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Capillary electrochromatography of proteins with polymer-based strong-cation-exchanger microspheres

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Abstract

Monodisperse poly(glycidyl methacrylate–divinylbenzene) microspheres were functionalized with propyl sulfonic acid moieties to obtain beads negatively charged in a wide pH range. They were packed into fused-silica capillary of 50 μm I.D. in order to separate proteins by capillary electrochromatography (CEC). Baseline separation of four basic proteins as well as three cytochrome *c* variants with an average column efficiency of 60 000 theoretical plates was obtained under isocratic elution conditions. The high efficiency is attributed to the uniformity of the column packing and the hydrophilic surface coverage of the polymer beads derived from the functionalization process. The effect of pH and salt concentration on protein separations was investigated and the results showed that the CEC separation mechanism is the combination of chromatographic retention and electrophoretic migration. Moreover, the column packed with the strongly acidic poly-(glycidyl methacrylate–divinylbenzene) beads was also suitable for protein separations by micro-HPLC with a salt gradient. The comparison between the two kinds of elution modes shows that the column described here exhibited higher peak efficiency with isocratic elution in CEC than with gradient elution in micro-HPLC.

Keywords: Electrochromatography; Stationary phases, electrochromatography; Proteins

1. Introduction

Fused-silica capillary columns packed with functionalized silica particles are most commonly employed in capillary electrochromatography (CEC) [1–7]. CEC with silica-based stationary phases is carried out at neutral or alkaline pH where the silanol groups are dissociated and electroosmotic flow (EOF) is generated upon application of a high electric field. Thus the separation of charged sample components, especially of basic compounds, is complicated because of the co-existing electrostatic and silanophilic interactions [8,9] between the deprotonated silanols and the charged solutes, and strongly tailing peaks are often seen on the electrochromatograms. Such untoward effects are frequently encountered in the chromatography of proteinaceous samples which are often adsorbed irreversibly by the siliceous support, with concomitant deterioration of the separating properties of the column [10].

Organo–polymeric stationary phases have found increasing applications in chromatography, one of the favorable attributes is the high chemical stability over a wide pH range [11,12]. In particular the versatility of the surface chemistry of polymers makes them well suited as column packing materials for the separation of charged large biomacromole-

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cules [13–15]. Vinyl–divinylbenzene polymers were introduced for gel permeation chromatography in 1964 [16] and have found numerous applications in various branches of high-performance liquid chromatography (HPLC) [13–15,17–19]. The chromatographic supports are most commonly acrylic or styrenic beads. Just recently columns with a macroporous monolithic stationary phases have generated some interest in CEC [20–23]. Nonetheless, stationary phases based on porous organo–polymeric support have rarely been used so far as the stationary phase for CEC [6,24]. In this study we describe the preparation of monodisperse microspheres by dispersion polymerization [25–30] and their functionalization for use in the CEC of proteins.

In modern liquid chromatography including CEC, the ideal size of particles for column packings is in the range of 3–5 μm. So far, methods for preparing polymer beads include suspension polymerization, emulsion polymerization [31], and dispersion polymerization [32]. Suspension polymerization leads to particles having broad particle size distribution even if the polymerization conditions are strictly controlled [33]. Although a size classification process can be employed to improve the particle size distribution to some degree, the involved fine particles and irregular size particles are still troublesome when they are used as column packings. Thus suspension polymerization is not suitable for preparing 3–5 μm monodisperse particles for CEC without classification. Particles produced from emulsion polymerization are monodisperse, but the particle size is usually too small for HPLC. To increase the final particle size in emulsion polymerization, Ugelstad and co-workers [34,35] developed a method of growing submicron particles using a two-step swelling technique. Lovelace et al. [36] also studied the growth of polymer particles to the μm-size range based on seeded emulsion polymerization. However, both multi-step approaches are rather tedious and time-consuming.

Dispersion polymerization was developed in the late 1960s [25] and has attracted increasing attention as a method for obtaining μm-sized, monodispersed particles in a single step [26–30]. Dispersion polymerization is actually a precipitation polymerization process in which the medium is miscible with the monomer but not the polymer. During the polymerization, free radicals formed by initiator decomposition grow in the continuous phase until their size reaches a critical chain length, at which point they precipitate in either a self- or aggregative nucleation process, forming nuclei. These nuclei aggregate with each other and finally become mature particles [32]. Usually a polymeric stabilizer is used for stabilizing the nucleation and aggregation or particle growth [37,38]. In some special cases, dispersion polymerization can be completed even without a steric stabilizer, resulting cleaner and purer monodisperse beads.

