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Electrokinetically-driven cation-exchange chromatography of proteins and its comparison with pressure-driven high-performance liquid chromatography

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Abstract

This paper examines protein ion-exchange behavior in electrokinetically-driven open-tubular chromatography with columns produced by immobilization of poly(aspartic acid) on capillary walls. Retention and selectivity are similar in the electrokinetic elution mode to that observed in HPLC. The separation mechanism was found to depend on the relationship of mobile phase pH to that of protein p*I* and ionic strength. Column efficiency in the electrokinetic elution mode was found to be 10–100-times higher than in HPLC. The best separations were achieved at intermediate ionic strength and high pH. The great advantage of these low-phase-ratio, high-efficiency open tubular columns is that isocratic separations in the electrokinetic elution mode were equivalent to gradient elution in the HPLC mode. Low phase ratio has the net effect of collapsing the chromatogram into a narrow elution window while the very high efficiency produces the requisite resolution.

Keywords: Stationary phases, LC; Electrochromatography; Proteins

1. Introduction

Capillary electrochromatography (CEC) is a hybrid of microcolumn liquid chromatography (LC) and capillary zone electrophoresis (CZE). The strong point of CEC is that it combines the high efficiency of CZE with the enhanced selectivity of LC [1,2]. A prerequisite in CEC is that either the support or stationary phase must have sufficient charge to induce the formation of an ionic double layer and cause electroosmotic flow (EOF) when voltage is applied to the column [3]. The magnitude of this flow is a function of the surface zeta potential and applied voltage. The fact that CEC columns have charge means that they are also capable of ion-exchange, a characteristic exploited in this work. EOF gives CEC systems several advantages relative to high-performance liquid chromatography (HPLC). One is that mobile phase can be convectively transported without a mechanical pump, as in HPLC. A second is that the flow profile is of greater radial uniformity in an EOF-driven system [4,5]. This diminishes band spreading arising from slow diffusion of analyte between streams of mobile phase moving at different velocities.

Whether to use packed or open tubular columns is

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an issue in CEC. Packed columns are much more widely used in LC than open tubular columns for several reasons. One is that specific surface area is 10–100-times greater in packed beds, enhancing both the phase ratio and loading capacity [6–9]. Another is that mobile phase mass transfer is generally faster in packed beds due to shorter diffusion distances. These same advantages apply to packed CEC columns. Finally, it is easier to apply bonded phase to a single lot of support and then use it to pack a large number of columns.

However, there are also negative aspects to packed beds, particularly in the case of packed capillary columns. One is that column frits are difficult to create and can be a major source of band spreading. Packing is another issue. Small particles are more difficult to pack in capillaries, especially when they are less than 2 μm in size. Yet another is frequent bubble formation [10]. Packed columns seem to be more likely to generate bubbles that disrupt current flow, cause baseline noise and induce band spreading [11–14]. The bubble problem is most frequently addressed by using positive pressure at the column ends. Flow-rate will then be the composite of hydraulic and electroosmotic pumping. Incorporating a hydraulic pumping system into a CEC system also has the advantage of allowing columns to be recycled more rapidly.

Many stationary phases have been prepared for CEC [15–23], most of which are for reversed-phase separations of small molecules. A few have been described for the separation of biopolymers [16,17,24]. Biopolymers provide several unique challenges in column and system design; all of which stem from the fact that large molecules adsorb to surfaces at multiple sites. This presents a dilemma in the case of electrokinetically-driven ion-exchange chromatography. Proteins and oligonucleotides frequently require up to 500 m*M* salt to effect elution from ion-exchange columns. Joule heating is excessive at this ionic strength. Very low-charge-density sorbents would perhaps circumvent this problem, but they are of limited resolution. The work reported here examines cation-exchange separations of proteins in the CEC mode using low-specific-surface-area, open tubular columns with a polymeric cation-exchange phase.

2. Experimental

2.1. Materials

D,L-Aspartic acid, β-alanine, and all proteins, trypsinogen (TRYN) (bovine pancreas), α-chymotrypsinogen A (CHYM) (bovine pancreas), ribonuclease A (RNase A) (bovine pancreas), cytochrome *c* (BCYT C) (bovine heart), cytochrome *c* (HCYT C) (horse heart), lysozyme (LYS) (chicken egg white), α-amylase (AMY) (*Bacillus licheniformis*), carbonic anhydrase (CA) (bovine erythrocytes), myoglobin (MYO) (horse skeletal muscle), were obtained from Sigma (St. Louis, MO, USA). Sodium phosphate monobasic, sodium phosphate dibasic heptahydrate, sodium chloride, diethyl ether and toluene were purchased from Mallinckcrodt (Phillipsburg, NJ, USA). (3-Aminopropyl)triethoxysilane (APS), *N*,*N*-dimethyformamide (DMF), β-alanine and triethylamine (TEA), tetrahydrofuran (THF), silver tetrahydrofuran (AgTHF), dithranol and mesityl oxide (MO) were obtained from Aldrich (Milwaukee, WI, USA). LiChrospher Si 1000 (10 μm, 1000 Å) was purchased from E. Merck (Darmstadt, Germany). Buffers were prepared with double distilled and deionized water.

2.2. Instrumentation

CEC was performed on an instrument designed and built in the laboratory. All high-voltage components of the system were contained in a Lucite cabinet fitted with a safety interlock that would interrupt electrical power to the instrument when the cabinet was open. A Spellman CZE 1000 R (Spellman High Voltage Electronics, Plainview, NY, USA) power supply was used to apply the electric field across the capillary. The power supply was connected to $6.35 \cdot 10^{-4}$ m (diameter) platinum electrodes immersed in 1.5-ml buffer reservoirs at with the capillary ends. Polyimide-coated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 75 μm I.D.×360 μm O.D. were used to prepare the columns. The columns were 40 cm in length with a separation length of 25 cm. Detection was achieved with a variable-wavelength UV absorbance detector (Model CV^4, Isco, Lincoln, NE,

USA). Detection was monitored at 200 nm for the proteins and 254 nm for MO. A Linear 1200 (Linear, Reno, NV, USA) was used to record the signal from the detector.

