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Journal of Chromatography A, 953 (2002) 239–249

JOURNAL OF CHROMATOGRAPHY A

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Capillary electrochromatography of peptides on a column packed with tentacular weak cation-exchanger particles

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Received 28 December 2000; received in revised form 15 January 2002; accepted 15 January 2002

Abstract

Silica-based, tentacular weak cation-exchanger particles were prepared for use as the stationary phase in the separation of positively charged sample components by capillary electrochromatography (CEC). Silica beads were first silanized with 3-(trimethoxysilyl) propyl methacrylate that served as a heterobifunctional linker, which reacted with 2-acrylamidoglycolic acid in a second step by radical polymerization in aqueous solution. Baseline separation of basic peptides with good column efficiency was obtained on packed capillary columns by isocratic elution CEC with NaCl as the mobile phase modulator. The retention mechanism in the electrochromatographic process was studied by examining the effect of salt concentration on the migration behavior of the peptides. The chromatographic retention factor k'_{lc} for charged sample components in the electrochromatographic process was estimated on the assumption that the overall migration rate of a charged migrant can be taken as the sum of the rate of chromatographic elution and the rate of electrophoretic migration. The estimated k'_{lc} values from experimental results were plotted against the molal salt concentration on a double logarithmic scale. The linear correlation is in good agreement with the prediction by the theory on the basis of traditional ion-exchange chromatography. The comparison of CEC results, obtained with open tubular and packed capillary columns having the same retentive functions as the stationary phase, supports the notion that variation of the phase ratio in the column offers an additional means to modulate the electrochromatographic migration behavior.

Keywords: Stationary phases, CEC; Weak cation-exchanger; Electrochromatography; Retention mechanisms; Peptides

1. Introduction

Capillary electrochromatography (CEC) has been introduced as a high efficient and high-resolution analytical technique which combines the features of high-performance liquid chromatography (HPLC) and high-performance capillary electrophoresis (HPCE) [1–12]. Employing electroosmotic flow (EOF) as the driving force to propel the mobile phase through the column, CEC generally offers higher peak capacity than HPLC does with isocratic elution because of the nature of EOF which is responsible for lesser band broadening [12–14].

So far, in most applications of CEC neutral and less polar compounds have been separated under conditions similar to those employed in reversed-phase HPLC. By using alkyl-silica stationary phases which were developed originally for reversed-phase

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)00070-5

HPLC, the residual silanol groups exposed to the mobile phase are dissociated at neutral or alkaline pH, the resulting fixed negative charges at the surface and zeta potential thus obtained [15] will generate relatively high EOF upon applying a sufficiently high electric field. The separation mechanism in CEC for neutral compounds is similar to that in reversed-phase HPLC which is based on solvophobic interactions between the non-polar hydrocarbonaceous moieties of the stationary phase and the sample components [16,17]. For charged analytes especially basic compounds, due to the interactions between the charged sample components and the silanophilic, negatively charged functions of the stationary phase, the separation mechanism is even more complicated, and design of the column and choice of the separation conditions simultaneously requires a careful consideration of several mechanistic features. Addition of a competing amine to the mobile phase at low pH has been proposed to mask the silanols and thus improve the separation performance in HPLC [18]. Such an approach was applied also in CEC to the separation of small basic compounds [19–21]. To circumvent the untoward effects associated with the use of high density residual silanols as the EOF generator, a mixture bed containing alkyl-silica and strong cation-exchanger (SCX) particles was proposed [22–24] for the separation of simple peptides. With employment of sulfonic acid functions at the SCX surface to generate the EOF, separation was carried out at low pH to diminish the dissociation of silanols and the alkyl-chains functioned as the hydrophobic retentive groups.

In CEC fixed charges at the chromatographic surface are required to have sufficiently high and stable zeta potential for the generation of EOF, consequently the stationary phases are in actuality ion-exchangers at the operating pH. For CEC to become a widely used analytical technique it is necessary to offer a means to separate complex mixtures containing charged biomacromolecules with a selectivity different from that obtained in HPLC or HPCE. This requires understanding of the separation mechanism and the methodology for synthesis of novel stationary phases that have to be tailor-made to meet the needs of CEC.

A family of new stationary phases and capillary columns has been developed recently in our laboratory for the separation of complex biomolecules. According to the column configuration, the columns are categorized into porous layer open tubular (PLOT) column [25], styrenic [26] and acrylic polymer based [27,28] monolithic columns, sintered silica based monolithic column [29] and siliceous particulate packed anion-exchanger column [30]. In order to separate positively charged biomolecules, the chromatographic surfaces of PLOT and monolithic columns investigated so far share the similar characteristics. They have quaternary ammonium or tertiary amine groups at the chromatographic surface which is also positively charged at low pH to generate EOF, the electrostatic repulsion between the surface and the sample components diminishes the electrostatic binding and the long hydrocarbonaceous chains grafted on the chromatographic surface function as the hydrophobic retentive sites. Other research groups also reported the separation of basic proteins on open tubular capillary columns with weak cation-exchanger [31] or alkyl chain coatings [32] by CEC. These newly developed columns have been successfully used for the separation of peptides or proteins under isocratic elution conditions. The separation mechanism entails elements from electrophoresis, reversed-phase or ion-exchange liquid chromatography. With the idea to use the fixed charges as both the EOF generator and the chromatographic retentive sites, silica based tentacular strong anion-exchanger [30] was synthesized. As reported in the study acidic proteins and protein variants could be resolved with high efficiency and high resolution by isocratic elution CEC. The aim of this paper is to report about the extension of the previous approach to synthesize a weak cation-exchanger stationary phase for the separation of positively charged biomolecules on packed capillary columns and gain further understanding on the mechanism of the differential migration processes underlying the separation.