In this work, typically 3–5 μm sized, monodispersed, highly crosslinked polymethacrylate microspheres were prepared by a simple dispersion polymerization procedure developed in our laboratory and described in another paper [39]. Those beads were subsequently functionalized by novel surface chemistry. The resulted beads provided significant advantages when used as a stationary phase for CEC of proteins.

2. Experimental

2.1. Materials

Fused-silica capillary tubing of 50 μm I.D.×375 μm O.D. with a polyimide outer coating was purchased from Quadrex Scientific (New Haven, CT, USA). Glycidyl methacrylate (GMA), epichlorohydrin, 3-(trimethoxysily)propyl methacrylate, 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), 1,3-propanesultone, 2-acrylamido-2-methyl-1-propanesulfonic acid, anhydrous tetrahydrofuran (THF), boron trifluoride diethyl etherate, tris(hydroxymethyl)-aminomethane (Tris), dimethyl sulfoxide (DMSO) and potassium persulfate were from Aldrich (Milwaukee, WI, USA). Divinylbenzene (DVB) (85%) was from Dow (Midland, MI, USA). Analytical-reagent grade monobasic, dibasic and tribasic sodium phosphates, sulfuric acid and hydrochloric acid were from J.T. Baker (Phillipsburg, NJ, USA) and azobisisobutyronitrile (AIBN) (98%) from Pfaltz and Bauer (Waterbury, CT, USA). Phosphoric acid (85%), sodium hydroxide and potassium hydroxide were of analytical-reagent grade from Mallinckrodt (Paris, KY, USA). HPLC-grade methanol, acetone

and acetonitrile (ACN) were purchased from Fisher (Fair Lawn, NJ, USA). Except DVB, the materials were used without further purification. Water was purified and deionized with a NANOpure system (Barnstead, Boston, MA, USA).

Ribonuclease A (bovine pancreas), myoglobin (horse skeletal muscle), cytochrome *c*, and α-chymotrypsinogen A (bovine pancreas) were purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of CEC columns

2.2.1. Functionalization of the microspheres by introducing sulfonic acid groups

The polymeric microspheres were prepared by a dispersion polymerization process described in Ref. [39]. The particle size distribution is measured by scanning electron microscopy (SEM) and the pore size distribution is measured by the BET method [39].

The chemical reactions involved in the functionalization process by introducing sulfonic acid moiety into the hydrophilized surface of the polymer beads are illustrated in Fig. 1. Hydrolysis of the epoxy groups of beads I into vicinal diol groups of beads II proceeded under catalysis with mineral acid. Beads I (1 g) were suspended in 10 ml of 0.1 mol/l aqueous sulfuric acid, stirred occasionally, and kept at 70 °C for 3 h. The resulting beads II were washed with water thoroughly, then re-dispersed in 10 ml of 50% aqueous KOH and stirred for 1 h. The liquid was removed, and the beads were transferred to 10 ml of an epichlorihydrin–water (1:1) mixture and stirred at room temperature for 3 h. The resulting beads III containing epoxy groups were washed with water and then hydrolyzed to beads IV using the same procedure described above with aqueous sulfuric acid. Beads IV were washed thoroughly with water and acetone. After drying at 60 °C for 60 min under nitrogen, beads IV were stirred with a mixture of boron trifluoride diethyl etherate and 1,3-propanesultone [40] in anhydrous THF at 60 °C for 5 h with a condenser and a nitrogen inlet. The resulted beads were then washed extensively with THF, methanol and water to afford beads V, which contain propyl sulfonic groups, as the strong cation exchanger.

