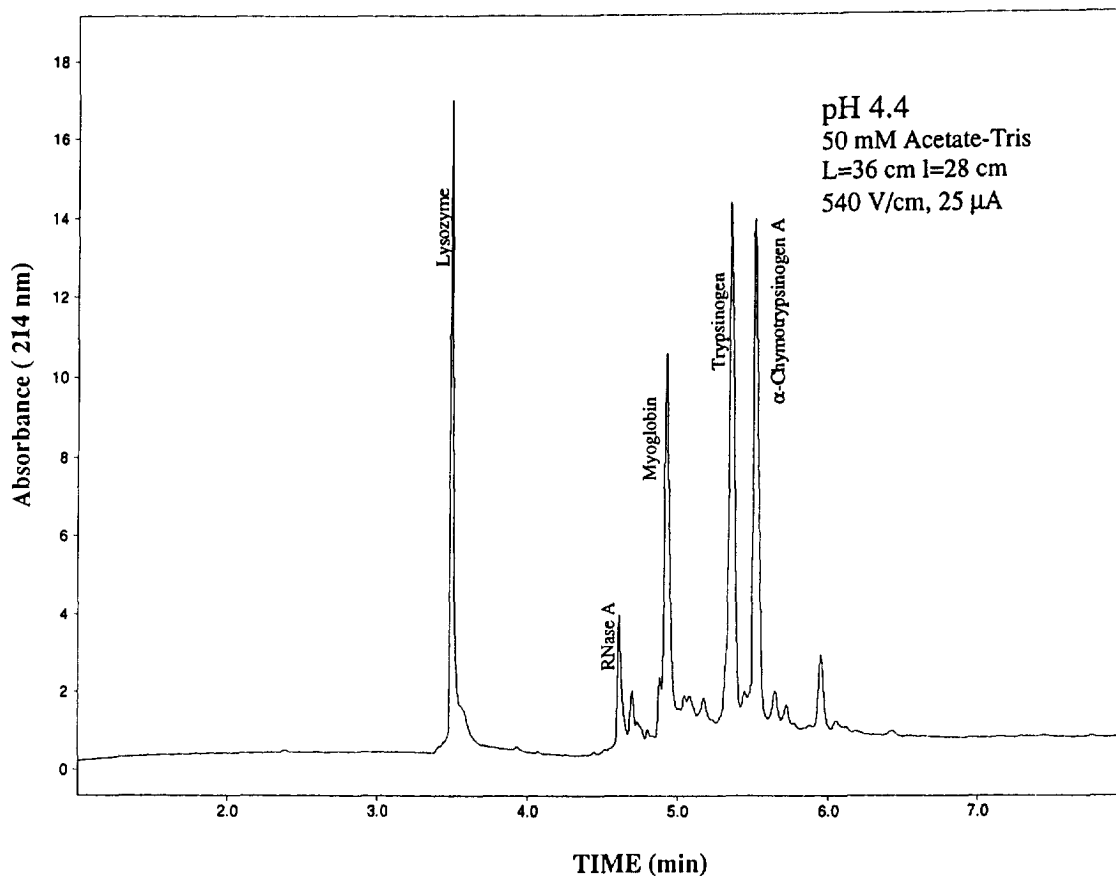


Fig. 6. Electropherogram of alkaline proteins obtained in a poly(AAEE)-coated capillary (36 cm total length, 28 cm inlet to window) at 25°C, using 540 V/cm (39  $\mu$ A) applied field, UV detection at 214 nm and 50 mM acetate–Tris (pH 4.4). Samples: 1 = lysozyme; 2 = ribonuclease A; 3 = myoglobin; 4 = trypsinogen; 5 =  $\alpha$ -chymotrypsinogen A. Anodic injection.

ible adhesion to the wall may occur since proteins expose additional binding sites. We therefore tried to separate alkaline proteins at neutral pH in a buffer of low ionic strength. As indicated by the electrophoretic titration curves, the selectivity reaches a maximum value at this pH. It has been reported by Schmalzing et al. [23] that when alkaline model proteins are analyzed at pH 7.0, a small amount of cationic additives is necessary to provide highly efficient separations. Spermine at a concentration of 1.5 mM was used as an additive (Fig. 7) and, similarly to what was seen previously [23], with a poly(AAEE) coating too the addition of such a cationic additive does

not lead to a reversal of EOF as observed in uncoated capillaries.