2. Experimental

2.1. Materials

Fused-silica capillary tubing of 50 μm I.D. and 375 μm O.D. with a polyimide outer coating was

purchased from Quadrex Scientific (New Haven, CT, USA). Silica beads (Spherisorb S5-W, 5 μm, 80 Å) were from Phase Separations (Norwalk, CT, USA). 3-(Trimethoxysilyl) propyl methacrylate was from Polysciences (Warring, PA, USA). 2,2-Diphenyl-1-picryhydrazyl hydrate (DPPH), 2-acrylamido glycolic acid monohydrate (96%) were from Aldrich (Milwaukee, WI, USA). Analytical reagent grade monobasic, dibasic and tribasic sodium phosphate, sodium bicarbonate, dimethylformamide (DMF) (99%) and hydrochloric acid were from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). Angiotensin I (acetate salt), angiotensin II (acetate salt), [Sar^1, Ala^8]-angiotensin II (acetate salt), [Phe^7]-bradykinin (acetate salt) were purchased from Sigma (St. Louis, MO, USA). Acrylamide (AA) was purchased from Bio-Rad Laboratories (Richmond, CA, USA). Phosphoric acid (85%), sodium hydroxide (98.8%) and potassium persulfate were of analytical reagent grade from Mallinckrodt (Paris, KY, USA). HPLC grade methanol, acetone, acetonitrile (ACN) were purchased from Fisher (Fair Lawn, NJ, USA). Water was purified and deionized with a Barnstead NANOpure system (Boston, MA, USA).

2.2. Stationary phase and column preparation

Since the use of mobile phase of very high eluent strength (i.e. high salt concentration) is undesirable in CEC because of the high rate of heat generation, a weak cation-exchanger with ionogenic (glycolic acid) functions was chosen to obtain a moderate binding of positively charged sample components by the chromatographic surface at an appropriate pH. The chemical reactions involved in the functionalization process are shown schematically in Fig. 1. The pretreatment and silanization procedures of the silica beads were described before [30]. Aqueous solution of 10% (w/w) 2-acrylamidoglycolic acid was prepared and neutralized with sodium bicarbonate. One gram of the silanized beads and 0.1% (w/w) $K_2S_2O_8$ were added in a 20-ml vial containing 10 ml of the above solution. The suspension was sonicated for 30 min and purged with helium for 10 min. Subsequently the vial was sealed by a cap and heated at 70 °C in an oil bath for 6–8 h. After cooling the vial to room temperature, the beads were centrifuged from the

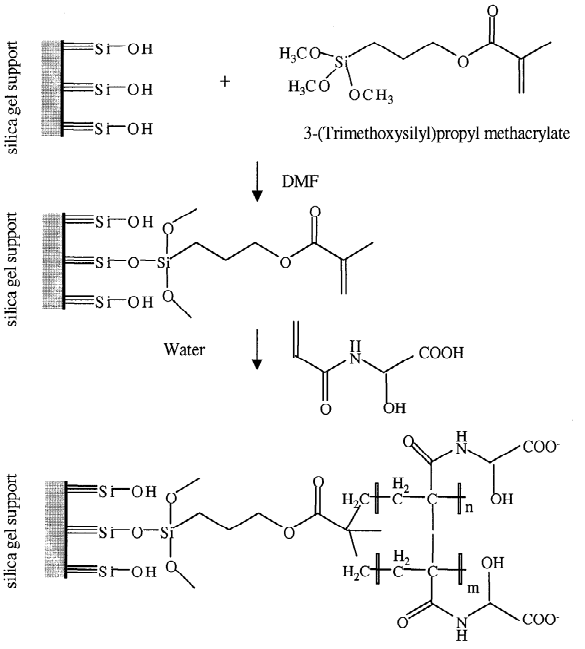

Fig. 1. Reaction scheme for the functionalization of the surface of silica gel particles employed in this study.

suspension, and were washed with methanol and deionized water.

The procedures of pretreatment and silanization of the inner wall of fused-silica capillaries were described previously [33]. The silanized capillary was filled with the neutralized 2-acrylamido glycolic acid solution, sealed both ends and heated at 70 °C in the oven of a Sigma 2000 gas chromatograph (Perkin-Elmer, Norwalk, CT, USA) for 6 h. Afterwards the capillary was flushed with methanol followed by deionized water and blown dry with nitrogen.

