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Capillary electrochromatography of proteins and peptides with porous-layer open-tubular columns

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Abstract

Capillary electrochromatography (CEC) of basic proteins and peptides was carried out with porous-layer open-tubular (PLOT) columns which had a functionalized rugulose polymeric porous layer grafted to the innerwall of 20 μm I.D. fused-silica capillaries. The porous layer was highly crosslinked and prepared by in situ polymerization of vinylbenzyl chloride and divinylbenzene in the presence of 2-octanol as a porogen inside a pretreated fused-silica capillary. The chloromethyl functions at the surface of the porous polymeric support layer were reacted with *N*,*N*-dimethyldodecylamine to obtain a positively charged chromatographic surface with fixed C_{12} alkyl chains. A mixture of lysozyme, cytochrome *c*, ribonuclease A and α-chymotrypsinogen A was separated isocratically by counterdirectional CEC with hydro–organic mobile phases containing acetonitrile and phosphate buffer, pH 2.5. The overall migration behavior of the four proteins was the result of an interplay of chromatographic retention and electrophoretic migration, and was different from that observed in capillary zone electrophoresis or in reversed-phase chromatography under similar conditions. The separation of three basic peptides by CEC also exhibited the same behavior. The stability of the PLOT column was tested by measuring electroosmotic mobility during continual use.

Keywords: Capillary columns; Porous-layer open-tubular columns; Electrochromatography; Proteins; Peptides

1. Introduction

Although the concept of capillary electrochromatography (CEC) was already introduced in the early 1970s [1], it has become practicable only upon the advent of fused-silica capillaries in chromatographic and electrophoretic separations [2]. CEC embodies many features of both capillary zone electrophoresis (CZE) and micro high-performance liquid chromatography (μ-HPLC), with one very important difference: it employs electroosmotic flow (EOF) of the mobile phase generated in high electric field. The major advantage of EOF over pressure-driven flow employed in HPLC is the reduced band spreading and concomitantly the potential for high peak capacity that is one of the main sources of the present interest in CEC [3]. Lack of understanding of the details of the separation mechanism and availability of suitable stationary phases are implicated to be the major roadblock in development of high-molecular-mass sample components.

So far CEC has been mostly used for separating small neutral molecules by reversed-phase chromatography with capillary columns packed with silica

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based stationary phase particles developed originally for HPLC [4–10] or with a porous monoliths [11]. CEC with octadecylated silica gel particles are usually carried out at neutral or alkaline pH so that the residual silanols at the stationary phase surface are dissociated and the fixed negative charges generate a vigorous EOF upon applying the electric field. However, this approach is not suitable for separation of proteins due to problems arising from electrostatic interactions between proteins and the dissociated silanol groups. Although some preliminary results are promising [12,13] the applicability of CEC to the separation of proteins and other biomacromolecules is yet to be demonstrated. This offers a particularly intriguing challenge to design novel stationary phases and columns for the separation by CEC of complex polyelectrolytes such as proteins.

This work is aimed at the preparation of open-tubular columns to separate proteins by CEC. In order to enhance the loading capacity of such columns and retain their high permeability a porous layer of the stationary phase was formed at the innerwall of fused-silica capillaries. Such porous-layer open-tubular (PLOT) columns have played a useful role in gas chromatography since the early 1960s [14,15], but like other kinds of open-tubular columns, they have been subject of only scattered investigation in liquid chromatography since the late 1970s [16–19]. It has been shown [20] that due to the slowness of diffusion in the liquid phase the capillary inner diameter ought to be less than 15 μm in order to obtain column efficiencies comparable to those in gas chromatography. However, CEC employs EOF and therefore band spreading is expected to be smaller in CEC than in conventional liquid chromatography with viscous flow under otherwise identical conditions although the beneficial effect of the flat (bulk) flow profile greatly diminishes when the tube wall is retentive and the retention factor is greater than 0.5 [21–25]. Thus CEC with open-tubular columns may represent a practicable proposition as demonstrated by some investigations [24,26]. In our approach a porous styrenic support layer is polymerized at the innerwall of 20 μm I.D. fused-silica capillaries. The surface of the support is functionalized in order to have fixed charges and appropriate chromatographic binding sites.

The use of PLOT columns was prompted by their high permeability that allows the use of CZE instrumentation for CEC without modification and by the relatively high loading capacity of PLOT columns in comparison to other open-tubular columns and their low phase ratio in comparison to packed capillary columns. Furthermore, PLOT columns do not require frits to retain the stationary phase, which is anchored to the innerwall of the fused-silica capillary. The goal of this study is to explore the utility of PLOT columns with fixed quaternary ammonium groups and dodecyl moieties at the chromatographic surface for the separation of basic proteins in a counterdirectional CEC process.

2. Experimental

2.1. Materials

Fused-silica capillary tubing with a polyimide outer coating, of 20 μm I.D.×375 μm O.D. was purchased from Quadrex (New Haven, CT, USA). 3-(Trimethoxysilyl)propyl methacrylate was purchased from Polysciences (Warrington, PA, USA), 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), *N*,*N*-dimethyldodecylamine and sodium trifluoroacetate (98%) from Aldrich (Milwaukee, WI, USA), monobasic, dibasic and tribasic sodium phosphate, dimethylformamide (DMF) (99.9%) from J.T. Baker (Phillipsburg, NJ, USA), divinylbenzene (DVB) (85%) and vinylbenzyl chloride from Dow (Midland, MI, USA), azobisisobutyronitrile (AIBN) (98%) from Pfaltz & Bauer (Waterbury, CT, USA). Lysozyme (chicken egg-white), cytochrome *c* (horse heart), ribonuclease A (bovine pancreas), α-chymotrypsinogen A (bovine pancreas), morphiceptin (hydrochloride salt), substance *P* (acetate salt), melittin (bee venom), and benzyl alcohol (approx. 99%) were purchased from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Burdick & Jackson (Muskegon, MI, USA). Phosphoric acid (85%) and sodium hydroxide (98.8%) were of analytical reagent grade from Mallinckrodt (Paris, KY, USA). HPLC-grade methanol, acetone, acetonitrile (ACN) and methylene chloride, and purified grade 2-octanol were purchased from Fisher (Fair Lawn, NJ, USA). Water was purified and deionized with a NANOpure system (Barnstead,

Boston, MA, USA). DVB was washed with 10% (w/v) aqueous sodium hydroxide to remove the inhibitors. The other materials were used without further purification.