For HPLC, sorbents were slurry packed into 50 mm×4.6 mm I.D. stainless steel columns using a high-pressure packing pump (Shandon Southern Instrument, Sewickley, PA, USA). Chromatographic evaluations were performed on a BioCAD liquid chromatograph (PerSeptive Biosystems, Framingham, MA, USA). Absorbance was monitored at 280 nm.

Mass measurements were performed on a Voyager mass spectrometer (PerSeptive Biosystems) in positive ion linear mode.

2.3. Synthesis and characterization of poly(succinimide)

A modification of the Alpert [25] method was used to synthesize poly(succinimide) (PSUCC). PSUCC was made by heating 10 g of D,L-aspartic acid in a crystallization dish at 190°C in an oven for 50 h. The resulting light tan powder was dissolved in 30 ml of DMF, gently heated and stirred with a glass rod until the solution turned light brown. The solution was then centrifuged to remove the small amount of insoluble solid. PSUCC was extracted by pouring the brown solution into 120 ml of diethyl ether with rapid stirring. The precipitate, PSUCC, was recollected by centrifugation, and lyophilize to yield a light tan powder.

The relative molecular mass (M_r) of PSUCC was obtained by matrix-assisted laser desorption ionization/time-of flight mass spectrometry (MALDI-TOF-MS). PSUCC was saturated in 2 μl of THF (solution A). The matrix solution was prepared by dissolving dithranol in THF at a concentration of 10^3 μg/μl (solution B). A metal, AgTHF, was dissolved in THF to enhance cationization at a final concentration of 10^5 μg/μl (solution C). Solutions A, B and C were mixed in the ratio of 10:10:1. 0.5 μl of the mixture containing solutions A, B and C was then deposited on a MALDI-TOF plate to form sample–matrix crystals. The mass spectrum was obtained at an accelerating voltage of 25 000 V. Grid and guide wire voltages were set at 90% and 0.050% of the accelerating voltage, respectively. Delay time for ion extraction was set at 150 ns, laser intensity was 2100 W cm^{-2}, and an average of 85 scans were taken at a pressure of $1.32 \cdot 10^{-7}$ Torr with the low mass gate set at 1500 V (1 Torr=133.322 Pa).

2.4. Column preparation

Capillaries were treated with 1.0 *M* NaOH for 15 min followed by 15 min of washing with deionized water. The capillaries were then placed in a GC oven and heated for 1 h at 100°C under a nitrogen pressure of 200 kPa. 1% (v/v) APS in toluene was then aspirated into the pretreated capillary for 24 h in a GC oven at 100°C under a nitrogen pressure of 200 kPa. The APS-derivatized capillaries were then removed from the GC oven and flushed with 2–4 ml toluene. The capillaries were filled with a solution of 5% (w/v) PSUCC in DMF and held at room temperature for 24 h followed a DMF wash. The columns were then filled with a solution of 3.2% (w/v) β-alanine in 4 ml of solvent (DMF–water–TEA, 24:16:1). After being held at room temperature for 24 h, the capillaries were flushed with water, then 50 m*M* HCl, and finally water again to convert PSUCC to poly(aspartic acid) (PAA).

PAA column packings for HPLC were prepared by the same procedure with the substitution of LiChrospher Si 1000 (10 μm, 1000 Å).

Carbon, hydrogen and nitrogen analyses on silica-based stationary phases were performed by H.D. Lee in the Microanalysis Laboratory, Department of Chemistry, Purdue University.

2.5. Analytes

Protein samples were made in such a way that the solution contained 1 mg/ml of each protein in a phosphate buffer equivalent to the mobile phase. Samples were injected into the capillaries by siphoning for a fixed time (1–3 s) at a fixed height (5–8 cm). MO was used as neutral mark to measure EOF.

2.6. HPLC

PAA immobilized silica was packed into a 50 mm×4.6 mm I.D. stainless steel column. The col-

umn was evaluated by the elution of proteins with a salt gradient 0–60% 1 *M* NaCl in 10 m*M* phosphate, pH 6 and pH gradient (pH 6 to pH 11 in 10 m*M* phosphate containing 100 m*M* NaCl) at a flow-rate of 1 ml/min. Proteins were also isocratically eluted within 25 min with 10 m*M* phosphate (pH 6, 7 and 8) containing 100 m*M* NaCl at a flow-rate of 1 ml/min.

Protein samples were prepared in concentrations of 1 mg/ml in phosphate running buffer. Columns were evaluated with TRYN, CHYM, BCYT C, HCYT C, RNase A, LYS, CA, MYO and AMY.

3. Results and discussion

3.1. Column design and elution

The following design concepts guided the preparation and elution of columns used in this work. Although cationic proteins readily adsorb to the anionic surface of fused-silica capillaries, desorption from silica is achieved with difficulty. This is perhaps due to the mixed ionic–hydrophobic character of the surface. A hydrophilic organic polymer layer was used to immobilize anionic stationary phase groups both to shield proteins from contact with the silica surface and to provide a more uniform, hydrophilic surface layer. This organic polymer layer was covalently attached to the surface at many sites to increase stability of the coating.

Open tubular capillaries were used to minimize specific surface area and phase ratio. Calculations show that specific surface area (m^2/ml) in the 50–100 μm I.D. open tubular capillaries used in these studies is 10^6-times smaller than in the average particulate sorbent used for ion-exchange chromatography of proteins. By supporting stationary phases on open tubular capillaries, k' is reduced orders of magnitude and proteins having k' values in the hundreds at low ionic strength on conventional packed columns should be easily eluted from capillaries. Specific loading capacity is also reduced in direct proportion to specific surface area, meaning that such open tubular columns will be exceptionally sensitive to overloading. This problem will be offset to a small extent by the fact that the amount of surface area required is reduced at low k' values because so little of the total analyte is adsorbed at any time.