The functionalized silica beads were dispersed in deionized water, which was also used as the packing solvent, and the suspension was sonicated for 30 min. The slurry packing technique was employed to pack the siliceous ion-exchanger particles into a functionalized fused-silica capillary, and the CEC column was fabricated with a procedure described previously [30]. The length of the packed segment of a packed CEC column is 240 mm.

2.3. Apparatus and separation conditions

A HP^{3D}CE capillary electrophoresis unit (Agilent

Technologies, Wilmington, DE, USA) was used with a P150 personal computer (Hewlett-Packard, Palo Alto, CA, USA). Windows 95 (Microsoft, Redwood, WA) and Chemstation V. 4.01 (Agilent Technologies, Wilmington, DE, USA) were installed to control the instrument functions and to process the data. The temperature in the cartridge where the CEC column was installed was set at 20 °C in all experiments and UV absorbance at 200 or 214 nm was stored.

A series of stock solutions containing NaCl at the concentration of (20, 30, 40, 50, 60, 70)$\times 10^{-3}$ m (molal concentration) were prepared by dissolving an appropriate amount of NaCl in 20 m*M* sodium phosphate solution, pH 4.5. Mobile phase solutions were prepared by mixing equal volume of the stock solution with acetonitrile. The pH value 4.5 reported for the mobile phase is that of the stock solution. Sample solution was made by dissolving peptides in 20 m*M* sodium phosphate solution, pH 4.5. The peptide sample solution contains 0.5 mg/ml [Sar^{1}, Ala^{8}]-angiotensin II, 0.5 mg/ml [Phe^{7}]-bradykinin, 0.3 mg/ml angiotensin I and 0.3 mg/ml angiotensin II. Sample solution was injected over 4 s at 3 kV. Cleansing solution contained 100×10^{-3} m NaCl in 20 m*M* sodium phosphate solution, pH 4.5 and 50% (v/v) ACN. Between runs the column was purged with the cleansing solution at 12.0 bar for 5 min. Afterwards the column was first equilibrated by pressurizing the running mobile phase from inlet to outlet at 12.0 bar until the baseline stabilized. Then with both ends pressurized at 12.0 bar the column was equilibrated electrokinetically at the operating voltage until a stable base line was obtained. The EOF marker, acrylamide, at a concentration of 1.0 μl/ml in deionized water, was injected over 4 s at 3 kV.

3. Results and discussion

3.1. Theory

Assuming that chromatographic elution and electrophoretic migration are independent to each other in the electrochromatographic process, the overall migration velocity of a charged sample component can be expressed with the pertinent migration velocities as

$$u_{\mathrm{cec}} = \frac{u_{\mathrm{eof}} \pm u_{\mathrm{eph,cec}}}{1 + k'_{\mathrm{lc}}} \tag{1}$$

where u_{cec} is the overall migration velocity that is obtained from the electrochromatogram, $u_{\mathrm{eph,cec}}$ is the electrophoretic velocity in the column proper and k'_{lc} is the chromatographic retention factor. Alternatively Eq. (1) can be written with corresponding mobilities as

$$\mu_{\mathrm{cec}} = \frac{\mu_{\mathrm{eof}} \pm \mu_{\mathrm{eph,cec}}}{1 + k'_{\mathrm{lc}}} \tag{2}$$

If the direction of the electrophoretic migration is the same as that of the EOF, the separation process is termed as co-directional, and in Eqs. (1) and (2) the sign in the nominator will be positive. In contradistinction the separation process is named as counter-directional when the electrophoretic migration takes place in the direction opposite to that of the EOF and in Eqs. (1) and (2) the sign in the nominator is negative. In this study both the sample components and the chromatographic surface are oppositely charged, the electrophoretic migration is in the same direction as the EOF so that it is a co-directional separation process and the sign in the nominator is positive in Eqs. (1) and (2) thereafter.

The overall mobility of a charged sample component can also be expressed as

$$\mu_{\mathrm{cec}} = \frac{\mu_{\mathrm{eof}}}{1 + k'_{\mathrm{cec}}} \tag{3}$$

where k'_{cec} is termed as the CEC migration factor [10] and can be correlated to the chromatographic retention factor k'_{lc} as

$$k'_{\mathrm{cec}} = \frac{k'_{\mathrm{lc}} - (\mu_{\mathrm{eph,cec}}/\mu_{\mathrm{eof}})}{1 + (\mu_{\mathrm{eph,cec}}/\mu_{\mathrm{eof}})} \tag{4}$$

In CEC the electrosmotic mobility of the mobile phase in a packed or open tubular column, when the thickness of the double layer is sufficiently small with regard to the characteristic length in the system, is conventionally expressed as

$$\mu_{\mathrm{eof}} = \frac{\varepsilon_{\mathrm{o}} \varepsilon_{\mathrm{r}} \zeta_{\mathrm{s}}}{\eta} \tag{5}$$

where ε_{r} is the dielectric constant of the medium, ε_{o} is the permittivity of vacuum, η is the viscosity of

the medium and ζ_s is the zeta potential at the surface of the packings. In turn the electrophoretic mobility of a charged sample component migrating in free solution is expressed by Hückel equation [34] and can be conveniently measured by a capillary zone electrophoresis (CZE) experiment:

$$\mu_{eph} = \frac{2\varepsilon_o \varepsilon_r \xi_p}{3\eta} \quad (6)$$

where ξ_p is the zeta potential of the migrant under consideration.