2.2. Apparatus and analytical procedure

Experiments were performed using a Model P/ACE 2200 capillary electrophoresis unit (Beckman, Fullerton, CA, USA) equipped with a UV detector and controlled by a NEC personal computer PowerMate SX/20 operated with a Beckman System Gold software.

In all experiments 10–40 m*M* aqueous sodium phosphate buffers, pH 2.5, containing acetonitrile of various concentrations were used. The four standard proteins were dissolved in deionized water to obtain a solution containing 1.0 mg/ml of each protein. Concentration of each peptide in deionized water was 0.3 mg/ml. The samples were injected at 0.5 p.s.i. for 15 s (1 p.s.i.=6894.76 Pa). Between runs the column was rinsed with acetonitrile for 5 min and then with the running mobile phase for at least 5 min at 20 p.s.i. inlet pressure. Benzyl alcohol, 2 μl/ml in water–ACN (1:1, v/v) was injected at 0.5 p.s.i. for 2 s. The EOF marker, DMSO, 1 μl/ml in water, was injected at 0.5 p.s.i. for 2 s. All samples were detected at 214 nm. All experiments were carried out at room temperature in the range from 20 to 23°C.

2.3. Column preparation

Two major steps are involved in the preparation of columns. The first entails the in situ formation of a porous annulus inside a silanized fused-silica capillary by using a modified version of a previously published method for the preparation of a styrenic inner lining in capillaries [27]. In the second step the surface of the polymeric porous support is functionalized to impart fixed charges and the desired retentive properties to the stationary phase.

2.3.1. Pretreatment

Fused-silica capillaries of 70 cm×20 μm I.D. were rinsed and filled with 1.0 *M* NaOH. The capillary ends were sealed by butane flame of a Veriflo Air-Gas Torch (Macalaster Bicknell, New Haven, CT, USA). The sealed tube was heated at 100°C for 2 h in the oven of a Sigma 2000 gas chromatograph (Perkin-Elmer, Norwalk, CT, USA). After cooling to room temperature the two ends of the tube were opened and the capillary was washed with 0.1 *M* HCl for 5 min, deionized water for 10 min, and acetone for 15 min, at 3.5 bar nitrogen pressure. Subsequently the open capillary was placed again in the oven at 120°C and purged with nitrogen for 1 h to remove residual water and acetone.

2.3.2. Silanization

A solution containing 30% (v/v) 3-(trimethoxysilyl)propyl methacrylate and 0.01% (w/v) DPPH in DMF was prepared, deaerated with helium for 15 min and filled into the pretreated capillary by carefully avoiding bubble formation. After both ends were sealed the tube was placed in the oven at 120°C for 6 h. Thereafter the capillary was taken out, washed extensively with DMF, methanol and methylene chloride and blown dry with nitrogen.

2.3.3. In situ polymerization

A mixture containing 60% (v/v) of 2-octanol, 20% (v/v) of vinylbenzyl chloride and 20% (v/v) of DVB, 0.1% (w/v) of the initiator AIBN was prepared and filtered through a cellulose membrane having 0.22 μm pores (Millipore, Bedford, MA, USA). The filtrate was degassed with helium for 15 min and filled into the silanized capillary. After both ends of the capillary were sealed it was heated at 65°C for 8 h and then 80°C for another 8 h, when a ca. 2 μm thick porous layer was formed at the innerwall of the capillary tube. Subsequently, the porogen (2-octanol) and some styrenic oligomers were removed by nitrogen purge and the capillary was further heated at 120°C for 1 h with a nitrogen stream. Finally the capillary with the macroreticular styrenic layer was washed with methanol and methylene chloride and blown dry with nitrogen.

2.3.4. Functionalization

The capillary containing the porous styrenic layer was filled with *N*,*N*-dimethyldodecylamine and with both ends sealed, it was heated at 55°C for 1 h and subsequently washed with methanol and water. The residual chloromethyl functions were hydrolyzed by rinsing the capillary with an aqueous solution con-

taining 0.1 *M* NaOH and 10% (v/v) methanol for 30 min. Finally, the capillary was washed again with water.

2.3.5. Preparation of the detection window

A small (2–3 mm) section of both the polyimide outer coating and the inner polymer lining of the column were burned off by using a butane fueled Archer Torch Model B microtorch (Radio Shack, New Haven, CT, USA), while the tube was purged with oxygen at 200 kPa inlet pressure. The capillaries were cut to have a length (inlet to outlet) of 47 cm with an effective length (inlet to the window) of 40 cm.

3. Results and discussion

3.1. Stationary phase design and column fabrication

The surface of the stationary phases in CEC has dual functionality. Fixed charges are needed for the generation of EOF upon applying the electric field, and the other functional groups serve as selective retaining sites for reversible binding of the sample components to be separated in the chromatographic process. The quaternary ammonium groups provide the fixed surface charges and the dodecyl chains act as binding sites. This stationary phase architecture was designed for the separation of proteins and peptides at low pH where they have positive net charge and thus the role of the fixed positive charges is not only to generate a vigorous EOF but also to reduce by coulombic repulsion the strength of protein or peptide binding.