Finally, variation in mobile phase pH and salt concentration was used to elute proteins. The logic for this strategy was that by varying the partition coefficient with pH, proteins could perhaps be eluted from ion-exchange columns at low ionic strength. The conventional high ionic strength, salt displacement approach used in HPLC would produce excessive Joule heating in a CEC system.

3.2. Synthesis and characteristics of the anionic stationary phase

PAA is a synthetic polypeptide that has been used to produce a cation-exchange chromatography sorbent for HPLC of proteins [25]. Immobilized PAA columns were prepared by reacting APS-silica surfaces with PSUCC according to the procedure seen in Fig. 1, scheme 2. PSUCC was synthesized by heating D,L-aspartic acid to 190°C for 50 h (Fig. 1, scheme 1). The average molecular mass of the resulting polymer was determined by MALDI-MS. The relative molecular mass was calculated from the MALDI mass spectrum (Fig. 2) according to the equations

$$M_n = \sum(N_n M_i)/N_i \text{ and } M_w = \sum(N_i M_i^2)/N_i M_i$$

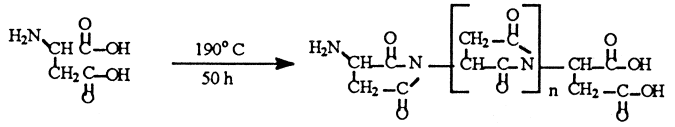
where M_n is the number-average molecular mass, M_w is the weight-average molecular mass, M_i is the mass of the *i*th oligomer, and N_i is the abundance of the *i*th oligomer. The average molecular mass was found to be 13 710.

Table 1 shows the elemental analysis of APS-silica and PAA-silica. The degree of coverage may be calculated from these values in several ways. One is the method of Brendsen and de Galan [26] in which the carbon percentage is related to surface coverage (α_R) by the equation

$$\alpha_R\ (\mu\text{mol}/\text{m}^2) = 10^6 p_c/(10^2 M_c n_c - p_c M_R) S_{BET}$$

The term p_c is the carbon percentage by relative molecular mass of the bonded material (after correction for any carbon present before bonding), n_c is the number of carbon atoms in the bonded group, S_{BET} is the specific surface area (m^2/g) of the hydride substrate, M_R is the molecular mass of the stationary

Scheme I



Scheme II

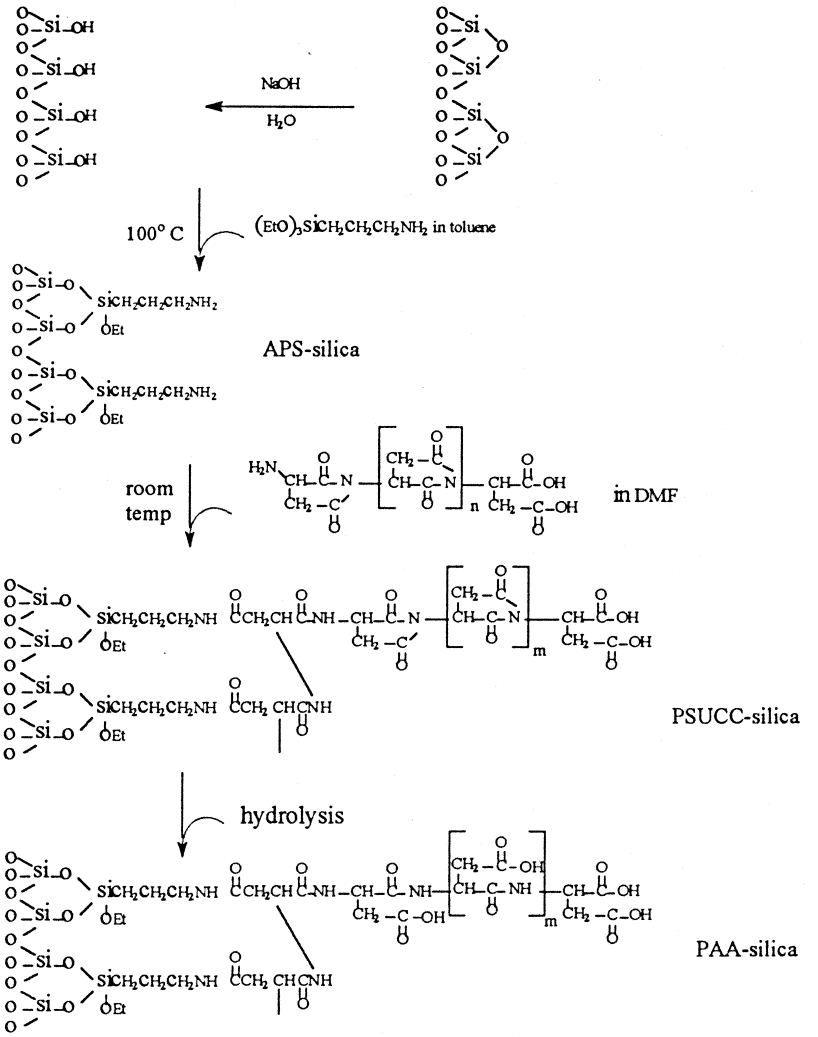


Fig. 1. Procedure for stationary phase synthesis.