3.2. Estimation of the electrophoretic mobility in a packed CEC column

In order to gain quantitative understanding of the chromatographic retention in a particular electrochromatographic process, assuming the overall migration velocity of a charged sample component can be expressed by Eq. (2), it requires the estimation of the electrophoretic mobility, $\mu_{eph,cec}$, of the charged migrant under the experimental conditions. One should be aware that in packed columns, the electrophoretic mobilities are not the same as those evaluated from a CZE experiment due to the porous nature of the sorbents, the tortuous flow path and the restricted diffusivity in the stationary phase [35]. Furthermore, due to the same structural differences there is a nonuniform electric strength in the packed column the magnitude of which are not the same as the apparent electric strength evaluated by dividing the applying voltage by the column length.

As a simplified approach, the electrophoretic mobility $\mu_{eph,cec}$ in a packed column can be estimated from the CZE value with a correction factor α as

$$\mu_{eph,cec} = \alpha \mu_{eph,cze} \quad (7)$$

where $\mu_{eph,cze}$ is the electrophoretic mobility of the charged migrant measured in a CZE experiment with an open fused-silica capillary having an inert coating on the inner wall to eliminate the interference by the siliceous surface.

The correction factor α includes the effect of the column structure and the mobile phase modulator such as commonly used salt and organic solvent. In estimating the effect of the packing structure on $\mu_{eph,cec}$, the EOF marker serves as a convenient reference assuming that the interparticular and intraparticular structure of the stationary phase in this study is equiaccessible to the sample components and the EOF marker. The electrosmotic mobilities are measured in an open tubular capillary column and in a packed capillary column having the same chromatographic surface with the same mobile phase preferably without mobile phase modulators and denoted as $\mu_{eof,open,neat}$ and $\mu_{eof,packed,neat}$, respectively. The ratio of the these two mobilities can be taken as a packing (structural) factor defined as

$$\alpha_1 = \frac{\mu_{eof,packed,neat}}{\mu_{eof,open,neat}} \quad (8)$$

In a similar way α_2 is defined as the correction factor for the effect of mobile phase modulator on $\mu_{eph,cec}$:

$$\alpha_2 = \frac{\mu_{eof,packed,mixed}}{\mu_{eof,packed,neat}} \quad (9)$$

It is convenient to consider α to be the product of the two correction factors, α_1 and α_2, which can be evaluated individually by experiments and with α thus obtained, $\alpha = \alpha_1 \alpha_2$, the $\mu_{eph,cec}$ value can be estimated by using Eqs. (7)–(9).

3.3. Effect of salt concentration on CEC of peptides

Salt is a widely used mobile phase modulator in electrostatic interaction chromatography. The effect of salt concentration on the overall migration behavior in CEC is twofold. It affects the magnitude of EOF as well as the retention of the sample components.

3.3.1. Effect of salt concentration on electrosmotic mobility

The effect of salt concentration on electrosmotic mobility in open and packed capillary columns is shown in Fig. 2. It can be seen from Fig. 2 that the measured electrosmotic mobility is higher in the open tubular column than in the packed column at the same salt concentration, and it decreases with increasing salt concentration faster in the open tubular column than in the packed column. In the

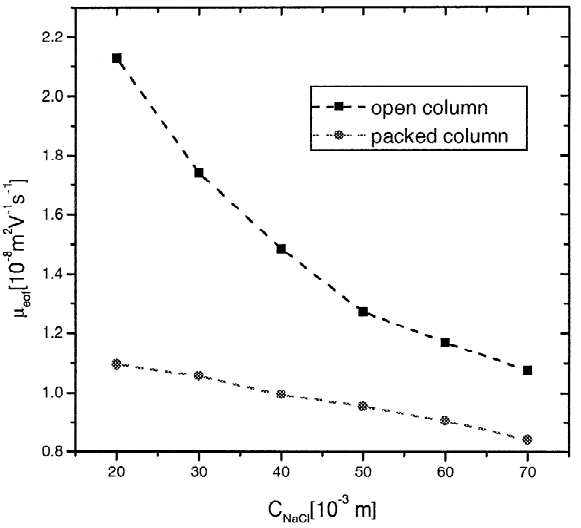

Fig. 2. Plots of the electrosmotic mobility against salt concentration for open tubular (■) and granular packed column (•). Packed column: 50 μm×326/241 mm, with 5 μm WCX beads; open tubular column: 50 μm×326/241 mm, with WCX functions on the inner wall; mobile phase: 20 m*M* phosphate solution, pH 4.5 containing 50% (v/v) ACN and 20, 30, 40, 50, 60, $70\cdot10^{-3}$ m NaCl; applied voltage 20 kV; UV detection at 214 nm.

following an attempt is made to establish a relationship between the salt concentration and the electrosmotic mobility.

