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Capillary Electrochromatography for Separation of Peptides Driven with Electrophoretic Mobility on Monolithic Column

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A mode of capillary electrochromatography for separation of ionic compounds driven by electrophoretic mobility on a neutrally hydrophobic monolithic column was developed. The monolithic column was prepared from the in situ copolymerization of lauryl methacrylate and ethylene dimethacrylate to form a C_{12} hydrophobic stationary phase. It was found that EOF in this hydrophobic monolithic column was very poor, even the pH value of mobile phase at 8.0. The peptides at acidic buffer were separated on the basis of their differences in electrophoretic mobility and hydrophobic interaction with the stationary phase; therefore, different separation selectivity can be obtained in CEC from that in capillary zone electrophoresis (CZE). Separation of peptides has been realized with high column efficiency (up to 150 000 plates/meter) and good reproducibility (migration time with RSD < 0.5%), and all of the peptides, including some basic peptides, showed good peak symmetry. Effects of the mobile phase compositions on the retention of peptides at low pH have been investigated in a hydrophobic capillary monolithic column. The significant difference in selectivity of peptides in CZE and CEC has been observed. Some peptide isomers that cannot be separated by CZE have been successfully separated on the capillary monolithic column in this mode with the same buffer used.

Capillary electrochromatography (CEC) is a hybrid method that combines features of both high-performance capillary electrophoresis (HPCE) and high performance liquid chromatography (HPLC).[1−3] To date, in most applications of CEC, neutral and hydrophobic compounds have been separated under conditions similar to those employed in reversed-phase HPLC. However, for CEC to become a widely used analytical technique, it is also necessary to offer a means to separate charged biomolecules with selectivity different from that obtained in HPLC or HPCE. Nonetheless, this aspect of CEC has not received much attention, probably because it requires novel separation systems especially tailored to take advantage of the peculiar features of CEC. Furthermore, an understanding of the separation mechanism greater than presently available is needed to exploit the full potential of CEC.

The most widely used columns in CEC are packed with an alkyl-silica stationary phase, and frits are prepared to prevent the loss of the stationary phase from the capillary. These stationary phases have an abundance of silanol groups, which are usually negatively charged in contact with neutral or alkaline mobile phase. They are responsible for the generation of an electroosmotic flow in a high electric field. However, a drawback with this kind of CEC column is that frits can result in the formation of bubbles, leading to loss of electroosmotic flow.[4−6] Although no frits are used in open tubular CEC (OT-CEC) columns, the relatively low phase ratio in OT-CEC restricts its further developments and applications. Recently, the monolithic columns[7−25] in CEC have attracted increasing attention because of their potential advantages, which could be easily prepared by in situ polymeri-

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zation. Because the rod of the stationary phase is directly bonded to the inner wall of the capillary through covalent bonds, no supporting frits are necessary. In addition, the pore size of the stationary phase could also be adjusted during the preparation procedure to obtain the optimal separation, especially in the analysis of biomolecules, such as peptides and proteins. Separation of peptides and proteins by CEC with monolithic column has already been demonstrated by several authors.[21−23]

In CEC, the mobile phase was driven by EOF, and it is believed that EOF is necessary for CEC separation. To generate strong EOF, packing beds with high charge density are preferred. For porous organic polymeric continuous beds, EOF is promoted by the incorporation of ionizable functional groups, such as acrylic acid, sulfonic acid, or ammonium monomers, within the polymerized mixture. For silica sol−gel monoliths and other inorganic continuous beds, EOF was attributed to the ionization of residual silanol groups. However, the separation of ionic compounds in these systems is always difficult. First, if the direction of EOF and that of electrophoretic migration of ionic compounds are different, these ionic compounds will migrate to the detection window in a long time or even cannot be loaded into the CEC column. For example, Huber et al.[26] have found that some acidic PTH-amino acids were subject to counterdirectional electrophoretic migration and did not enter the ODS column at pH 7.55. Second, electrostatic interaction between the charged groups on the packing beds, which is necessary for generation of EOF, and the ionic compounds will inevitably take place. This kind of secondary interaction between the stationary phase and solutes may result in poor efficiency or peak tailing. It was assumed that the interaction of the fully dissociated carboxylic group with an unbounded silanol group on the surface of the ODS packing materials results in the poor efficiency.[27,28] Because of the interaction of basic solutes with ionized silanol groups, peak tailing for basic solutes in reversed-phase CEC with silica-based ODS packing was often reported.[29−31] To improve the peak symmetry, a competing base was typically added in the mobile phase.[29−31]