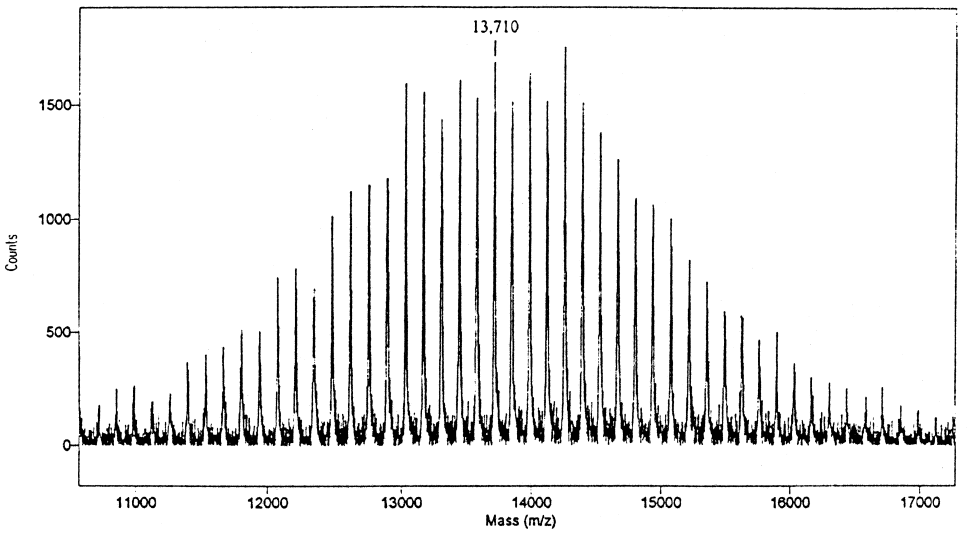


Fig. 2. Positive-ion MALDI mass spectrum of poly(succinimide). The spectrum obtained at an accelerating voltage of 25 000 V, grid voltage 90.000%, and guide wire voltage 0.050%. Delayed time 150 ns, laser intensity 2100 W cm^{-2}, scans averaged 85, pressure $1.32 \cdot 10^{-7}$ Torr, low mass gate 1500 V.

phase, and M_c is the atomic mass of carbon. Using data from elemental analysis and the manufacturers measured specific surface area for the silica sorbent (30 m^2/g), this equation estimates alkyl amine surface coverage on the silica to be 4.69 $\mu mol/m^2$. This relatively high surface coverage provides ample sites for coupling PSUCC to the surface. Subsequent to polymer immobilization, the surface coverage of the polymer was found to be 0.11 $\mu mol/m^2$ (1.5 mg/m^2). Assuming a density of one, i.e., close packing as in the dry state of the polymer, the minimum thickness of the PAA polymer layer on the silica would be about 10 Å. Obviously, hydration and charge repulsion within the polymer will cause it to swell in solution. The extent to which swelling occurs also depends on mobile phase ionic strength and the number of covalent linkages between the polymer and the support surface. Swelling will cause the coating to be much thicker than 10 Å.

Table 1
Elemental analysis of coated silica used in the study

Coating	C (%)	H (%)	N (%)
APS-silica	0.83	0.08	0.23
PAA-silica	2.40	0.40	0.66
Net PAA	1.57	0.32	0.43

It is assumed that the polymer thickness and surface coverage in the capillaries is identical to that on the porous silica support. This leads to the conclusion that because the size of proteins used in these studies was greater than 10 Å (Table 2) they probably do not penetrate through the anionic stationary phase to the support surface of open tubular capillary columns.

As noted above, EOF is essential to transport mobile phase in CEC. EOF in uncoated fused-silica capillaries was on the order of $4 \cdot 10^{-4}$ $cm^2/V\,s$ with 10 m*M* phosphate at pH 6 (Fig. 3a). The dramatic increase in EOF with pH should be noted. This phenomenon is obviously associated with the ionization of surface silanols. In contrast, EOF in the coated column was relatively constant at $2 \cdot 10^{-4}$ $cm^2/V\,s$ from pH 5 to 9 with 10 m*M* phosphate buffer (100 m*M* NaCl) (Fig. 3b). This suggests that (i) the coating process has sequestered and masked most silanols at the capillary wall and (ii) the requisite charge for EOF comes from the ionization of carboxyl groups in the PAA coating. The pK_a of carboxyl groups in PAA is about 5.

3.3. Elution of proteins in the HPLC mode

The coating described above was applied to a

Table 2
Protein retention as a function of pH[a]

Protein	p*I*	M_r[b]	Dimension (Å)	Elution time (min) at different pH		
				pH 6	pH 7	pH 8
Carbonic anhydrase	7.3	27 020	47×41×41	19.86	20.79	20.99
Myoglobin	7.5	17 500	–	16.15	17.20	19.56
Amylase	8.4	55 000	–	17.29	17.97	16.40
Trypsinogen	8.7	25 000	–	15.75	15.92	16.22
Ribonuclease A	8.8	13 690	38×28×22	Not eluted	18.72	16.40
Chymotrypsinogen A	9.0	23 660	50×40×40	18.52	19.18	16.90
Mesityl oxide[c]	–	–	–	17.82	17.14	17.14

[a] The separation system was a PAA-coated column of 40 cm (effective length 25 cm)×75 μm I.D. operated at 100 V/cm with a mobile phase of 10 m*M* phosphate buffer containing 0.1 *M* NaCl. Detection was achieved at 200 nm.
[b] M_r was taken from J. Chromatogr. 127 (1976) 1.
[c] Mesityl oxide is an EOF marker. Its migration times are given for comparison with those of proteins.

conventional 1000 Å pore diameter, 10 μm diameter support and examined in the cation-exchange mode with HPLC. The logic behind initially examining coatings and elution methods with HPLC is as follows. First, HPLC testing protocols are well established in the HPLC mode and it is easier to test columns. Second, sorbents found to be inferior in HPLC are likely to be inadequate in the CEC mode as well.

Ionic strength gradient elution chromatograms of six cationic proteins from the PAA sorbent in the HPLC mode are seen in Fig. 4. The separation mechanism in this case is one of ion displacement [27,28]. Although suitable resolution is obtained, the high concentrations of salt used at the end of the gradient would cause excessive Joule heating in CEC. An alternative would be to use pH gradient elution at low ionic strength. Separations in this case