According to the Debye–Hückel theory, the potential distribution near a flat surface can be described as

$$\varphi = \varphi_o \exp(-\kappa x) \tag{10}$$

where φ_o is the surface potential, κ is the Debye screening parameter, $1/\kappa$ is the double-layer ''thickness'' and x is the distance from surface. We also assume that the surface charge density is constant for a given chromatographic surface in contact with a particular mobile phase. Surface charge density is related to the surface potential as

$$\sigma_s = -\varepsilon_o\varepsilon_r\left(\frac{d\varphi}{dx}\right)_{x=0} = \varepsilon_o\varepsilon_r\varphi_o\kappa \tag{11}$$

Assuming that the zeta potential can be approximated by the potential at $x=\kappa^{-1}$, we obtain upon combining Eqs. (5), (10) and (11) the following expression for the electrosmotic mobility:

$$\mu_{eof} = \frac{\sigma_s}{e\kappa\eta} \tag{12}$$

κ is expressed as a function of the concentration of simple salt solution

$$\kappa = \left[\left(1000e^2N_A/\varepsilon_o\varepsilon_r k_B T\right)\sum_i z_i^2 m_i\right]^{1/2} \tag{13}$$

where N_A is the Avogadro's number, k_B is the Boltzmann constant, T is the temperature in Kelvin, z_i is the valence of the salt and m_i is the molal concentration. For a univalent salt, combining Eqs. (13) and (14) we obtain the following relationship between μ_{eof} and m:

$$\log\mu_{eof} = \log\left(\frac{\sigma_s}{e\eta(2000e^2N_A/\varepsilon_o\varepsilon_r k_B T)^{1/2}}\right) - 0.5\log m \tag{14}$$

which shows a linear correlation between $\log\mu_{eof}$ and $\log m$. Replotting the electrosmotic mobility data on a double logarithmic scale, it is seen in Fig. 3 that the data measured with the open tubular capillary column show good linear relationship between $\log\mu_{eof}$ and $\log m$ and the slope of the regression

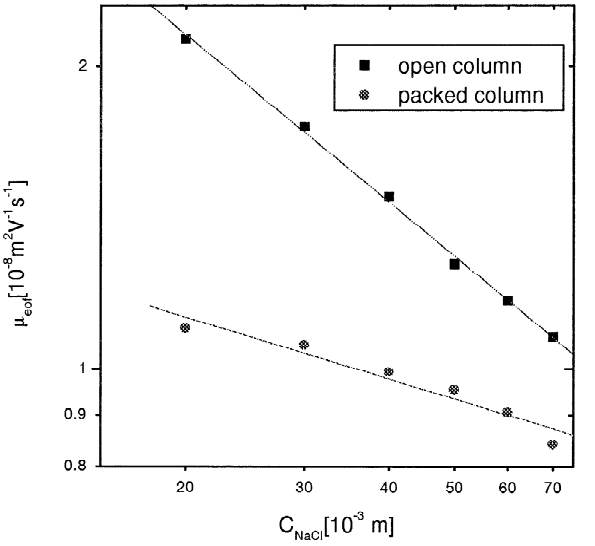

Fig. 3. Double logarithmic plots of electrosmotic mobility against salt concentration. Conditions same as Fig. 2. The linear regression formula for data measured on the open tubular column is: log EOF = 1.05 − 0.55 log *C*.

line agrees well with the theoretical prediction in Eq. (14). On the other hand the data measured on the packed capillary column don't have such a good agreement. This observation is not unexpected in view of the more complex electric and flow field distribution in the packed column than in the open cylindrical tube.

3.3.2. Effect of salt concentration on chromatographic retention

The chromatographic part of the separation mechanism in CEC is expected to involve both electrostatic and/or hydrophobic interactions that can be modulated by adding salt and/or organic modulator to the mobile phase, respectively. The effect of salt on the retention behavior of proteins and peptides in electrostatic interaction chromatography has been described by the three-parameter equation [36]:

$$\log k'_{lc} = A - B \log m_s + Cm_s \quad (15)$$

where k'_{lc} is the chromatographic retention factor and m_s is the molal salt concentration in the eluent. Parameter B expresses the magnitude of electrostatic interactions and depends on the characteristic charge of the eluite and the salt counterion. Parameter C