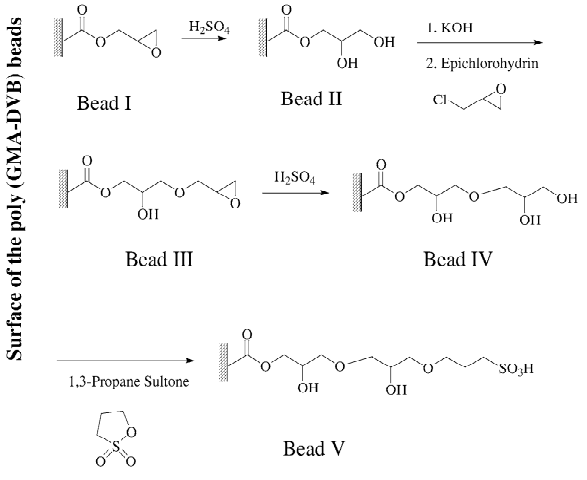

Fig. 1. Functionalization of the surface of the poly(GMA–DVB) beads to form the SCX bead V.

2.2.2. Pretreatment of the capillary inner wall

The capillary was washed and filled with 1 *M* NaOH, sealed at both ends using a butane flame burner (Veriflo Air-Gas Torch, Macalaster Bicknell, New Haven, CT, USA) and heat-treated in an oven at 120 °C for 2 h. Thereafter, the capillary was washed with deionized water then methanol and dried at 120 °C with nitrogen stream.

The NaOH treated capillary was silanized with a hetero bifunctional linker, 3-(trimethoxysilyl)propyl methacrylate, according to the procedure described in the literature [41]. After that an aqueous solution of 2-acrylamido-2-methyl-1-propanesulfonic acid (10%, w/w) was prepared, neutralized with sodium hydrogencarbonate and then 0.1% (w/v) of potassium persulfate was added to the monomer solution. The silanized capillary was filled with the above prepared solution. After both ends were sealed, the capillary was heated in the oven at 90 °C for 6 h. Then it was washed with deionized water, methanol and dried with a nitrogen stream.

2.2.3. Column packing procedure with anionic microspheres

The CEC column was packed with the functionalized anionic microspheres (Bead V), the strong cation exchanger (SCX) using a modified literature procedure [39,42]. Typically a capillary of 350 mm length was employed. The capillary was tapped in a

vial containing 5 μm dry silica particles to fill a ca. 0.5 mm length into the capillary. An Archer Torch Model B (Radio Shack, New Haven, CT, USA) microtorch fueled with butane was used to make a retaining frit at the end of the column by sintering the silica particles at elevated temperature. A 5% (w/v) slurry of the functionalized bead V was made in deionized water and sonicated for 30 min. Then, the capillary was connected to a cylindrical stainless steel reservoir (30×4.7 mm) which was filled with the slurry and connected to a Constametric III metering pump (Thermo Separation Products, San Jose, CA, USA). Deionized water was employed as the packing solvent and the flow-rate was set at 0.4 ml/min. Once the front of the packings reached 200 mm, the flow-rate of the packing solvent was turned down to zero in order to let the pressure gradually release. After reaching near atmospheric pressure the column was disconnected from the reservoir and washed at the same flow-rate with deionized water for 4 h. Then another retaining frit was prepared by placing a thin layer of 5 μm silica on the top of the polymeric beads which was then sintered with butane flame while keeping the packing solvent pumped through. A 3–4 mm long detection window was also formed during sintering. The column was stabilized for another 2 h, then the flow-rate was turned to zero to gradually release the pressure in the column.

2.3. Chromatography: apparatus and procedure

2.3.1. Micro high-performance liquid chromatography (μ-HPLC)

μ-HPLC experiments were performed using a modified Hewlett-Packard (Wilmington, DE, USA) HP 1090 Series liquid chromatography, equipped with an autosampler, a Model DRV 5 high-performance pumping system, a Model 2000 UV detector with a Model 9550-0155 on column capillary cell (Thermo Separation Products, Fremont, CA, USA). In order to operate the instrument in the flow-rate range required for capillary columns, the mobile phase flow was split after the injection valve by using a T-fitting from Upchurch Scientific (Oak Harbor, WA, USA) and a restrictor capillary. The column was mounted at right angels to the fluid inlet of the T piece with the restrictor capillary placed opposite to the fluid inlet. Split ratios were typically in the order of 4000:1 and adjusted by altering the length and diameter of the restrictor capillary. Data were collected using the DOS version 1.1 of the Hewlett-Packard ChemStation software, which was installed on a Hewlett-Packard Vectra 486DX33 IBM-compatible computer running Windows 3.1 operating system. The UV detector was connected to the computer through a Model 35900 analog/digital interface by Hewlett-Packard.