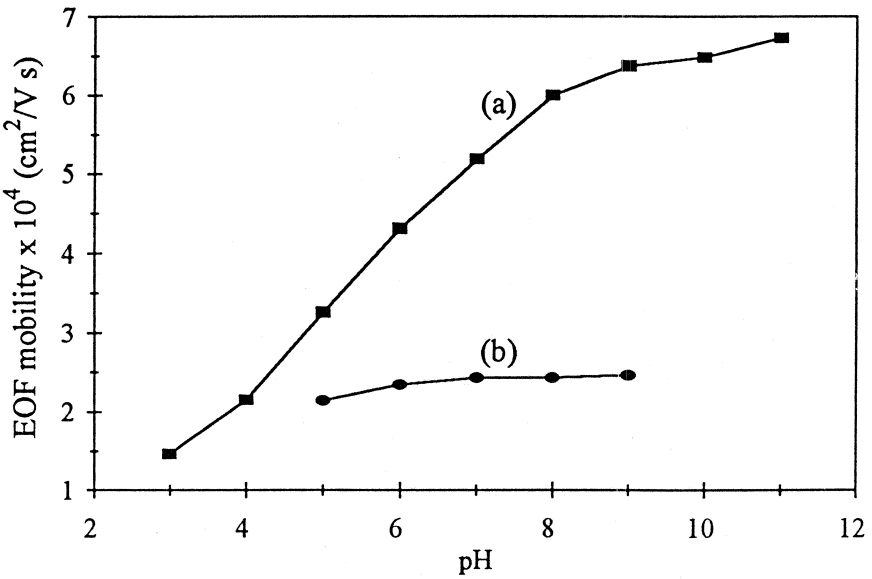


Fig. 3. Dependence of electroosmotic flow on pH. (a) Untreated capillary. Capillary 50 cm×75 μm I.D.; mobile phase: 10 m*M* phosphate; detection at 254 nm. (b) PAA-coated capillary. Capillary 40 cm (25 cm separation length)×75 μm I.D.; mobile phase: 100 m*M* NaCl in 10 m*M* phosphate; detection at 254 nm.

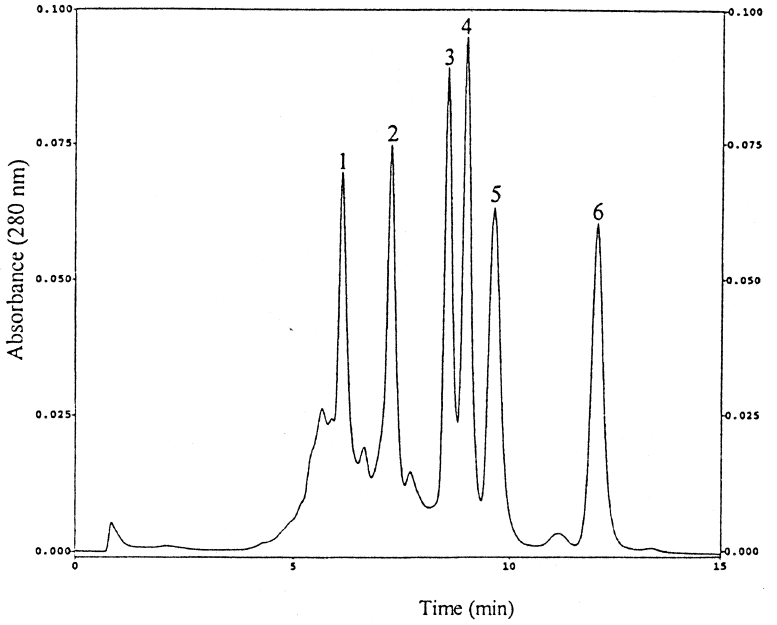


Fig. 4. Salt gradient elution of (1) trypsinogen, (2) α-chymotrypsinogen A, (3) bovine cytochrome *c*, (4) horse cytochrome *c*, (5) ribonuclease A, (6) lysozyme in the HPLC mode. Salt gradient: 0–60% 1 *M* NaCl in 10 m*M* phosphate, pH 6, 15 min. Column: 50×4.6 mm I.D.; detection: 280 nm.

are achieved by initially adsorbing proteins to the cation-exchange column below their isoelectric point (p*I*) (where they have a net positive charge) and then gradually increasing the pH to a point that is greater than the p*I* of a protein. Proteins acquire a net negative charge above their p*I* and are repelled from the anionic surface of cation-exchange columns in this elution mode. Elution at low ionic strength is attractive for CEC because it would reduce joule heating. Even though pH gradient elution chromatography of proteins has been known for decades, it has never been widely used [29–31]. Perhaps it is because the resolution is very poor at low ionic strength, as was found in these studies (data not shown). Resolution and recovery with pH gradient elution were much better with 100 m*M* buffer (Fig. 5). At this ionic strength the separation mechanism will be a combination of displacement and pH gradient elution. Most proteins will elute from cation-exchange columns before their p*I*, i.e., while they are still positively charged, in this elution mode [31].

Isocratic elution of the PAA column in the HPLC mode was relatively ineffective (Fig. 6). MYO, AMY and CA were poorly retained and could not be separated at any pH. This is attributed to their relatively low isoelectric point. They are just not strongly adsorbed (Table 2). More cationic proteins, such as TRYN, RNase A and CHYM were retained sufficiently to be separated isocratically. Finally, very basic proteins, such as ribonuclease A, were totally adsorbed at pH 6 (Fig. 6a). As the pH was increased to 7 (Fig. 6b) RNase A eluted as a very broad peak; well resolved from CHYM. At pH 8 (Fig. 6c) RNase A eluted as a sharp peak and was still partially resolved from CHYM. The efficiency of the PAA column was poor in all cases (Table 3).

The results above lead to the following conclusions relating to the separation of proteins in the HPLC mode with high-surface-area, high-phase-ratio PAA columns. One is that it is necessary to use gradient elution to separate mixtures in which the components vary widely in charge. Isocratic elution was only useful in the separation of proteins closely related in p*I*. A second is that even in the isocratic elution mode, at the p*I* extremes protein are either non-retained and fail to be resolved or are totally retained. A third conclusion is that a purely salt