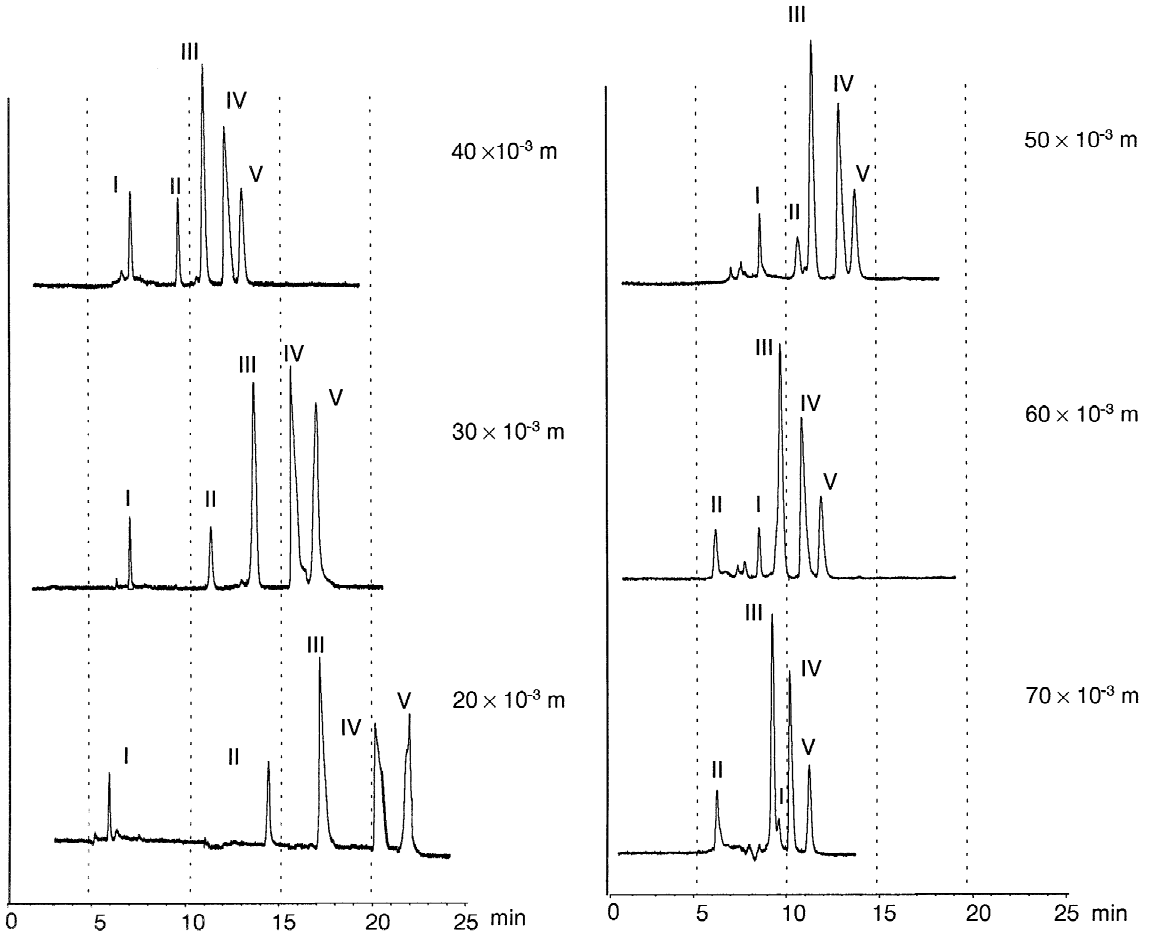

Fig. 4. Electrochromatograms of the peptides obtained at different salt concentrations. Column: 50 μm×326/241 mm, packed with 5 μm WCX beads; mobile phase: 20 m*M* phosphate solution, pH 4.5 containing 50% (v/v) ACN and 20, 30, 40, 50, 60, $70 \cdot 10^{-3}$ m NaCl; applied voltage 20 kV; UV detection at 214 nm; Sample components: I-EOF marker, II-angiotensin II, III-[Phe^{7}]bradykinin, IV-[Sar^{1},Ala^{8}]angiotensin II, V-angiotensin I.

represents hydrophobic interactions and its magnitude depends on the hydrophobic contact area of the sample components upon binding at the surface of the stationary phase and the properties of the salt. The C term reflects the effect of hydrophobic moieties at the chromatographic surface. At relatively low salt concentrations, the hydrophobic effect is usually negligible and its magnitude can also be reduced by adding organic solvent to the mobile phase. It is customary in HPLC to illustrate the salt effect by plots of $\log k'_{lc}$ against $\log m_s$. Such plots are quasi-straight lines with negative slopes when the C term in Eq. (15) is negligible. It can be seen from Eq. (4) that in CEC the magnitude of the overall migration factor k'_{cec} would also decreases monotonically with the salt concentration if the chromatographic retention is dominated by electrostatic interactions.

The electrochromatograms in Fig. 4 illustrate the effect of salt concentration in CEC of peptides with the tentacular WCX column. The mobile phase contained 50% (v/v) ACN to diminish the retention from hydrophobic interactions. It is seen that with increasing salt concentration from 20 to 70×10^{-3} m the migration times were reduced in spite of the attenuation of the EOF as shown in Fig. 2. Decreasing migration time is most likely a manifestation of weakening electrostatic binding of the basic peptides to the negatively charged chromatographic surface. This is supported by plots of the overall migration factor k'_{cec} against the salt concentration shown in Fig. 5. Since k'_{cec} decreases monotonically with incremental salt concentration we may infer that the migration is governed by a single retention mechanism. It also suggests that the role of k'_{cec} can be extended beyond its serving solely as a dimensionless peak locator and it could become as a ''diagnostic tool'' for the investugation on separation mechanism in CEC as proposed before [28].