2.3.2. Capillary electrochromatography

The experiments were conducted using a Model MDQ P/ACE system capillary electrophoresis unit equipped with a P/ACE system MDQ UV detector (Beckman, Fullerton, CA, USA). A Model 6588-12U IBM personal computer with Beckman MDQ capillary electrophoresis software version 2.2. and Windows 95 (Microsoft, Redmond, WA, USA) was used to control the instrument and to acquire and process the data.

For separation of proteins, the mobile phase was made by dissolving appropriate amounts of NaCl in 20 m*M* aqueous sodium phosphate buffer in a pH range from 6 to 8. The concentration of NaCl in the mobile phases ranged from 50 to 200 m*M*. The proteins were dissolved in deionized water to obtain a solution containing 1 mg/ml of each protein. The EOF marker, DMSO, at a concentration of 2 μl/ml in water, was injected electrokinetically at 2 kV for 1 s. The protein samples were injected at 8 kV for 2 s. After each run the column was rinsed with 500 m*M* NaCl for 8 min which was followed by the reconditioning with the running mobile phase for 40 min at 100 p.s.i. inlet pressure (1 p.s.i.=6894.76 Pa). Then with both ends pressurized at 100 p.s.i. the column was equilibrated electrokinetically at the operating voltage for 10 min. All samples were detected at 214 nm. All experiments were carried out at 25 °C unless otherwise specified.

3. Results and discussion

3.1. Functionalization of the chromatographic surface

In the chromatography of proteins mild conditions are preferred in order to improve the binding kinetics

and minimize denaturation. Therefore, the use of eluents having high organic strength and/or extreme pH has to be avoided. Columns packed with ion exchangers have been used to separate peptides and proteins since the early 1950s [43,44]. These hydrophilic sorbents with fixed ionogenic functions facilitated the separation of proteins and other biopolymers without denaturation and with relatively high selectivity and resolution. When polymer beads are employed as the support for the stationary phase it is necessary to hydrophilize the surface in order to attenuate the untoward hydrophobic effect between proteins and the stationary phase.

In this paper we introduce a novel column packing with narrow particle size distribution that are obtained by the copolymerization of glycidyl methacrylate and divinyl benzene. By employing the plurality of the built-in reactive glycidyl groups, the surface of the porous particles can be easily functionalized to have a hydrophilic layer and ionizable groups. Fig. 1 shows the reaction scheme for the functionalization of the poly(GMA–DVB) beads and the formation of the chromatographic surface of a strong cation exchanger. It can be seen from the reaction scheme that after complete hydrolysis of the epoxy groups at the surface of Bead I, the formation of the chromatographic surface includes another hydrophilization step by treatment of the hydroxyl groups of Bead II with epichlorohydrin followed by the hydrolysis of the newly introduced oxyrane groups [45]. We believe that this additional hydrophilization step is expected to offer enhanced shielding to prevent close contact of the protein molecules with the hydrophobic spots on the chromatographic surface. It is noted that this is a particular case that the functional groups serve as a hydrophilic shield over the underlying polymer surface and the sulfonic groups are attached to the flexible alkoxide chain. It is believed that with this ''soft'' hydrophilic shield, interactions between the hydrophobic polymer matrix and the sample molecules are hindered with concomitantly favorable sorption kinetics and high mass recovery of proteins.

3.2. Salt gradient elution of proteins in the μ-HPLC mode

The chromatogram of the four basic proteins eluted on the SCX column with a salt gradient in the μ-HPLC mode is shown in Fig. 2. The separation mechanism in this case is essentially ion exchange. It can be seen from Fig. 2 that a suitable resolution is obtained with 500 m*M* NaCl used at the end of the gradient. The result is promising because it indicates the potential of such columns for the separation of proteins in the μ-HPLC mode. Meanwhile it is interesting to see that the development for stationary phases in CEC leads to an efficient separation media for proteins in HPLC.