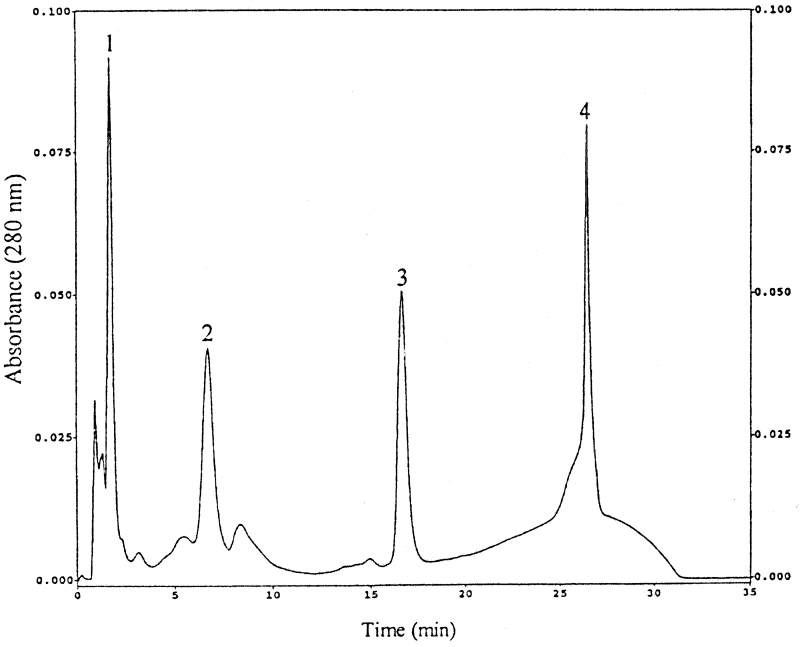


Fig. 5. pH gradient elution of (1) trypsinogen, (2) α-chymotrypsinogen A, (3) ribonuclease A, (4) bovine cytochrome *c* in the HPLC mode. pH gradient: pH 6 to pH 11, 10 m*M* phosphate in 0.1 *M* NaCl. Column: 50×4.6 mm I.D.; detection: 280 nm.

displacement mode of elution at a fixed pH cannot be used with CEC because the requisite ionic strength is too high. Finally, it is concluded that manipulation of pH at a fixed, intermediate ionic strength is more likely to be successful in CEC with cation-exchange columns for proteins.

3.4. Electrokinetic elution of proteins

Based on the HPLC studies above, it was concluded that salt concentrations of 50–100 m*M* were required for isocratic elution of proteins from the PAA column. It is seen in Fig. 7 that the protein mixture used above can be separated isocratically in PAA-coated capillary columns when eluted with 10 m*M* phosphate buffer containing 100 m*M* NaCl. This is in contrast to the HPLC mode. It is also noted that selectivity is different than in HPLC and varies with pH. Because the stationary phase is the same in both cases, these differences must be due to differences in separation mechanism in the two systems. Charged analytes are transported through CEC columns by a combination of EOF and electrophoretic mobility. The additional electrophoretic mobility contribution will alter the selectivity of CEC relative to HPLC. Charged analytes that are either non-partitioning or very weakly adsorb to the column walls could be separated either partially or totally by electrophoretic mobility [32–34].

Indeed, MYO and CA are separated at pH 8.0 where they have a weak net negative charge and are not expected to adsorb electrostatically to the negatively charged cation-exchange column (Fig. 7c). Separation of these species at this pH is probably the result of differences in their electrophoretic mobility, which in this case is opposite to the oncoming EOF. Thus, they elute later than neutral species transported by EOF alone. The prospect that the separation might be due to non-electrostatic interactions with the column must also be considered, but is much less likely. When mobile phase pH is reduced from 8 to 6 or 7, MYO and CA acquire a weak positive charge. This could impact their electrokinetic transport in a cation-exchange column in two ways. Proteins with a net positive charge will migrate with the EOF and could emerge from the column before neutral species. On the other hand, they can also interact electrostatically with the negatively charged station-

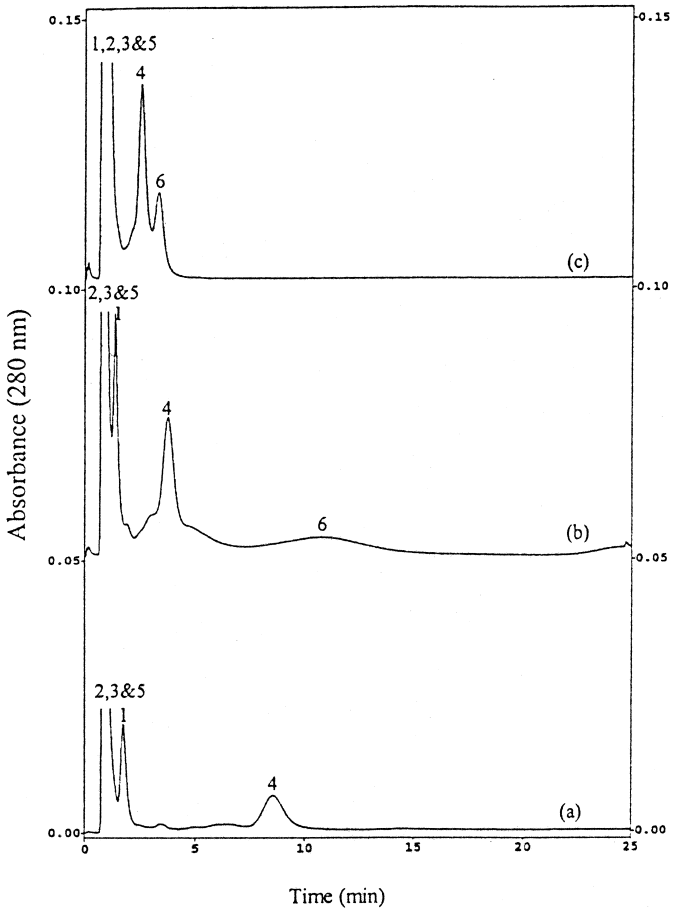


Fig. 6. Isocratic elution of (1) trypsinogen, (2) myoglobin, (3) α-amylase, (4) α-chymotrypsinogen A, (5) carbonic anhydrase, (6) ribonuclease A in the HPLC mode at (a) pH 6, 10 m*M* phosphate in 0.1 *M* NaCl, 6 not eluted; (b) pH 7, 10 m*M* phosphate in 0.1 *M* NaCl; (c) pH 8, 10 m*M* phosphate in 0.1 *M* NaCl. Column: 50×4.6 mm I.D.; detection: 280 nm.

ary phase and be retained by the column. The magnitude of their net positive charge and the extent to which they interact with the column determines their elution position. It is seen that the elution time of MYO becomes increasingly shorter as pH decreases (Fig. 8), as would be expected from their decreasing electrophoretic mobility. The fact that the elution time of MYO is near that of the neutral marker indicates that the extent of electrostatic interaction with the column is small. This conclusion is confirmed by the weak interaction of myoglobin with the PAA columns in the HPLC mode using the same mobile phase.