In fact, as for separation of ionic compounds, the CEC system can be without EOF. The ionic compounds will be driven by their electrophoretic mobilities, and the separation can be obtained according to the differences in the electrophoretic mobilities and interactions of them with the stationary phase. In this work, a neutral hydrophobic monolithic column was prepared for separation of peptides. It was found that EOF in this system is very low, and the migration of the peptides mainly resulted from their electrophoretic mobility. Separation of peptides was obtained with high column efficiency and good reproducibility.

EXPERIMENTAL SECTION

2.1. Materials. Peptides were purchased from Serva (Heidelberg, Germany). Lauryl methacrylate (LMA), ethylene dimethacrylate (EDMA), and γ-methacryloxypropyltrimethoxysiliane (γ-MAPS) were purchased from the Sigma Chemical Co. (St. Louis, MO). 1-Propanol, 1,4-butanediol, and azobisisobutyronitrile (AIBN) were obtained from the Fourth Shanghai Reagent Plant (Shanghai, China). HPLC grade acetonitrile (ACN) was supplied by the Yuwang Chemical Plant (Zibo, Shangdong Province, China). Lauryl methacrylate and ethylene dimethacrylate were extracted with 5% aqueous sodium hydroxide solution and dried by anhydrous magnesium sulfate. All test compounds were of analytical grade. Water used in all of the experiments was doubly distilled and purified by a Milli-Q system (Millipore Inc., Milford, MA). Capillaries with 75 μm i.d. and 365 μm o.d., from which about a 1-mm portion of the polyimide coating was removed for UV detection, were purchased from the Yongnian Optic Fiber Plant (Hebei, China).

2.2. Instrumentation. Electrochromatographic experiments were carried out on a Beckman MDQ system (Beckman, Fullerton, CA) equipped with a UV detector. Data acquisition and processing were performed with the Beckman ChemStation software. A HPLC pump was used to flush the columns.

2.3. Samples and Solution. The peptides used in this study were first dissolved in water, then were diluted to the appropriate concentration with the mobile phase before injection. The stock solution of phosphate buffer (200 mM) was prepared by dissolving 3.12 g NaH_2PO_4 in about 90 mL of water, then adjusting to various values of pH by either on or both of sodium hydroxide and phosphoric acid and transferring the solution to a 100-mL flask. The running buffers were prepared by mixing 5 mL of phosphate buffer and the appropriate volume of acetonitrile and water. Before running, the running buffers were degassed in an ultrasonic bath for about 15 min.

2.4. Preparation of the Monolithic Polymer Capillaries. The procedure for vinylization of the capillary was similar to that reported in the literature.[15] The capillary was rinsed first with 0.1 M NaOH solution for 1 h and then with water until the pH value of the outlet solution was 7.0. After subsequent flushing with methanol for 10 min, it was dried by passage of nitrogen gas. γ-MAPS dissolved in methanol in a volume ratio of 1:1 was injected into the capillary with a syringe. It was then kept at 35 °C overnight with both ends sealed with rubber. Finally, the capillary was rinsed with methanol and water successively to flush out the residual reagents. Thus, the layer of γ-MAPS was introduced onto the inner wall of the capillary.