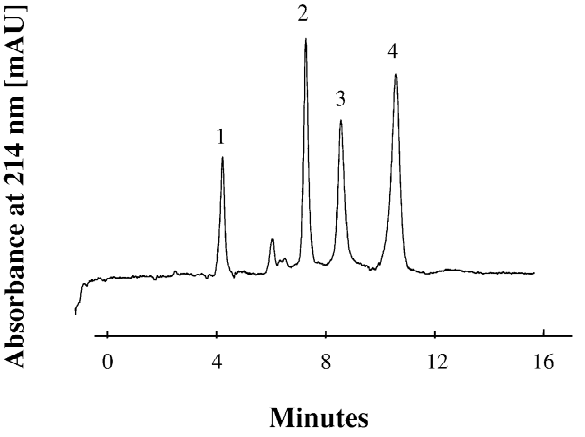

Fig. 2. Salt gradient elution of proteins in μ-HPLC mode. SCX column, 30 cm (effective length 20 cm)×50 μm I.D.; mobile phase, (A) 20 m*M* sodium phosphate buffer, pH 6.0, (B) A+500 m*M* NaCl, linear gradient: 0–80% B over 10 min; flow-rate, 0.2 μl/s; detection, 214 nm. Sample: (1) myoglobin, (2) ribonuclease A, (3) α-chymotrypsinogen, (4) cytochrome *c*.

3.3. Effect of pH on CEC separation of basic proteins with the SCX column

Upon applying high electric field, negatively charged sulfonic groups generate the EOF. The velocity of EOF should be independent of the pH of the mobile phase in the range of measurement, which was verified experimentally. The electroosmotic mobility was measured with DMSO as the neutral marker in the pH range of 2.5 to 8 with 20 m*M* phosphate buffer containing 150 m*M* NaCl. The EOF mobility ranges from $1.33 \cdot 10^{-8}$ m^2 s^{-1} V^{-1} at pH 2.5 to $1.36 \cdot 10^{-8}$ m^2 s^{-1} V^{-1} at pH 8.0.

A mixture of four proteins, i.e., myoglobin, ribonuclease A, α-chymotrypsinogen, and cyto-

Table 1
Isoelectric points and molecular masses of the proteins investigated

Protein	p*I*	M_r
Myoglobin	7.1	17 400
Ribonuclease A	8.8	13 500
α-Chymotrpsinogen	9.5	25 000
Cytochrome *c* (horse heart)	10.6	12 500

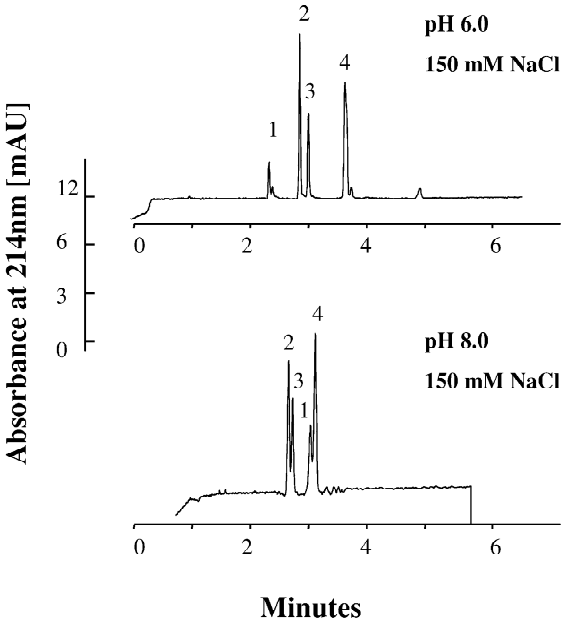

Fig. 3. Effect of pH on CEC of proteins. SCX column, 30 cm (effective length 20 cm)×50 μm I.D.; mobile phase, 150 m*M* NaCl in 20 m*M* sodium phosphate buffer, (a) pH 6.0, (b) pH 8.0; applied voltage, 15 kV; detection, 214 nm. Sample: (1) myoglobin, (2) ribonuclease A, (3) α-chymotrypsinogen, (4) cytochrome *c*.

chrome *c*, was subjected to CEC with the SCX column. The isoelectric point (p*I*) values of the proteins and molecular masses are listed in Table 1. The separation by CEC was performed with 150 m*M* NaCl in 20 m*M* phosphate buffer, pH 6.0 and the resulting chromatogram is shown in Fig. 3a. The peak efficiencies for each protein, as measured with the 30 cm (effective length 20 cm) column, were evaluated and listed in Table 2. It is seen that the column efficiency was on the average of 60 000 theoretical plates which was considered to be quite high in protein chromatography with isocratic elution. Ultimately, the relatively low band spreading, i.e., high peak capacity, is the dominant reason for the success in the isocratic protein separation by this approach. The elution order of proteins followed the same pattern that was observed in the μ-HPLC test.