Table 3
Number of theoretical plates using the PAA column in the HPLC mode

Protein	Number of theoretical plates/m		
	pH 6	pH 7	pH 8
Trypsinogen	2700	2200	–
Ribonuclease A	–	460	3100
Chymotrypsinogen A	5400	3500	3500

Although CA is very similar to MYO in terms of its p*I*, it shows very different elution behavior under the same conditions (Fig. 7a–c). The elution time of CA changed little with decreasing pH (Table 2). This is interpreted to mean that in contrast to MYO, electrostatic interaction of CA with the column is sufficient to negate decreases in electrophoretic

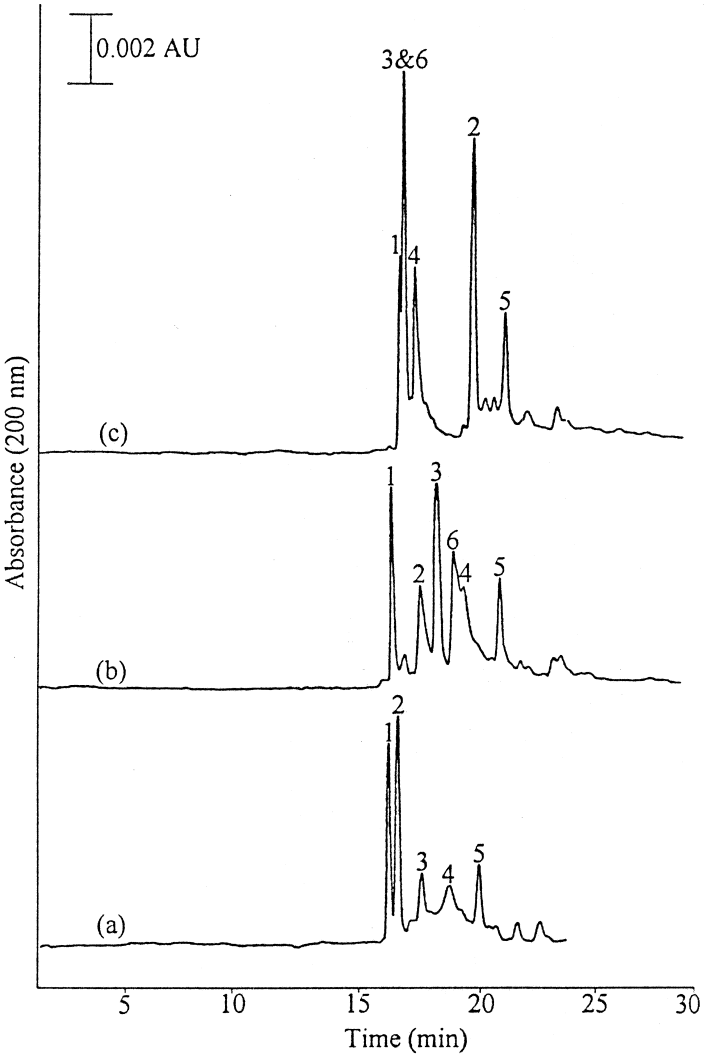


Fig. 7. Isocratic elution of (1) trypsinogen, (2) myoglobin, (3) α-amylase, (4) α-chymotrypsinogen A, (5) carbonic anhydrase, (6) ribonuclease A in the electrokinetic mode at (a) pH 6, 100 m*M* NaCl in 10 m*M* phosphate; (b) pH 7, 100 m*M* NaCl in 10 m*M* phosphate; (c) pH 8, 100 m*M* NaCl in 10 m*M* phosphate. Capillary: 40 cm (effective length 25 cm)×75 μm I.D.; voltage: 100 V/cm; current: 50 μA; detection: 200 nm.

mobility with reductions in pH. Again, this conclusion is confirmed by HPLC (Fig. 6). As mobile phase pH was decreased from 8 to 6, electrostatic interaction with the PAA sorbent increased. This is probably due to the fact that CA has a weak net positive charge at pH values slightly below its pI, allowing it to interact with the cation-exchange column below its p*I* as seen as in HPLC.

RNase A was the only species examined that failed to elute at pH 6.0 (Fig. 7a). This is attributed to a very strong electrostatic interaction of the protein with the PAA column, as observed in the HPLC mode (Fig. 6a). Electrostatic interaction of this protein with the column decreased as pH was increased (Fig. 7b and c), again as expected from HPLC (Fig. 6b and c).

The behavior of CHYM and AMY are very similar to that of RNase A. Retention time was longest at pH

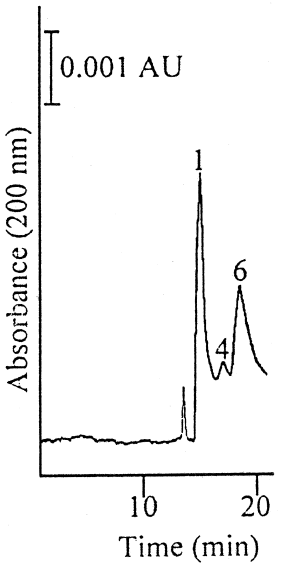


Fig. 8. Step salt gradient elution of (1) trypsinogen, (4) α-chymotrypsinogen A, (6) ribonuclease A in the electrokinetic mode. Loading buffer: 10 m*M* phosphate, pH 9.0, after 1 min run, applied elution buffer: 50 m*M* NaCl in 10 m*M* phosphate, pH 9.0. Current: 25 μA. Other conditions as in Fig. 7.