AIBN (5 mg, ∼0.5wt % with respect to the monomers) was dissolved in the solution consisting of 500 μL of EDMA and 600 μL of LMA, respectively. The porogenic solvent contained 45 wt % 1-propanol and 55 wt % 1,4-butanediol. Monomer and porogenic solvent in the volume percentages of 50% were mixed together. After ultrasonication for 15 min and being sparged with nitrogen for 10 min, a small part of the mixed solution was removed for capillary preparation using a 100-μL syringe. A pretreated 35-cm capillary was attached to the syringe inlet, and the polymerization mixture was sucked into the capillary for a certain length. The capillary was plugged at both ends with rubber stoppers and was hung in a GC oven at 60 °C for 12 h. The prepared monolithic capillary column was washed with methanol and water by a HPLC pump to flush out the residual reagents. No movement of the monolithic bed was observed. The detection window was then created at the end of the continuous polymer bed. Finally, the capillary with the monolithic polymer stationary phase was

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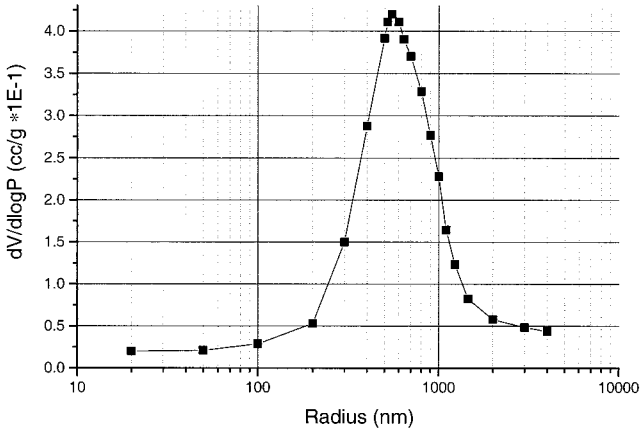

Figure 1. Pore size distribution profile of the uncharged monolithic column.

equilibrated with the running buffer by applying 95 psi for ~4 h before separation of the samples.

2.5. Electrochromatographic Experiments. The monolithic capillary column was placed in the CE instrument and equilibrated by applying a voltage of 1 kV under a pressure of 95 psi at both ends until the baseline signal was stabilized. The injections were made by applying a voltage of 2 kV for 2 s in CEC. The temperature was kept at 25 °C, and the detection wavelength was set at 214 nm. Separation of peptides in the CEC experiment was performed using a capillary of 30 cm (10 cm inlet to detector) × 75 μm i.d. × 365 μm o.d.

RESULTS AND DISCUSSION

3.1. Performance of the Monolithic Column. In this study, the monolithic columns were prepared by in-situ polymerization of LMA with EDMA. The permeability of the porous polymeric monolith depends on its porous property. The typical pore size distribution profile of the monolithic column was measured by mercury porosimetry and is shown in Figure 1. The average pore diameter of the monolithic column is ~550 nm. Thiourea could be eluted at ~4.2 min with inlet pressurized at 95 psi. This means that the monolithic bed has good permeability, and its flow resistance is low.

There are no ionizable moieties existing on the uncharged monolithic bed, so there should be no strong EOF generated on this type of columns. The effect of the eluent pH on the electroosmotic mobilities in CEC with a monolithic column was investigated, and the obtained results are shown in Table 1. As can be seen, the electroosmotic flow in such a monolithic column is quite poor, even when the eluent pH is 8.0. This result indicates that the EOF in a neutral monolithic column is negligible, and the silanol groups in the inner wall of the capillary are almost completely covered by the monolithic bed. The weak electroosmotic mobilities may be attributed to ionization of the residual silanol groups on the unpacked capillary wall.

Under the usual CEC, the mobile phase and neutral solutes are driven only by the EOF, and the electrophoretic mobility also contributes to the migration of the ionic compounds. In our case, when the ionic solutes are separated on the neutral monolith column, they are driven mainly by the electrophoretic mobility, as in CZE, but the interaction of ionic solutes with the monolithic bed provides the different separation selectivity from conventional

Table 1. Effect of pH on Electroosmotic Flow in Monolithic Capillary Column[a]

pH of mobile phase	Electroosmotic mobility ($\times 10^8$ $m^2v^{-1}s^{-1}$)
8.0	0.106
7.0	0.085
6.0	0.083
4.5	0.075
3.0	0.034

[a] Experimental conditions: buffer, 2 mM Phosphate, 40%(v) acetonitrile; column, 100 μm i.d. × 365 μm o.d., effective length 10 cm (total length 20 cm).