The four proteins were also separated at pH 8 and the resulting electrochromatogram is depicted in Fig. 3b. It can be seen that the selectivity is different compared to that at pH 6. Ribonuclease A and α-chymotrypsinogen almost coeluted and their migration velocities were increased. At this pH, the two proteins carry very weak positive charges, therefore the strength of electrostatic interactions with the negatively charged chromatographic surface became attenuated, and thus the chromatographic retention. On the other hand, elution time of myoglobin became longer as pH increases. This could be explained by its decreasing electrophoretic mobility at a pH close to its p*I*. The results shown here support our earlier postulate that charged analytes migrate across CEC columns by a combination of electrophoretic migration and chromatographic retention which in this case is due to the electrostatic interaction.

Table 2
Column efficiencies obtained in the separation of proteins on a 30 cm (effective length 20 cm) SCX column with 150 m*M* NaCl in 20 m*M* sodium phosphate buffer, pH 6.0; applied voltage, 15 kV

Protein	50 m*M* NaCl		100 m*M* NaCl		150 m*M* NaCl	
	t (min)	*N*	*t* (min)	*N*	*t* (min)	*N*
Myoglobin	3.301	56 397	3.052	58 940	2.305	54 219
Ribonuclease A	NE	NE	3.207	62 000	2.798	63 814
α-Chymotrypsinogen	NE	NE	3.649	63 838	3.047	65 391
Cytochrome *c*	NE	NE	NE	NE	3.652	51 038

NE stands for ''not eluted within 20 min''.

3.4. Effect of salt concentration on the separation of proteins

In our case the chromatographic surface is functionalized with sulfonic acid groups as shown in Fig. 1. As a result, positively charged sample components will be bound electrostatically to the negatively charged sulfonic acid groups of the stationary phase. Therefore, relatively high salt concentration is needed to bring about the elution of the positively charged sample components. However, the magnitude of EOF depends on the density of the fixed charge at the stationary phase surface and on the electrolyte concentration in the eluent. According to the Debye–Hückel theory, in open tubes the magnitude of the EOF mobility has a linear dependence on the reciprocal square root of the ionic strength in the mobile phase [46,47]. However, when the electroosmotic mobility, as measured with our SCX column, is plotted against the salt concentration in Fig. 4, the EOF mobility has a linear relationship with the salt concentration instead of the reciprocal square root of the ionic strength in the mobile phase. The EOF decreased from $1.71 \cdot 10^{-8}$ to $1.24 \cdot 10^{-8}$ $m^2 s^{-1} V^{-1}$ with increasing ionic strength in the range from 50 to 200 m*M*. This is not unexpected since in a packed column the electric field and the flow field are more complex than those in the an open cylindrical tube [47].

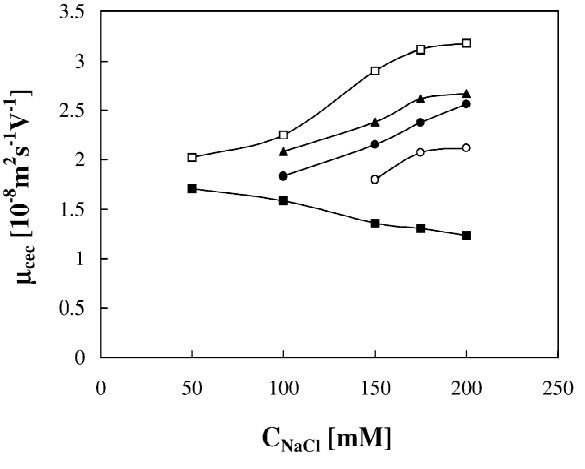

Fig. 4. Effect of ionic strength on the mobility of EOF (■), myoglobin (□), ribonuclease A (▲), α-chymotrypsinogen (●) and cytochrome *c* (○). SCX column, 30 cm (effective length 20 cm)×50 μm I.D.; mobile phase, 50 to 200 m*M* NaCl in 20 m*M* sodium phosphate buffer, pH 6.0; applied voltage, 15 kV; detection, 214 nm.