Before the formation of the porous support layer by in situ polymerization, the silanol groups at the innerwall of the fused-silica capillary were reacted with 3-(trimethoxysilyl)propyl methacrylate. Such silanization of the surface facilitates the anchoring of the polymeric layer by covalent bonds via the vinyl functions. As explained in a previous publication [27], the inhibitor, DPPH, was used to prevent the free radical polymerization of the hetero-bifunctional silanizing agent at elevated temperatures.

The porous support layer at the silanized innerwall was formed by copolymerization of a mixture of equal volumes of vinylbenzyl chloride and DVB in situ. Fig. 1 shows scanning electron micrographs of a PLOT column prepared in this way. It is shown in Figs. 1d and 1e that adjacent to the siliceous capillary innerwall there is a dense fluid impervious polymeric annulus which is between the porous stationary phase layer and the fused-silica so that the mobile phase cannot contact the innerwall of the capillary. It is noted that there is no crack between the capillary innerwall and the polymer layer upon fracturing PLOT columns and this shows that there exists strong bonding between the silanized innerwall and the polymer layer.

The functionalization of the surface of the porous polymer layer was greatly facilitated by the abundance of chloromethyl groups which originate from the comonomer vinylbenzyl chloride. The scheme of the functionalization reactions is shown in Fig. 2. The reaction of the *N*,*N*-dimethyldodecylamine with the chloromethyl groups at the surface results in the formation of strongly positively charged quaternary ammonium functions and at the same time the anchoring of the dodecyl chains to the surface. Subsequently (not shown in Fig. 2) the unreacted chloromethyl groups were hydrolyzed by sodium hydroxide to hydroxymethyl groups that impart improved wetting properties of the surface by the aqueous mobile phase.

3.2. Electroosmotic mobility and column stability

Fig. 3 shows that under conditions of reversed-phase chromatography, at acidic pH over the concentration range of acetonitrile from 0 to 60%, the electroosmotic mobility in our PLOT columns with positively charged chromatographic surface is greater than that in the raw fused-silica capillary serving as the reference. As stated above the electroosmotic mobility and velocity were measured with DMSO. The hydro–organic mobile phase was aqueous sodium phosphate, pH 2.5, containing acetonitrile. The results depicted in Fig. 3 lend strong support to the surface architecture of the stationary phase as illustrated in Fig. 2. The high electroosmotic mobility of the PLOT column indicates high surface concentration of the quaternary ammonium groups which result in a high zeta potential. The EOF is anodic,