The chromatographic retention factor k'_{lc} is estimated by using Eqs. (2), (7)–(9) and the results were listed in Table 2. The logarithmic chromatographic retention factors k'_{lc} of the four peptides (angiotensin I, [Phe^7]-bradykinin, [Sar^1,Ala^8]-angiotensin II and angiotensin II) are plotted against $\log C$ and are shown in Fig. 6. Good linear correlation was observed for these peptides in a certain range of salt concentration. It is noticed that the slopes of the

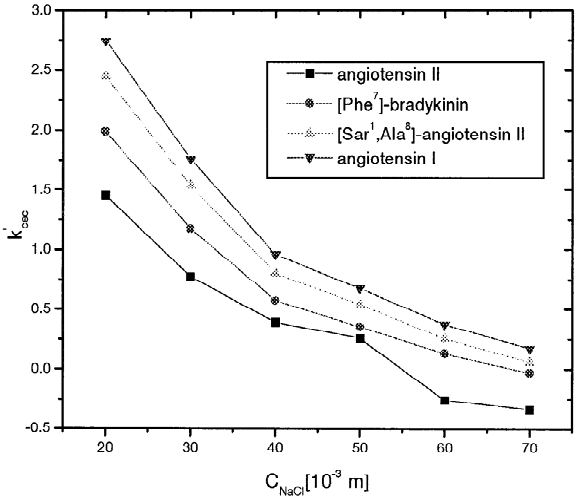

Fig. 5. Plots of the overall migration factors k'_{cec} in CEC against the salt concentration.

regression lines of angiotensin I, [Phe^7]-bradykinin and [Sar^1,Ala^8]-angiotensin II are close to each other. According to the literature [36], the electrostatic interaction parameter B in Eq. (15) is evaluated as, $B = Z_p/Z_s$, where Z_p and Z_s are the charac-

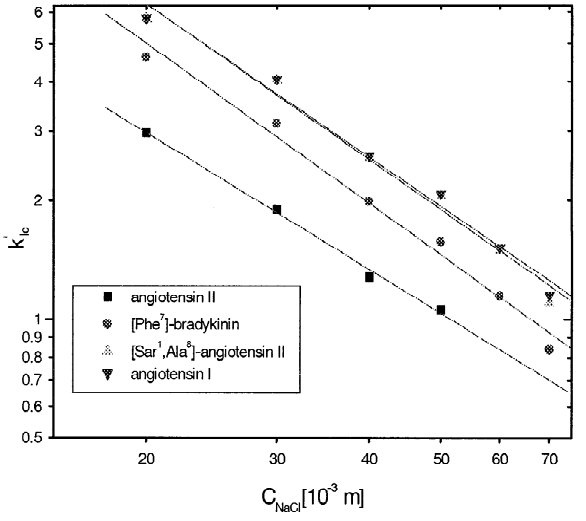

Fig. 6. Double logarithmic plots of the estimated chromatographic migration factors k'_{lc} against the salt concentration. The linear regression formula is: angiotensin II, $\log k'_{lc} = 1.97 - 1.15 \log C$; [Phe^7]-bradykinin, $\log k'_{lc} = 2.46 - 1.35 \log C$; [Sar^1,Ala^8]-angiotensin II, $\log k'_{lc} = 2.51 - 1.31 \log C$; angiotensin I, $\log k'_{lc} = 2.47 - 1.29 \log C$.

Table 1
Properties and structure of the peptides employed in this study

	Formula weight (w)	Net charge (q) at pH 4.5	q/w ($\times 10^{-3}$)
Angiotensin I	1296.5	+2	1.543
Angiotensin II	1046.2	+1	0.956
[Sar^1,Ala^8]angiotensin II	926.1	+2	2.160
[Phe^7]bradykinin	1110.3	+2	1.801

Angiotensin I: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu.
Angiotensin II: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe.
[Sar^1,Ala^8]angiotensin II: Sarcosine-Arg-Val-Tyr-Ile-His-Pro-Ala.
[Phe^7]bradykinin: Arg-Pro-Pro-Gly-Phe-Ser-Phe-Phe-Arg.

teristic charges of the migrant and the salt counter-ion, respectively. It is seen from Table 1 that angiotensin I, [Phe^7]-bradykinin and [Sar^1,Ala^8]-angiotensin II have similar net charges. Angiotensin II has the least net charge, the linear regression line shows also the smallest slope. The data from the regression analysis are consistent with the prediction based on qualitative net charge analysis and therefore it supports the usefulness of the method suggested for the evaluation of the k'_{lc}.

3.4. Effect of phase ratio on the separation of peptides by CEC

The phase ratio, ϕ, relates the chromatographic retention factor k'_{lc} to K, which is the chromatographic equilibrium constant for the distribution of the migrant between the mobile phase and the stationary phase:

$$k'_{lc} = K\phi \quad (16)$$

In most cases the equilibrium constant is conveniently adjusted by the composition of the mobile phase. However, the column architecture (packed or open tubular) usually determines the practical range of the phase ratio. In view of Eq. (16), if K remains unchanged, reduction of the phase ratio attenuates the retention factor with concomitant reduction of the eluent strength that is necessary to facilitate the separation.