The electrochromatograms depicted in Fig. 5 show the effect of salt concentration on the separation of the four standard proteins on the SCX column in CEC. It is seen that when the mobile phase contained 50 m*M* NaCl, only myoglobin was eluted. This may be because myoglobin's p*I* value is close to 6.0 and at the neighborhood of this pH it is only weakly bound to the stationary phase. In contradistinction the elution of the more strongly bound proteins requires relatively higher ionic strength. The increase

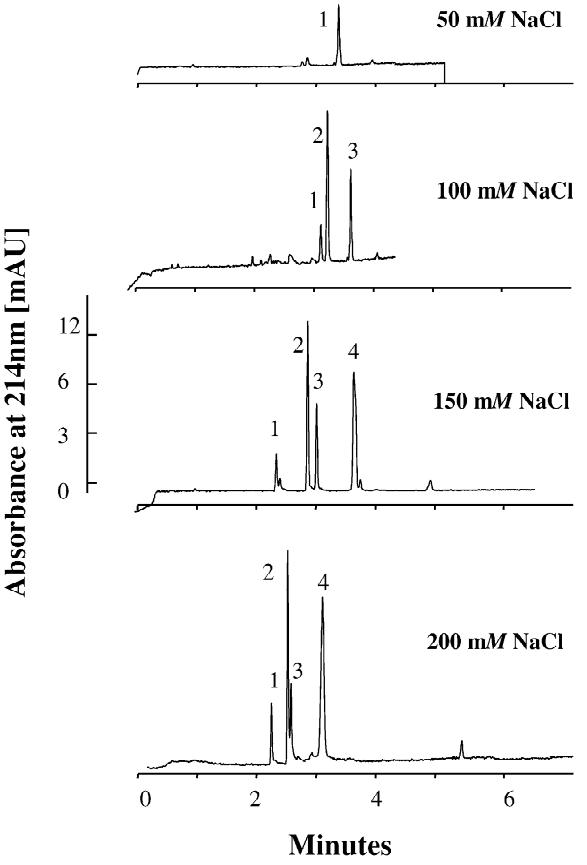

Fig. 5. Effect of salt concentration on CEC of proteins. SCX column, 30 cm (effective length 20 cm)×50 μm I.D.; mobile phase, pH 6.0, 20 m*M* sodium phosphate buffer in addition of (a) 50 m*M* NaCl; (b) 100 m*M* NaCl; (c) 150 m*M* NaCl; (d) m*M* 200 NaCl; applied voltage, 15 kV; detection, 214 nm. Sample: (1) myoglobin, (2) ribonuclease A, (3) α-chymotrypsinogen, (4) cytochrome *c*.

of NaCl concentration to 100 m*M* has brought about the elution of ribonuclease A and α-chymotrypsinogen. The separation of all components was completed by an apparently isocratic elution with 150 m*M* NaCl in the eluent. The plots of the overall mobility of each protein against the salt concentration are shown in Fig. 4. It is also seen that the overall mobilities of proteins increase significantly upon increasing the salt concentration from 50 to 200 m*M* NaCl even though the EOF mobility decreases with increasing salt concentration. Another way of illustrating the effect of salt concentration is depicted in Fig. 6 where the overall migration factor, k'_{cec}, was plotted against the salt concentration in the eluent. It is seen that the k'_{cec} values decrease with the salt concentration in a non-linear fashion. It is believed that the increase in the mobilities of proteins and the decrease in the CEC migration factors as increasing salt concentration are both due to the attenuation of the strength of protein binding to the chromatographic surface by the electrostatic effect.

As shown in Fig. 5, the elution behavior of the proteins in the CEC system under investigation is similar to that usually exhibited in ion-exchange chromatography, i.e., the order of elution approximately follows the order of strength of their electrostatic interaction with the sulfonic acid residues.