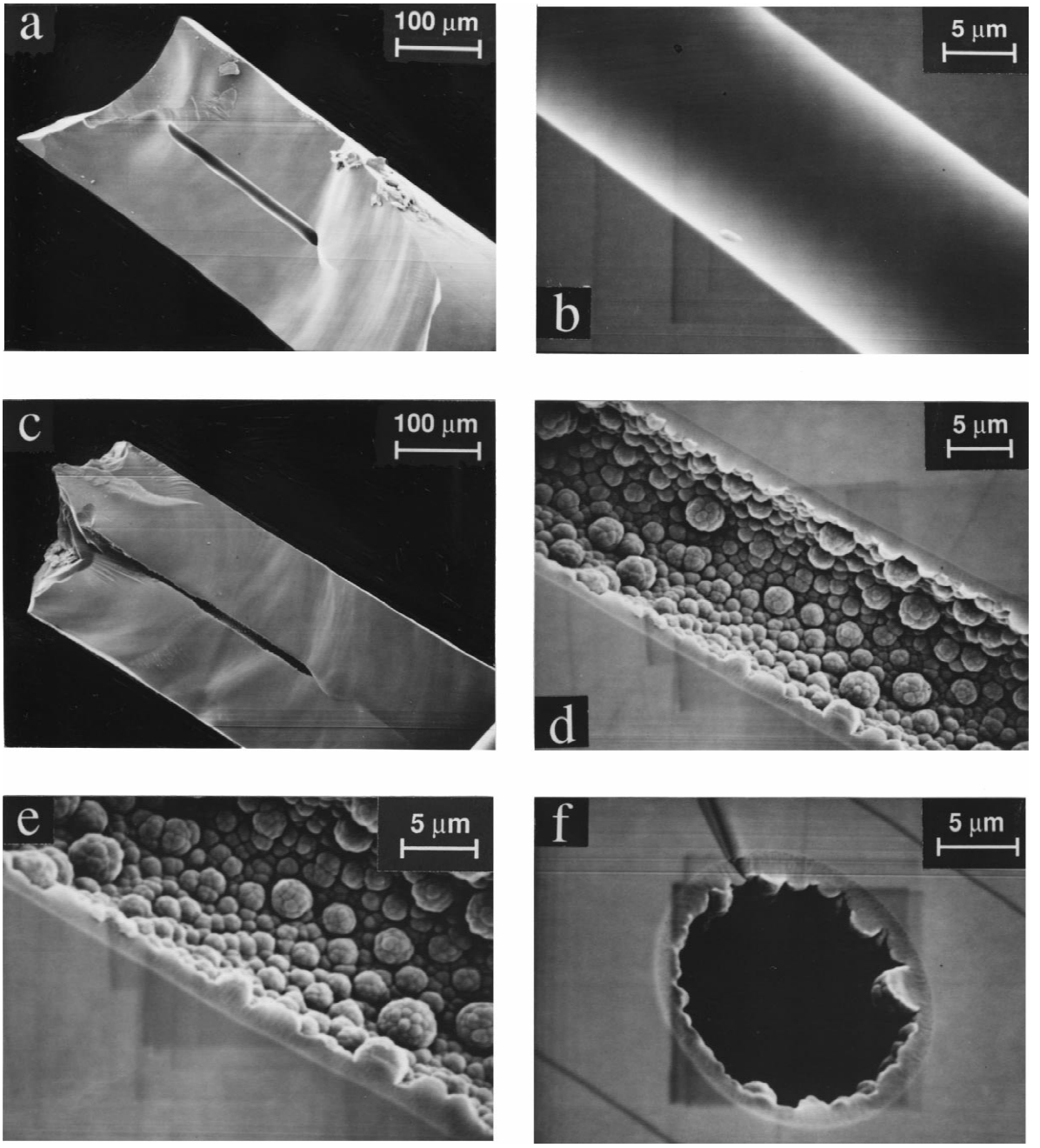

Fig. 1. Typical scanning electron micrographs of the raw fused-silica capillary and a PLOT column used in this study. (a) Fractured end of raw fused-silica capillary of 20 μm I.D.; (b) enlarged lumen of the raw fused-silica capillary shown in (a); (c) fractured end of a PLOT column; (d) the rugulose porous layer in the capillary column shown in (c); (e) the rugulose porous layer at higher magnification than in (d); (f) cross-section of PLOT column.

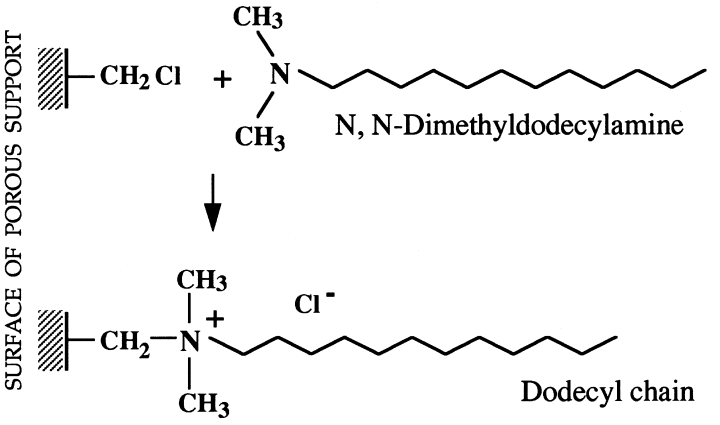

Fig. 2. Reaction scheme for the functionalization of the surface of the highly crosslinked porous poly(vinylbenzyl chloride–divinylbenzene) support layer with dodecyltrimethylammonium groups.

therefore the PLOT column was operated at ''reversed polarity''.

The effect of the sodium phosphate concentration on the electroosmotic mobility was also investigated and the results are shown in Fig. 4. It is seen that the electroosmotic mobility is the highest in neat aqueous sodium phosphate buffer decreases with increasing salt concentration. The decrease in mobility per unit salt concentration is significantly greater than that obtained with packed capillary columns of comparable chromatographic surface [9,23] and this may be taken as a manifestation of the ''openness'' of the column.

The stability of the PLOT columns used in this study was tested by measuring daily the electroosmotic mobility under the same conditions. A

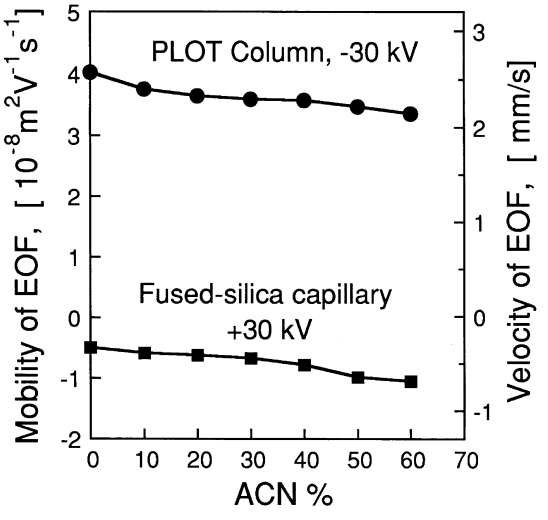

Fig. 3. Plots of the electroosmotic mobility and velocity as measured with DMSO as the unretained neutral marker against the acetonitrile concentration in 20 m*M* aqueous sodium phosphate, pH 2.5, in (1) raw fused-silica capillary, 47 cm (effective length 40 cm)×20 μm, and (2) PLOT column, 47 cm (effective length 40 cm)×20 μm fused-silica capillary, with a ca. 2 μm thick polymer layer having dodecyltrimethylammonium functions at the surface as the stationary phase. Applied voltage, +30 kV for fused-silica capillary and −30 kV for PLOT column.

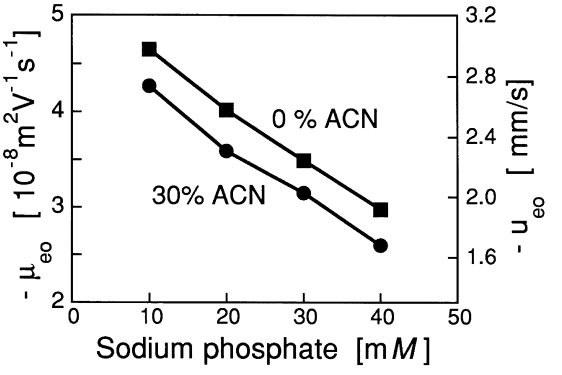

Fig. 4. Plots of the electroosmotic mobility and velocity of DMSO in a PLOT column against the concentration of sodium phosphate in the buffer. Column, 47 cm (effective length 40 cm)×20 μm fused-silica capillary, with a ca. 2 μm thick polymer layer having dodecyltrimethylammonium functions at the surface as the stationary phase. Mobile phase, 10, 20, 30, or 40 m*M* aqueous sodium phosphate, pH 2.5, containing no or 30% (v/v) acetonitrile. Applied voltage, −30 kV.