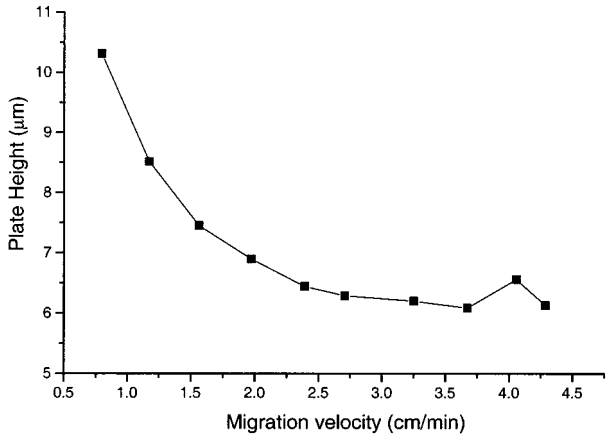

Figure 2. Plot of the plate height (H) versus the linear velocity of the eluent (u) for Lys-Ser-Trp. Experimental conditions: column, effective length 10 cm (total length, 30 cm) with 75 μm i.d. and 365 μm o.d.; mobile phase, 40 mM phosphate buffer (pH 2.1); UV detection wavelength, 214 nm; electrokinetic injection, 5 kV for 5s.

CZE. The performance of the monolithic column was evaluated by separation of peptides at various voltages. The current (I) across the monolithic column (effective length, 10 cm; total length, 30 cm) linearly increased when the applied voltage (V) ranged from 3 to 10 kV, with correlation coefficient of 0.9998, which means that the Joule heating could be neglected. The reproducibility of migration times for six peptides on such monolithic columns is evaluated by 10 consecutive separations, and the relative standard deviations observed for t_r of the six peptides is <0.5%.

The efficiency of the monolithic column up to 150 000 plates/m can be obtained for the separation of peptides. The dependence of the plate height (H) on the migration velocity for Lys-Ser-Trp through the capillary is shown in Figure 2. It can be seen that the plate height decreases steeply when increasing the migration velocity, and then it becomes almost constant at velocity higher than 2.5 cm/min. Apparently, with low velocities, the axial diffusion plays the major role, and mass transfer contribution to the plate height is small, even at higher velocity. This is because the absence of interparticular volume in the continuous column forces all of the mobile phase to flow through the separation medium rather than around it,[32] and the mass transfer of the molecules is enhanced by convection, a process that has a positive effect on the separation.[33] Usually, the electroosmotic flow (EOF)

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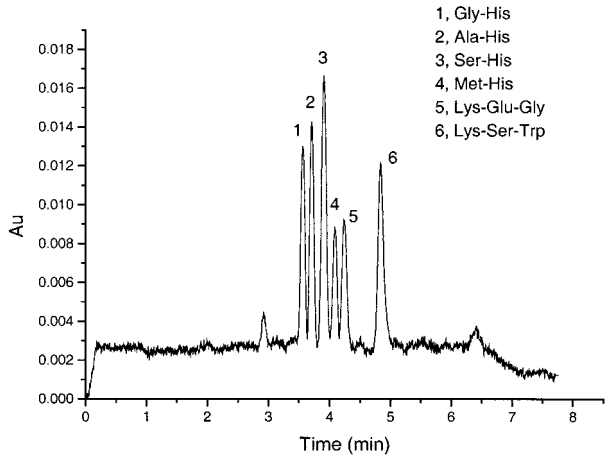

Figure 3. Electrochromatogram for separation of basic peptides on the monolithic column. Experimental conditions: applied voltage, 5 kV. Other conditions are the same as those in Figure 2.