#### 4. Conclusion

From the foregoing experiments, it can be concluded that the lifetime of capillaries coated with poly(AAEE) bonded to the wall through direct Si–C linkages is at least doubled. With this new type of phase, the EOF was suppressed to a negligible value. Highly efficient separations of acidic and basic proteins were achieved at different pHs and low ionic strength, owing to efficient

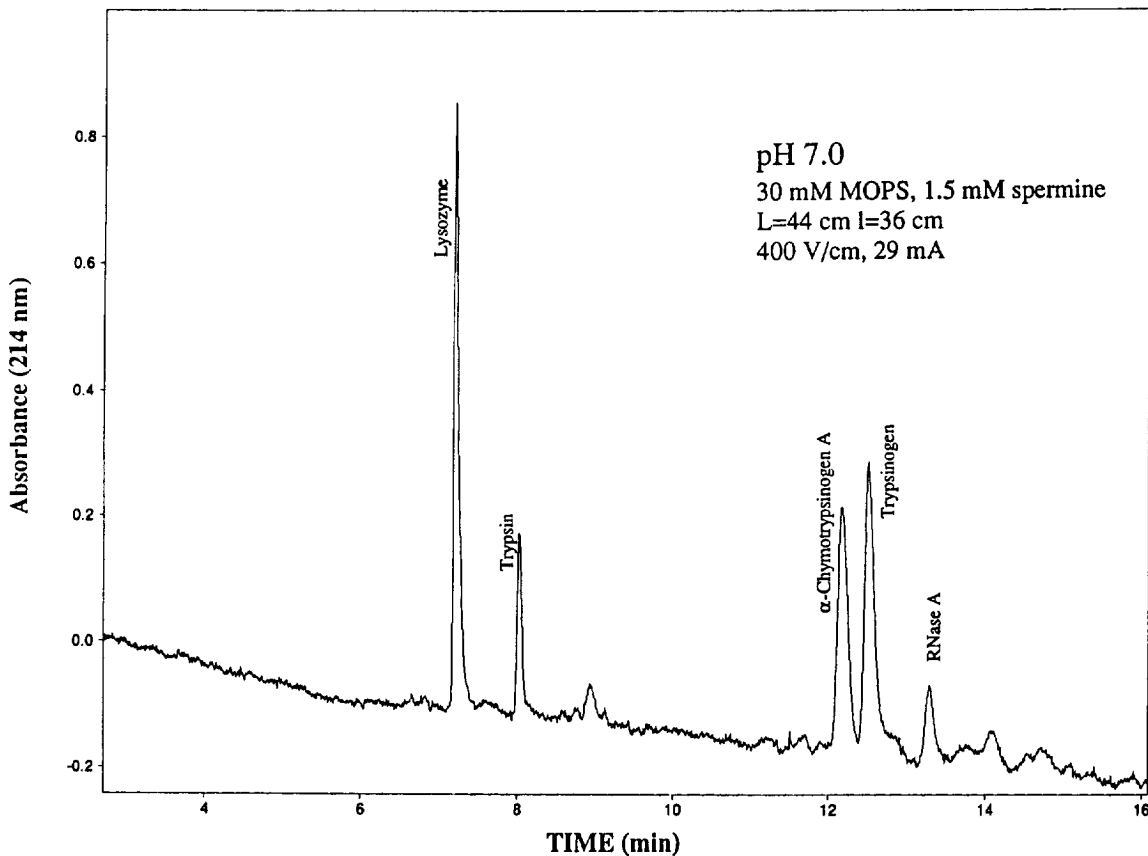


Fig. 7. Electropherogram of alkaline proteins obtained in a poly(AAEE)-coated capillary (44 cm total length, 36 cm inlet to window) at 25°C, using 400 V/cm (29  $\mu$ A) applied field, UV detection at 214 nm and 30 mM MOPS-Na (pH 7.0) and 1.5 mM spermine. Samples: 1 = lysozyme; 2 = trypsin; 3 =  $\alpha$ -chymotrypsinogen A; 4 = trypsinogen; 5 = ribonuclease A. Anodic injection.

surface shielding and to the hydrophilic character of the polymer chains attached to the wall.

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