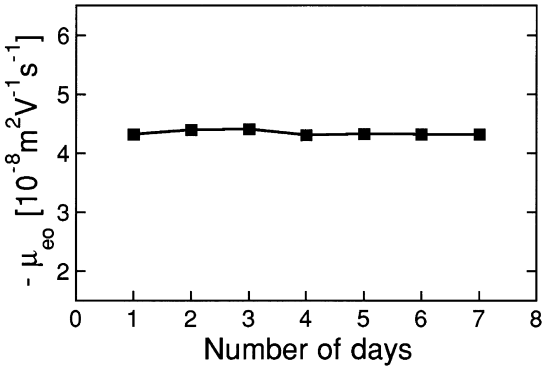

Fig. 5. Plot of the electroosmotic mobility of DMSO in PLOT column against the number of days in operation. PLOT column, 47 cm (effective length 40 cm)×20 μm fused-silica capillary, with a ca. 2 μm thick polymer layer having dodecyltrimethylammonium functions at the surface as the stationary phase. Mobile phase, 20 m*M* neat aqueous sodium phosphate, pH 2.5. Applied voltage, −30 kV. The daily average mobility was calculated from data of three runs.

typical test included measurement of the migration time of DMSO at least three times during a 4 h run of the column. Fig. 5 shows the plot of the migration time against the number of days. No untoward changes in the chromatographic behavior of the columns were seen and the migration times remained about the same during a week test period.

3.3. Protein separations

The PLOT columns with the positively charged stationary phase and dodecylated chromatographic surface were employed at pH 2.5 for the separation of the four basic proteins by CEC. As shown by a typical electrochromatogram in Fig. 6 and by the data in Table 1, the electrochromatographic system was successfully used to separate the four basic proteins. It should be emphasized that the separation was carried out by counterdirectional CEC with isocratic elution and the electric field strength and temperature were also kept constant. The order of elution suggests that the separation is the result of both chromatographic retention and electrophoretic migration. Unlike in reversed-phase HPLC, where the elution order of proteins corresponds to their hydrophobicity, in CEC of proteins with our PLOT columns the elution order is similar to the migration order in CZE although the distribution of the peaks is

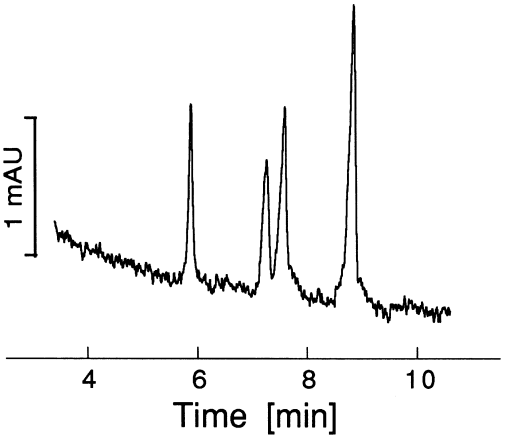

Fig. 6. Electrochromatogram of four basic proteins obtained under isocratic elution conditions by using a PLOT column. Column, 47 cm (effective length 40 cm)×20 μm fused-silica capillary, with a ca. 2 μm thick polymer layer having dodecyltrimethylammonium functions at the surface as the stationary phase. Mobile phase, 20% (v/v) acetonitrile in 20 m*M* aqueous sodium phosphate, pH 2.5. Applied voltage, −30 kV. Peaks: (1) α-chymotrypsinogen A, (2) ribonuclease A, (3) lysozyme, (4) cytochrome *c*. Mobility of EOF measured with DMSO, $\mu_{eo} = -3.46 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}; migration time of DMSO, 3.10 min. Theoretical plate numbers for each protein are shown in Table 1.

quite different. It is not surprising that the retention behavior of the proteins in CEC is much more complicated than that of small neutral molecules, since it is expected to be the result of the interplay of EOF, chromatographic retention and electrophoretic migration barring any changes in the energetics of retention in the high electric field.

In order to gain insight in the ''retention'' mechanisms, the four proteins were separated not only in CEC by using the PLOT columns but also in CZE by using a raw fused-silica capillary under otherwise identical conditions. As shown in Fig. 3, the electroosmotic mobility is much higher in the PLOT column than in the fused-silica capillary, since at pH 2.5 the silanols at the tube innerwall are protonated [28]. Separation of the four basic proteins by CEC and CZE is shown by the corresponding chromatograms and electropherograms in Fig. 7. In the counterdirectional migration process in CEC with the PLOT column, the proteins were eluted in the same direction as the EOF. It is also noted that in CEC with the PLOT column, α-chymotrypsinogen A, the most hydrophobic of the four proteins could not be

Table 1
Retention and plate efficiency (per column) in CEC of proteins with 0, 10, 20 and 30% (v/v) acetonitrile in the mobile phase (PLOT column and conditions as in Fig. 7a)

Protein	0% ACN			10% ACN			20% ACN			30% ACN		
	t (min)	*N*	*H* (μm)	*t* (min)	*N*	*H* (μm)	*t* (min)	*N*	*H* (μm)	*t* (min)	*N*	*H* (μm)
Chymotrypsinogen A	–	–	–	–	–	–	5.89	42 900	9.3	7.80	26 400	15.2
Ribonuclease A	5.06	38 100	10.5	5.56	23 200	17.3	7.27	20 320	19.7	11.09	38 000	10.5
Lysozyme	5.22	44 900	8.9	5.78	28 900	13.8	7.61	33 440	12.0	12.05	30 300	13.2
Cytochrome *c*	6.13	31 800	12.6	6.50	52 300	7.7	8.87	41 150	9.7	14.24	8 300	46.6

eluted when the acetonitrile concentration in the mobile phase was less than 20% (v/v). As expected in CZE with the raw fused-silica capillary at pH 2.5, all four proteins were eluted regardless of acetonitrile concentration, although the separation efficiency by CZE is inferior to that obtained by CEC and this is likely due to the uncontrolled effect of wall adsorption.