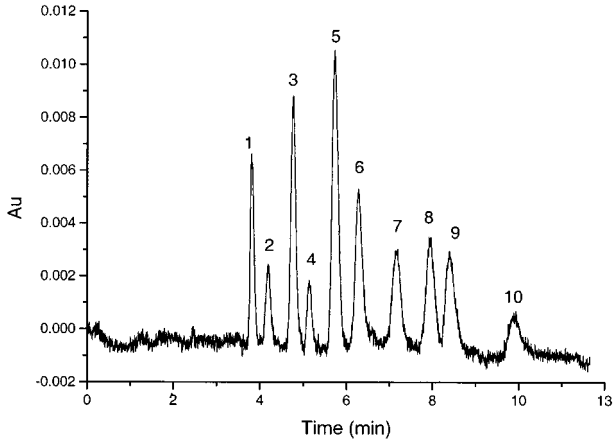

Figure 4. Electrochromatogram for separation of the peptide mixture on the monolithic column Experimental conditions are the same as those in Figure 3. Peaks: 1, Val-Lys; 2, Lys-Glu; 3, His-Phe; 4, Lys-Thr-Tyr; 5, Gly-Ser; 6, Gly-Ile; 7, Met-Val; 8, Met-Met; 9, Met-Leu; 10, Met-Tyr.

is used to drive the mobile phase instead of hydrodynamic flow in CEC. Because of the flat, plug-like profile of the EOF, CEC offers greatly enhanced separation efficiencies relative to HPLC. However, the electroosmotic mobilities in this system are very low, and the movement of ionized solutes in the capillary should be mainly attributed to electrophoretic mobility, which also results in the flat profile for migration of ionized solutes to account for the relatively low plate height at high migration velocity.

The application of the C_{12} monolithic column was demonstrated by CEC separation of peptide mixtures under an eluent of 40 mM sodium phosphate buffer (pH 2.1), and the obtained electrochromatograms are shown in Figures 3 and 4. It can be seen that the baseline separation for mixture peptides was obtained, and all of the basic peptides containing amino acids of His and Lys showed very good peak symmetry.

3.2. Comparison of Separation Selectivity on CEC with CZE. CZE can be used to separate peptides on the basis of the differences in electrophoretic mobility of the solutes. The peptides in the neutral monolithic CEC column are also driven by their electrophoretic mobility; however, the selectivity may be different

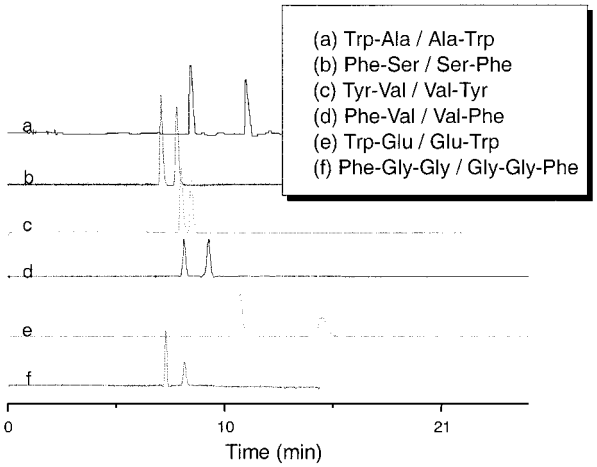

Figure 5. Electrochromatograms for separation of peptide isomers on monolithic column in CEC. Experimental conditions are the same as those in Figure 3.

Table 2. Comparison of Peptide Isomer Separation in CZE and CEC[a]

peptide isomers	resolution in CZE[b]	resolution in CEC[b]
Trp-Ala/Ala-Trp	unseparated	4.078
Phe-Ser/Ser-Phe	unseparated	2.206
Tyr-Val/Val-Tyr	−1.065	1.191
Phe-Val/Val-Phe	−1.260	3.760
Glu-Trp/Trp-Glu	unseparated	8.274
Phe-Gly-Gly/Gly-Gly-Phe	unseparated	3.535