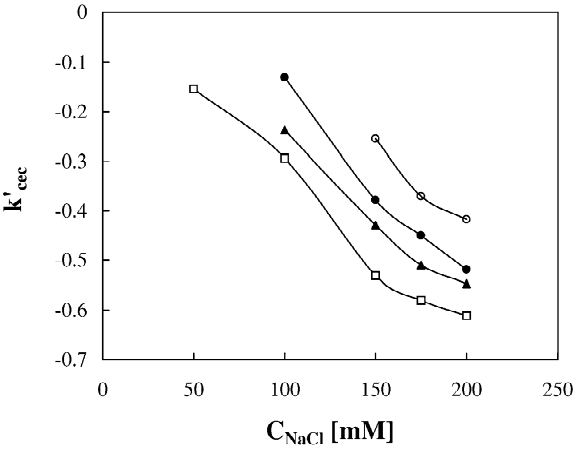

Fig. 6. Effect of salt concentration on the migration factor, k'_{cec}, of myoglobin (□), ribonuclease A (▲), α-chymotrypsinogen (●) and cytochrome *c* (○). SCX column, 30 cm (effective length 20 cm)×50 μm I.D.; mobile phase, 50 to 200 m*M* NaCl in 20 m*M* sodium phosphate buffer, pH 6.0; applied voltage, 15 kV; detection, 214 nm.

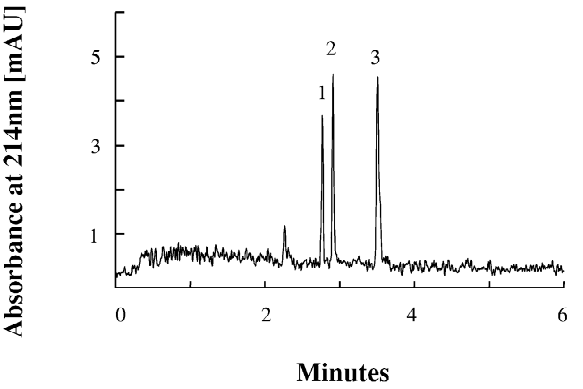

Fig. 7. Isocratic CEC of cytochrome *c* variants. SCX column, 30 cm (effective length 20 cm)×50 μm I.D.; mobile phase, 150 m*M* NaCl in 20 m*M* Tris–HCl buffer, pH 7; applied voltage, 15 kV; detection, 214 nm. Sample: (1) bovine, (2) horse, (3) rabbit.

3.5. CEC of cytochrome c variants on the SCX column 1

Cytochrome *c* variants of three species were separated in CEC with the SCX column using a mobile phase containing 20 m*M* Tris–HCl buffer, pH 7.0, and 150 m*M* NaCl. The electrochromatogram is shown in Fig. 7 and it demonstrates the excellent separation resolution of our SCX stationary phase for analytes that have closely related molecular structure. The cytochrome *c* of bovine and rabbit differ in four amino acid residues whereas the cytochrome *c* of bovine and equine differ only in three residues in their amino acid sequences [48–50].

4. Conclusions

The new platform, based on monodisperse porous polymethacrylate microspheres prepared from a simple one-step dispersion polymerization process, appears to be particularly well suited for engineering a new generation of capillary columns for use in CEC and μ-HPLC. The stationary phase, which was formed according to the novel reaction pathway, has negative charges attached to oligomeric chains that are anchored to the surface of the support. This novel packing has been successfully employed for the separation of proteins in isocratic CEC. Since the oligomeric chains are neutral and hydrophilic,

electrostatic interactions between the proteins and the sulfonic acid functions dominated the chromatographic retention at near neutral pH where the proteins were positively charged.

Finally it is concluded that the major advantage of the CEC system is that protein mixtures may be separated in the apparently isocratic elution mode with a resolution approaching to that achieved in the gradient elution mode of μ-HPLC. The ultimate reason for this phenomenon is postulated as following. For the isocratic separation of proteins, the eluent strength of the mobile phase is kept at a relatively high level so that the magnitude of the chromatographic retention factors of the sample components is relatively small. Therefore, they will elute in a narrow elution window and are ultimately baseline separated because of the high peak efficiency due to the use of electroosmotic flow.

Acknowledgements

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