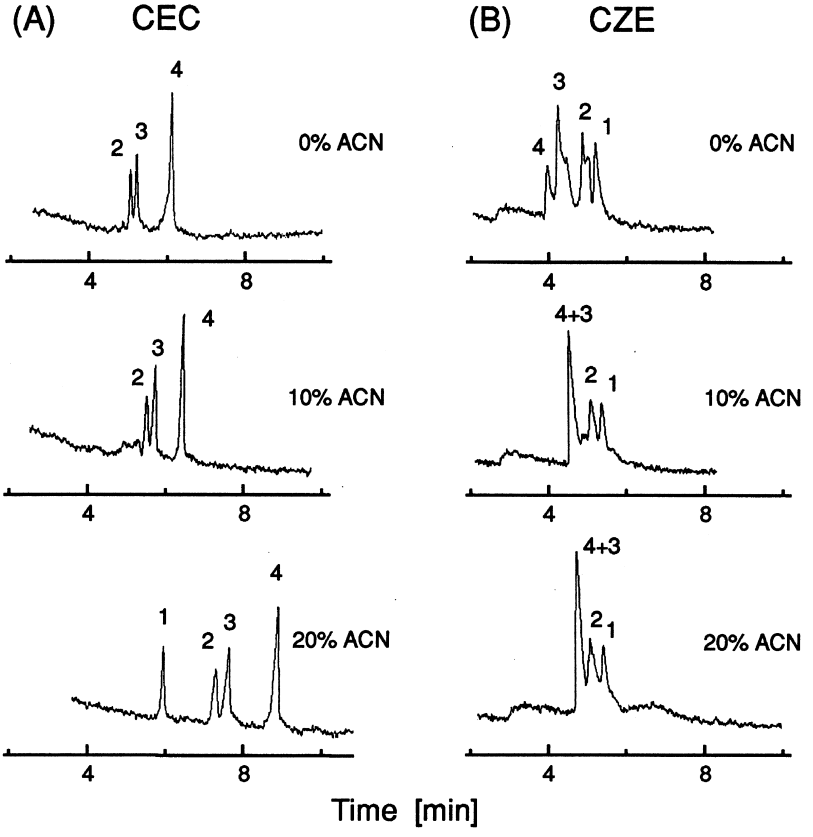

Fig. 7. Effect of acetonitrile concentration on the separation of four basic proteins by CEC and CZE. (A) CEC, PLOT column, 47 cm (effective length 40 cm)×20 μm fused-silica capillary with a ca. 2 μm thick polymer layer; (B) CZE, 47 cm (effective length 40 cm)×20 μm raw fused-silica capillary. Mobile phase, compositions are given in % (v/v) acetonitrile in 20 m*M* aqueous sodium phosphate, pH 2.5, as indicated in both CEC and CZE. Applied voltage, −30 kV for CEC and +30 kV for CZE. Peaks: (1) α-chymotrypsinogen A, (2) ribonuclease A, (3) cytochrome *c*, (4) lysozyme. The EOF mobilities at different acetonitrile concentrations are shown in Fig. 3 in both PLOT column and in raw fused-silica capillary.

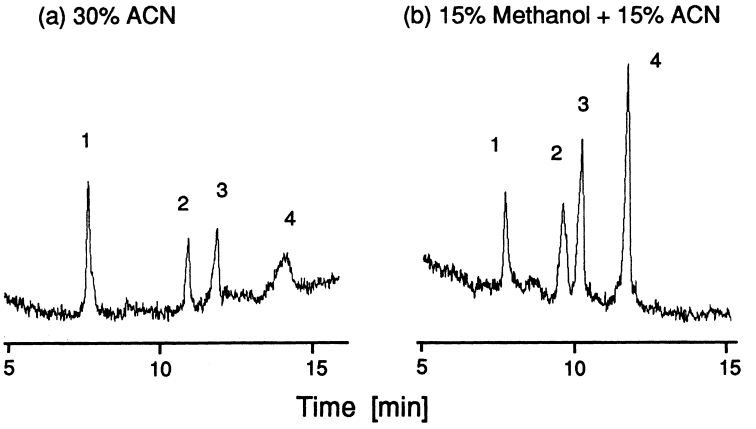

Fig. 8. Electrochromatograms of four basic proteins obtained by isocratic elution with (a) 30% (v/v) acetonitrile and (b) 15% (v/v) methanol and 15% (v/v) acetonitrile in 20 m*M* sodium phosphate buffer (pH 2.5) as the mobile phase. PLOT column, 47 cm (effective length 40 cm)×20 μm fused-silica capillary, with a ca. 2 μm thick polymer layer having dodecyltrimethylammonium functions at the surface as the stationary phase. Applied voltage, −30 kV. Peaks: (1) α-chymotrypsinogen A, (2) ribonuclease A, (3) cytochrome *c*, (4) lysozyme. For both mobile phases, the mobility of EOF measured with DMSO were equal, $\mu_{eo} = -2.75 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}; migration time of DMSO, 3.88 min.