[a] Experimental conditions: buffer, 40 mM phosphate, pH 2.1; CZE, capillary with 75 μm i.d. × 365 μm o.d., total length 30 cm (effective length 20 cm); CEC, capillary with 75 μm i.d. × 365 μm o.d., total length 30 cm (effective monolithic length 10 cm). [b] Where resolution (R_s) is calculated with equation as follows: $R_s = (t_{r(2)} - t_{r(1)})/(W_{1/2(2)} - W_{1/2(1)})$, the negative R_s values in CZE means that the order of eluted peptides is reversed from that in CEC.

from that in CZE because of the involvement of the chromatographic mechanism. Six pairs of dipeptides isomers, including Trp-Ala/Ala-Trp, Phe-Ser/Ser-Phe, Tyr-Val/Val-Tyr, Phe-Val/Val-Phe, Trp-Glu/Glu-Trp, and Trp-Ala/Ala-Trp, and a tripeptide isomer of Gly-Gly-Phe/Phe-Gly-Gly were selected as test solutes. They were separated in CEC with the monolithic column and in CZE under an identical buffer. In the former case, both electrophoretic and chromatographic mechanisms contribute to the migrations of peptides; but in the later case, only electrophoretic mechanism contribute to their migrations. The obtained results for the separation of the above peptide isomers by CZE and CEC modes are listed in Table 2. Figure 5 shows the typical chromatograms for separation of peptides isomers in CEC. As shown in Figure 5, good separation of all six pairs of peptide isomers on CEC was obtained; however, only two pairs of peptide isomers, Tyr-Val/Val-Tyr and Phe-Val/Val-Phe, were separated or partially separated on CZE, and their elution orders in CZE were reversed to those in CEC. The peptide isomers have a similar mass/charge ratio and may be difficult to be separate in CZE mode at highly acidic or basic buffer, because the separation of solutes in CZE is based only on the difference of electrophoretic mobility as mentioned above. However, these peptide isomers could be well-separated in capillary electrochromatography as a result of their different

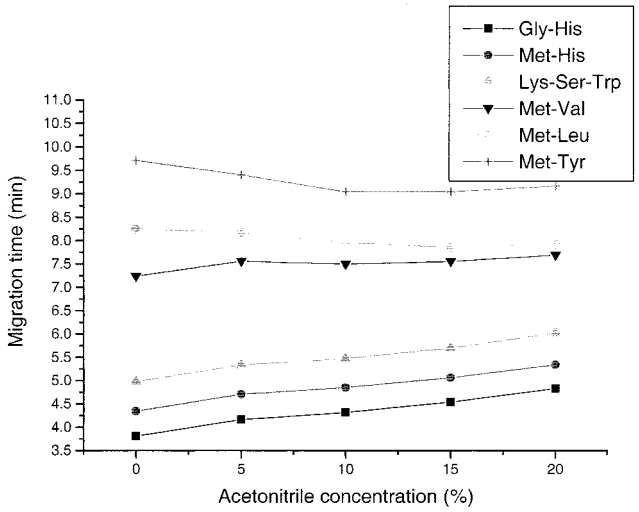

Figure 6. Effects of acetonitrile concentration on the migration time of peptides. Experimental conditions: mobile phase, 40 mM phosphate buffer (pH 2.1) with acetonitrile concentration varied from 0 to 20%. Other conditions are the same as those in Figure 3.

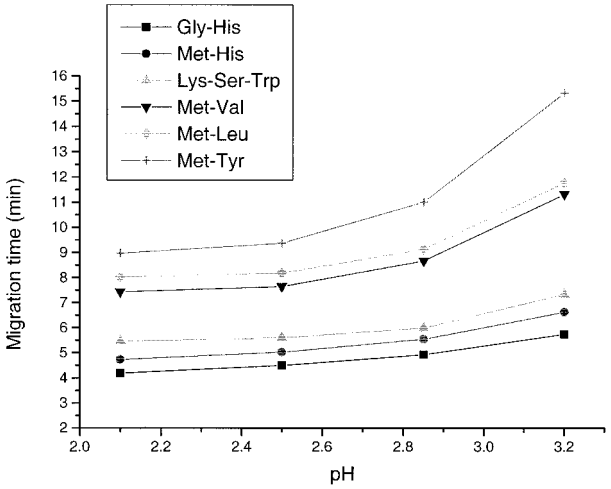

Figure 7. Effect of eluent pH on the migration time of peptides. Experimental conditions: mobile phase, 40 mM phosphate buffer (pH 2.1) with pH varied from 2.1 to 3.2. Other conditions are the same as those in Figure 3.