CEC of the basic peptides was carried out with an open tubular capillary column having inner wall coated with the same ion-exchanger functions as the stationary phase particles used in a corresponding packed capillary column. Since the retentive groups were the same in both the open tubular and the packed capillary columns, the equilibrium constant was assumed to be the same in both columns and the different electrochromatographic results are attributed to the difference on phase ratio. As seen in Fig. 7a the four peptides were well separated with the open tubular capillary column by using an eluent containing 20×10^{-3} m NaCl. In contrast, as seen in Fig. 4 with the packed capillary column under the same eluent strength the peaks were broad and tailing. It is also noted that the elution order of the four peptides is the same as that in CZE that is shown in Fig. 7b although the selectivities are not identical. This observation suggests that under such an eluent strength the electrophoretic migration might dominate the separation process of the peptides on the open tubular capillary column while the peptides are relatively strongly retained on the packed capillary column.

4. Conclusions

The results of this study demonstrate that the tentacular weak cation-exchanger described here is a suitable stationary phase for the separation of basic substances like peptides by isocratic elution CEC with satisfactory peak efficiency and resolution under appropriate conditions. The results have shown that the introduction of novel stationary phases, which are tailor-made to the particular separation problems, can be advantageous and most likely necessary for the further development and applications of CEC.

The chromatographic contribution to the sepa-

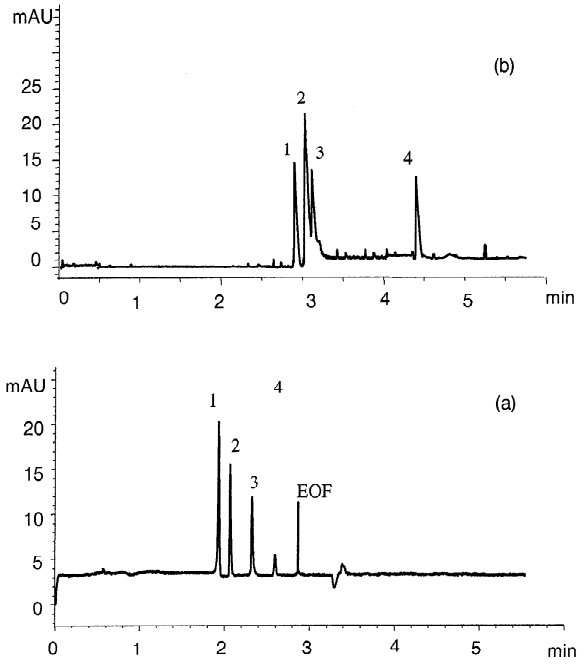

Fig. 7. Comparison of open tubular CEC and CZE of peptides. (a) Open tubular CEC column: 50 μm×326/241 mm, with WCX functions on the inner wall; mobile phase: mobile phase: 20 m*M* phosphate solution, pH 4.5 containing 50% (v/v) ACN and 20×10^{-3} m NaCl; (b) PVA coated fused-silica CZE column: 50 μm×326/241 mm; mobile phase: 20 m*M* phosphate solution, pH 4.5; applied voltage 20 kV; UV detection at 214 nm. Sample components: 1-[Sar^1,Ala^8]angiotensin II, 2-[Phe^7]bradykinin, 3-angiotensin I, 4-angiotensin II.

ration mechanism of peptides by CEC was estimated. The overall electrochromatographic migration rate was decomposed into chromatographic elution and electrophoretic migration. The usefulness of the method to estimate the chromatographic retention factor was supported by the results of the study on the effect of salt concentration on the k'_{lc} values and a qualitative net charge analysis. As seen in the electrochromatograms (Fig. 4) and the estimated chromatographic retention factors (Table 2), the packed WCX column exhibits relatively strong retention for these basic peptides in a wide range of the mobile phase modulator. The separation selectively obtained in CEC is different from that obtained in HPLC or CZE where the separation mechanism is solely chromatographic retention or electrophoretic migration, respectively. It is expected to make CEC a complementary bioanalytical method for biological and biomedical research.

Acknowledgements

This work was supported by Grant No. GM 20993 from the National Institute of Health, US Department of Health and Human Services, and a grant from the National Foundation of Cancer Research.

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Table 2
Effect of salt concentration on the chromatographic retention factors of the four basic peptides

Salt concentration [10^{-3} m]	Estimated chromatographic retention factor, k'_{lc}			
	Ang II	[Phe^7]Brd	[Sar^1,Ala^8]Ang II	Ang I
20	2.971	4.620	5.773	5.804
30	1.900	3.138	4.034	4.062
40	1.280	1.992	2.574	2.592
50	1.060	1.573	2.057	2.079
60	0.212	1.146	1.496	1.522
70	0.084	0.840	1.100	1.154

Ang II, angiotensin II; [Phe^7]Brd, [Phe^7]bradykinin; [Sar^1,Ala^8]Ang II, [Sar^1,Ala^8]angiotensin II; Ang I, angiotensin I.

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