3.4. Migration behavior in CEC of charged sample components

It was shown in Fig. 8a that when the ACN concentration was increased to 30% (v/v), some of the proteins were not eluted any longer as sharp peaks. However, by using a mobile phase with an organic modifier containing 15% (v/v) methanol and 15% (v/v) acetonitrile, the proteins could be well separated as seen in Fig. 8b. The most remarkable result is as shown in Figs. 7A and 8a that in the range from 0 to 30% ACN the retention of the four basic proteins increased with increasing acetonitrile concentration in the mobile phase. At higher acetonitrile concentrations (40% and above), the four basic proteins could not even be eluted. Plots of the elution time of the four proteins against the ACN concentration are presented in Fig. 9 that illustrates even more pronouncedly such behavior of the CEC system: the migration velocities of the proteinaceous sample components decreased with increasing acetonitrile concentration. This behavior is just the opposite of that observed in reversed-phase chromatography.

Figs. 6 and 7A also illustrate another remarkable feature of the electrochromatographic process underlying the separation of the four proteins that requires gradient elution in all interactive chromatographic methods using viscous flow. Here, the separation takes place by isocratic elution with a rather narrow

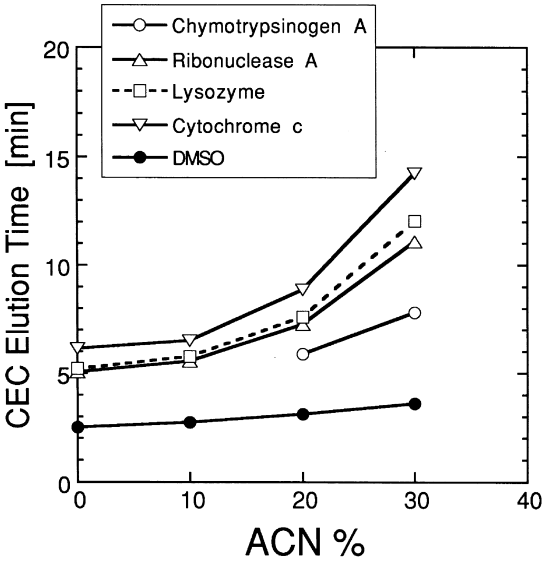

Fig. 9. Plots of CEC elution time of proteins against the acetonitrile concentration in 20 m*M* sodium phosphate buffer, pH 2.5. PLOT column, 47 cm (effective length 40 cm)×20 μm fused-silica capillary, with a ca. 2 μm thick polymer layer having dodecyltrimethylammonium functions at the surface as the stationary phase. Applied voltage, −30 kV.

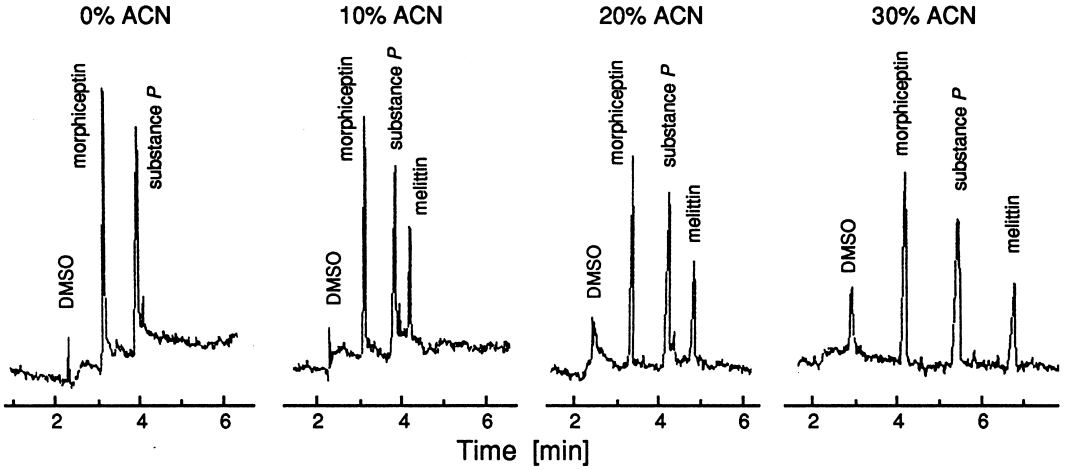

Fig. 10. Electrochromatograms of CEC separation of three peptides under isocratic elution conditions. PLOT column, 47 cm (effective length 40 cm)×20 μm fused-silica capillary, with a ca. 2 μm thick polymer layer having dodecyltrimethylammonium functions at the surface as the stationary phase. Mobile phase, compositions are given in % (v/v) acetonitrile in the buffer of 10 m*M* sodium phosphate and 10 m*M* sodium trifloroacetate, pH 2.5. Applied voltage, −30 kV.

elution window which is weakly dependent on the organic strength of the mobile phase. Evidently the complex interplay of EOF, electrophoretic migration and chromatographic retention in a counterdirectional CEC scheme of charged sample components is responsible for this effect which was also observed with the separation of peptides as shown below in Figs. 10 and 11.