6.0 and shortest at pH 8.0 as expected from HPLC (Fig. 6a–c). This is attributed to decreasing positive charge as pH is increased. The fact that retention decreases less at pH 7.0 than was observed with RNase A is thought to be due to differences in the p*K* values of various functional groups in these proteins. It is well known that proteins of very similar p*I* have different titration curves; a phenomenon widely exploited in ion-exchange chromatography and electrophoresis [35].

The only apparent anomaly in the results shown in Fig. 7 is the behavior of TRYN. Although this protein has a net positive charge (p*I* = 8.7) under all the elution conditions examined and would be expected to adsorb strongly to the cation-exchange column, there was no apparent electrostatic interaction with the column in any case. This was also noted in the HPLC mode. Apparently the charge density in this protein is sufficiently low up to 1.5 pH units below the p*I* that it does not adsorb to cation exchangers in 10 m*M* phosphate buffer containing 100 m*M* NaCl.

The number of theoretical plates observed in the isocratic elution mode of CEC (Table 4) was roughly 100-times greater than in the HPLC mode (Table 3). Efficiency was highest at pH 8 and tended to decrease as pH was decreased. This is as would be expected. Interaction of cationic proteins with the column increases with decreasing pH. Proteins that interact electrostatically most strongly with the column, i.e., RNase A and CHYM, showed the greatest loss in efficiency at low pH. Even in the most extreme case of retention, efficiency in the CEC mode was 10-times larger than in HPLC.

Gradient elution in the CEC mode was also examined using the three basic proteins TRYN, CHYM and RNase A. The proteins were first adsorbed onto the PAA-coated capillary at pH 9 (10 m*M* phosphate buffer). After 1 min of elution with the pH 9 loading buffer the mobile phase was switched to 50 m*M* NaCl in 10 m*M* phosphate, pH 9.0. All three proteins were eluted as shown in Fig. 8 in the same order observed in HPLC (Fig. 6). It will be noted that the peaks are substantially broader than in the isocratic elution mode. This is attributed to the fact that the capacity of the open tubular PAA column is so low it is overloaded at pH 9 during the loading step. It is likely that most of the band spreading occurred during this phase of the separation.

Table 4
Number of theoretical plates using the PAA column in the CEC mode

Protein	p*I*	Number of theoretical plates/m		
		pH 6	pH 7	pH 8
Carbonic anhydrase	7.3	140 000	300 000	270 000
Myoglobin	7.5	140 000	73 000	190 000
Amylase	8.4	51 000	200 000	150 000
Trypsinogen	8.7	190 000	250 000	340 000
Ribonuclease A	8.8	–	28 000	180 000
Chymotrypsinogen A	9.0	7600	51 000	75 000

3.5. A strategy for low-phase-ratio CEC

It has been noted above that the surface area of open tubular CEC columns is substantially smaller than that of columns packed with porous particles. This has a substantial impact on the way columns may be operated. When comparing systems in which the distribution coefficient (K_D) is the same, capacity factor (k') and the amount of analyte in the stationary phase (n_s) are proportional to the surface area (A_s) of the column

$$k' = \frac{n_s}{n_m} = K_D \cdot \frac{A_s}{V_m}$$

where V_m is the volume of the mobile phase, and n_m is the amount of analyte in the mobile phase. In view of the fact that the surface area of open tubular columns is orders of magnitude smaller than that of columns packed with columns, analytes that would elute from packed columns with k' values in the hundreds will elute with a k' of one or less from an open tubular column. The equation

$$R_s = \left(\frac{N^{1/2}}{4}\right)\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'_2}{1 + k'_2}\right)$$

would suggest that resolution (R_s) in open tubular systems operating at low k' should be inferior to packed columns operating at higher k' values. This is true if the efficiency (N) of both systems is the same. But it was shown above that efficiency in the open tubular CEC systems can be substantially larger than that of packed HPLC columns operated at the same K_D. Taking a hypothetical case in which k'_{hplc} is 100-times larger than k'_{cec} and assuming $\alpha = 2$, $N_{hplc} = 4000$, $N_{cec} = 100\,000$, $k'_{hplc} = 10$, and $k'_{cec} = 0.1$, the resolving power of the CEC system is still 50% that of the HPLC system. When N_{cec} rises to 200 000 plates the resolving power of the CEC system is 75% that of the HPLC system. At $N_{cec} =$ 400 000 plates the two systems are equal. Efficiency data from Tables 3 and 4 show the values chosen for the hypothetical cases above to be realistic. Open tubular CEC systems differ from high phase ratio HPLC columns in yet another way. Mixtures in which the components vary widely in K_D must be gradient eluted from high-phase-ratio HPLC systems (Figs. 4 and 5), but they may be eluted isocratically from low-phase-ratio, high-efficiency CEC systems (Fig. 7). In effect, the mixture is collapsed into a narrower separation window but still resolved because of the higher efficiency of the open tubular CEC column. Although the open channel CEC ion-exchange columns examined are of lower phase ratio, lower loading capacity, and provided slightly lower resolution of specific components than HPLC columns, they have the great advantage of providing isocratic instead of gradient elution separations.

4. Conclusions

It may be concluded that proteins are separated in electrokinetically-driven cation-exchange chromatography by a combination of differential electrophoretic mobility and adsorption. When the net charge of a protein is large and opposite to that of the column, adsorption dominates the rate at which it is transported. In contrast, when its net charge is the same as that of the column, transport will be exclusively by a combination of EOF and electrophoretic migration against the EOF. Analytes will elute later than a neutral marker.

It is further concluded that optimum separations in electrokinetically-driven cation-exchange chromatography will be achieved in the isocratic elution mode with salt concentrations of 50–100 m*M*. Below 50 m*M* recovery is poor and above 100 m*M* there is excessive Joule heating.

Finally it is concluded that the major advantage of electrokinetically-driven cation-exchange chromatography is that protein mixtures may be separated in the isocratic elution mode with resolution approaching that achieved in the gradient elution mode by HPLC.

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