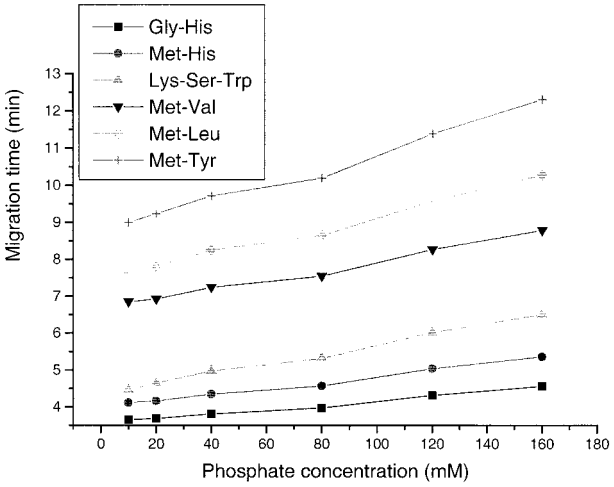

Figure 8. Influence of phosphate concentration on the migration time of peptides. Experimental conditions: mobile phase, phosphate buffer (pH 2.1) with concentration varied from 10 to 160 mM. Other conditions are the same as those in Figure 3.

interactions with the hydrophobic stationary phase, but the analytes were propelled mainly by electrophroetic mobility.

3.3. Effect of Mobile Phase Compositions. In the capillary electrochromatography, the capacity factor (k') used in chromatography is no longer valid for describing the migration process of an ionic compound because of the presence of electrophoretic mobility and chromatographic interaction. The migration time of the analytes was chosen in order to investigate the effects of the mobile phase compositions on the separation.

The effect of the acetonitrile concentration on the peptide separation was investigated by using phosphate buffer at pH 2.1, and the obtained results are shown in Figure 6. It can be seen that the migration time of the early-eluted peptides (Gly-His, Met-His, and Lys-Ser-Trp) increases, but that of the late-eluted peptides (Met-Val, Met-Leu and Met-Tyr) slightly decreases when increasing the concentration of acetonitrile from 0 to 20%. This can be explained by the fact that the former three dipeptides are weakly retained on the monolithic stationary phase, and the dominant mechanism contributing to their migration is electrophoretic mobility. Therefore, as in CZE, the increase of acetonitrile concentration will decrease their electrophoretic mobility and lead to the longer migration time. The latter three peptides are strongly retained on the stationary phase, and the dominant mechanism contributing to their migration is chromatographic interaction. As in reversed phase chromatography, the higher the acetonitrile concentration, the weaker their retention on the stationary phase, thus resulting in faster migration under a high acetonitrile concentration.

The effects of the eluent ion strength and pH on the separation were studied by using phosphate buffer containing 10% acetonitrile, and the obtained results are shown in Figures 7 and 8, respectively. As can be seen from Figure 7, the migration times of all six peptides increased when the pH increased from 2.1 to 3.2. This may be the result of two things: First, the positive charge of the peptides decreases with increasing of pH, which results in the decrease of electrophoretic mobility and, thereby, a long migration time. Second, the hydrophobicity of peptides may also be increased because of the decrease of peptide charge, which results in strong chromatographic interaction and long migration time. The effect of the eluent ionic strength on the migration of peptides in this CEC mode may be similar to that in CZE. The increase in migration time for peptides may result from the decrease in the electrophoretic mobility of peptides with the increase of eluent ionic strength. Another reason contributing to the increase of the migration may be that the hydrophilicity of mobile phase will increase with an increasing ionic strength, which will lead to the strong chromatographic interaction with stationary phase and longer migration time.