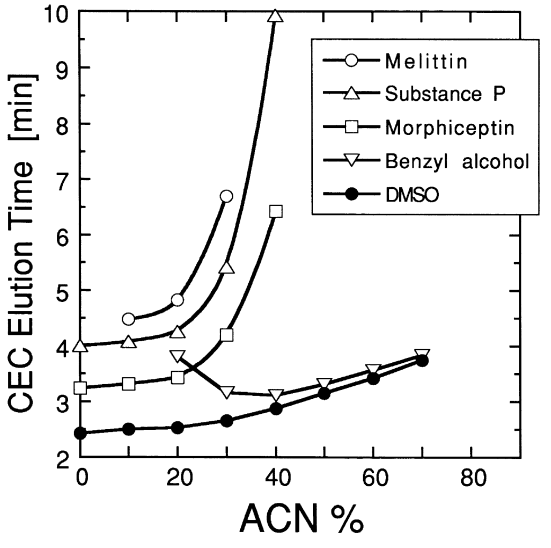

Fig. 11. Plots of CEC elution time of three basic peptides and benzyl alcohol against the acetonitrile concentration in the mobile phase containing 10 m*M* sodium phosphate and 10 m*M* sodium trifloroacetate, pH 2.5. PLOT column, 47 cm (effective length 40 cm)×20 μm fused-silica capillary, with a ca. 2 μm thick polymer layer having dodecyltrimethylammonium functions at the surface as the stationary phase. Applied voltage, −30 kV.

The migration behavior of three peptides and benzyl alcohol, an uncharged low-molecular-mass substance, was investigated under similar conditions that were employed for separating proteins on the PLOT column. Fig. 10 shows chromatograms of a mixture containing DMSO, morphiceptin, substance *P* and melittin, at different concentrations of ACN in the mobile phase. Plots of the elution time of each component in this mixture against the acetonitrile concentration are shown in Fig. 11. It is seen from Figs. 10 and 11 that in CEC with our PLOT columns the migration behavior of the peptides is similar to that of proteins as shown in Fig. 9, and their ''retention'' increases with the acetonitrile concentration. However, benzyl alcohol, the uncharged migrant, exhibited a retention behavior very similar to that observed in reversed-phase μ-HPLC [11].

4. Conclusions

Isocratic CEC of proteins and peptides was carried out at acidic pH by using a PLOT column with a

positively charged hydrocarbonaceous porous layer as the stationary phase and with acetonitrile as the mobile phase modulator. Whereas benzyl alcohol exhibited a retention behavior typical in reversed-phase chromatography as far as the dependence of the retention on the acetonitrile concentration is concerned, the migration velocities of peptides and proteins decreased with increasing organic strength of the mobile phase. The unique interplay of chromatographic retention, electrophoretic migration and EOF in the counterdirectional CEC system effects the separation in a way different from that we find in other chromatographic techniques. The observation of the effect of acetonitrile on the migration pattern of basic peptides is similar to that of basic proteins under isocratic conditions suggests a mechanistic similitude which requires a more detailed study to reveal the mechanism of separating complex biomolecules by CEC.

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References

[1] V. Pretorius, B.J. Hopkins, J.D. Schieke, J. Chromatogr. 99 (1974) 23.
[2] J.W. Jorgenson, K.D. Lukacs, J. Chromatogr. 218 (1981) 209.
[3] H. Poppe, J. Chromatogr. A 778 (1997) 3.
[4] S. Kitagawa, T. Tsuda, J. Microcol. Sep. 6 (1994) 91.
[5] C. Yan, R. Dadoo, H. Zhao, R.N. Zare, D.J. Rakestraw, Anal. Chem. 67 (1995) 2026.
[6] N.W. Smith, M.B. Evans, Chromatographia 38 (1994) 649.
[7] M.M. Dittmann, G.P. Rozing, J. Chromatogr. A 744 (1996) 63.
[8] C. Fujimoto, Y. Fujise, E. Matsuzawa, Anal. Chem. 68 (1996) 2753.
[9] G. Choudhary, Cs. Horváth, J. Chromatogr. A 781 (1997) 161.
[10] C.G. Huber, G. Choudhary, Cs. Horváth, Anal. Chem. 69 (1997) 4429.
[11] R. Asiaie, X. Huang, D. Farnan, Cs. Horváth, J. Chromatogr. A 806 (1998) 251.
[12] Cs. Horváth, G. Choudhary, X. Huang, in: Proceedings of the 19th International Symposium on Capillary Chromatography and Electrophoresis, Wintergreen, VA, 1997, p. 60.
[13] K.D. Altria, LC·GC 16 (1998) 548.
[14] I. Halász, Cs. Horváth, Anal. Chem. 35 (1963) 499.
[15] I. Halász, Cs. Horváth, Nature 197 (1963) 71.
[16] T. Tsuda, K. Hibi, T. Nakanishi, T. Takeuchi, D. Ishii, J. Chromatogr. 158 (1978) 227.
[17] T. Tsuda, M. Novotny, Anal. Chem. 50 (1978) 632.
[18] J.H. Knox, M.T. Gilbert, J. Chromatogr. 186 (1979) 405.
[19] T. Tsuda, K. Nomura, G. Nakagawa, J. Chromatogr. 248 (1982) 241.
[20] G. Guiochon, in: Cs. Horváth (Ed.), HPLC – Advances and Perspectives, Academic Press, New York, 1980, p. 1.
[21] J.H. Knox, I.H. Grant, Chromatographia 24 (1987) 135.
[22] J.H. Knox, Chromatographia 26 (1988) 328.
[23] J.H. Knox, I.H. Grant, Chromatographia 32 (1991) 317.
[24] G.J.M. Bruin, P.P.H. Tock, J.C. Kraak, H. Poppe, J. Chromatogr. 517 (1990) 557.
[25] E. Wen, R. Asiaie, Cs. Horváth, J. Chromatogr. A, in press.
[26] J.J. Pesek, M.T. Matyska, L. Mauskar, J. Chromatogr. A 763 (1997) 307.
[27] X. Huang, Cs. Horváth, J. Chromatogr. A 788 (1997) 155.
[28] J. Kohr, H. Engelhardt, J. Microcol. Sep. 3 (1991) 491.