3.4. Effect of the Applied Voltage. The influence of the applied voltage on the migration behavior of peptides was also investigated. There is no doubt that the migration time of all peptides decreases seriously with when the applied voltages are increased. As was addressed previously, the Joule heating in this system is negligible; therefore, the applied voltage has almost no influence on electrophoretic mobility. However, the influence of voltage on the retention factor of peptides is difficult to determine,

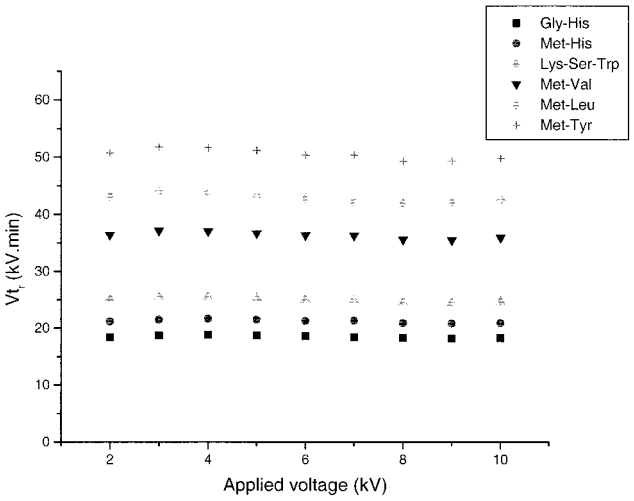

Figure 9. Relationship of the product of migration time of peptides and the applied voltages with the applied voltages. Experimental conditions: applied voltage varied from 2 to 11 kV; other conditions as in Figure 2. Solutes: 1, Gly-His; 2, Met-His; 3, Lys-Ser-Trp; 4, Met-Val; 5, Met-Leu; 6, Met-Tyr.

because both electrophoretic and chromatographic mechanisms contribute to the migration of the charged solutes.

The following equation can be derived from chromatographic theory,

$$Vt_r = (1 + k')\frac{L_t L_{id}}{\mu_{ep}} \quad (1)$$

where k' is the retention factor contributed only by the interaction of the solute with the stationary phase, L_{id} is the column length from injection end to detection window, L_t is the total length of the capillary monolithic column, μ_{ep} is the electrophoretic mobolity of the solute, t_r is the migration time of the solute, and V is the applied voltage for separation.

The value for μ_{ep} should be a constant for a given solute if the Joule heating is negligible; therefore, any change in the value of Vt_r result from the changing of k' at different voltage. This means that the influence of voltage on k' is negligible if Vt_r is kept at a constant. The experimental data at different voltages are treated, and obtained results are shown in Figure 9. The values of Vt_r are almost not affected by the applied voltages, which indicates that the chromatographic retention factors of the tested peptides (k') contributed mainly from the hydrophobic interaction with the neutral monolithic bed are not changed during the CEC separation. These results are in agreement with that obtained in reversed-phase CEC packed with ODS particles in which little influence of voltage on the retention of neutral solutes was found.[34] However, the retention of acidic compounds was found to increase when the applied voltage in ion-exchange CEC is increased.[28]

CONCLUSION

This is the first attempt to use the copolymer (LMA and EDMA) as a support material for CEC separation of peptides. The EOF on a monolithic column was many times less than that in a bare silica capillary, because the residual silanol groups on the capillary wall were reacted or covered, or both, by the MAPS and copolymer. Separation of peptides was realized with elelctrophoretic mobility as the driving force on the basis of their differences not only in the electrophoretic mobility, but also in the hydrophobic interaction between the peptides and the monolithic bed. The existence of hydrophobic interaction between the peptide ions and hydrophobic monolithic bed can provide the additional separation selectivity that is different from conventional CZE. Symmetric peaks, high column efficiency, and good reproducibility were obtained for the separation of all of the peptides, including basic peptides. The CEC mode developed in this work may provide a complementary technique for separation of charged solutes from CZE because of the involvement of the chromatographic mechanism.

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