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Open-Tubular Capillary Electrochromatography Coupled with Electrospray Ionization Mass Spectrometry for Peptide Analysis

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In this study, the open-tubular electrochromatographic (OT-CEC) migration behavior of various peptides has been characterized using etched and chemically (*n*-octadecyl- and cholesterol-) modified capillaries, interfaced to an electrospray ionization mass spectrometer through a sheath liquid configuration. The stationary phases were fabricated by etching the inner wall of the fused-silica capillary and then chemically modifying the new surface through a silanization/hydrosilation reaction. Unlike some other OT-CEC stationary-phase preparation methods, leaching of the immobilized stationary phase and subsequent contamination of the electrospray ion source was largely avoided with this novel surface modification technology. The influence of the immobilized organic phases and those of the buffer electrolytes (pH, the type and content of organic solvent) on the retention and separation of the selected peptides was investigated. Significant peptide retention was found even at very low pH with both types of stationary phases, under conditions whereby the electrophoretic migration dominated the separation process. Due to the effective coverage of the etched surface by a silanization/hydrosilation reaction, adverse adsorption of charged analytes onto the capillary wall was minimized. As a result, very efficient and highly reproducible peptide separations were achieved over a broad pH range. Moreover, peptide-specific multizoning effects were observed. The origin of this novel phenomenon was explored. Compared to capillary electrophoresis electrospray ionization mass spectrometry system, much higher detection sensitivity could be obtained, since a larger amount of sample could be injected and stacked at the head of the open-tubular capillary column without deteriorating the separation performance. On the basis of these observations, these procedures have been adapted to allow the analysis of tryptic peptides generated from proteins.

Capillary electrochromatography (CEC) is a hybrid technique of capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC),[1−6] with a recognized potential in the separation of peptides.[7,8] As in capillary electrophoresis, the application of an electric field across a capillary generates an electroosmotic flow (EOF), which drives the mobile phase through the capillary. In contrast to the parabolic flow profile produced by a mechanical pump in HPLC, a flat plug flow profile is achieved for the EOF with electrically driven systems, resulting in narrower peaks and higher separation efficiencies.[4,9] Resolution in CEC occurs via differential solute−stationary-phase interactions, and in the case of charged analytes, via differential electrophoretic mobilities as well.[5,10] Thus, CEC combines the attributes of both chromatographic and electrophoretic separation and possesses the advantages of high separation efficiency found with capillary electrophoresis and the high selectivity of liquid chromatography.[11]

Packed capillaries are commonly used in CEC. Generally, a fused-silica capillary with an internal diameter of 50−100 μm is employed, packed with reversed-phase chromatographic sorbents of 1.5−10-μm particle size. The packing material is held in place by two retaining frits, usually created by thermal fusion of the packing material itself.[12] An advantage of packed capillaries is the high stationary-phase to mobile-phase ratio, and thus high retention and capacity. However, several limitations need to be addressed before this conventional form of CEC becomes a viable alternative to HPLC or CE. The most challenging problem is to retain the packing material with reproducibly fabricated frits, while maintaining unrestricted flow through the capillary. Another problem of packed capillaries is the tendency of bubble formation around the frits, which often results in unstable baseline, variable migration times, and current breakdown. In addition, basic

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compounds tend to interact irreversibly with the ionized silanol groups on the surfaces of silica-based stationary phases, because of the lower surface coverage of the particles, leading to peak tailing and deterioration of separation.

An alternative approach to packed-column capillary electrochromatography is open-tubular (OT)-CEC. In the OT-CEC format, the stationary phase is immobilized onto the inner walls of fused-silica capillaries; thus, problems associated with the use of retaining frits and silica-based particles can be eliminated. To date, several methods have been developed for the preparation of stationary phases in OT-CEC, such as physical adsorption, covalent bonding and cross-linking, porous layers, sol−gel technology, and molecular imprinting.[13] Among these methods, physical adsorption of charged compounds to the capillary wall is the simplest way to create a stationary phase in OT-CEC. This can be achieved by rinsing the capillary with buffers containing a monomeric or polymeric surfactant to form single- or multiple-layer coatings.[14,15] However, these coatings were primarily created for the purpose of reducing the adverse analyte−capillary wall interactions rather than inducing chromatographic retention per se.[16] The lifetime of these coatings is also usually short because of the release of the coating materials into the buffer electrolyte.[17] As a result, when these types of OT-CEC columns are coupled directly to an electrospray ionization mass spectrometer, the introduction of the nonvolatile surfactant carried with the buffer electrolyte into the mass spectrometer creates a high background signal and interferes with detection of the analytes of interest. Moreover, a further drawback of the OT-CEC columns prepared with the above methods is the low phase ratio, and this results in weak chromatographic retention and low selectivity. In order to address these problems, we developed a new approach that involves first, etching the inner wall of the fused capillary and, second, chemical modification of the new surface via silanization/hydrosilation reactions.[18] As a result of the etching procedure, the surface area of the inner capillary wall is increased significantly by up to 1000-fold.[18,19] Moreover, this particular format of OT-CEC has proved to be well-suited for the separation of basic compounds,[18−21] since ~95% of silanols can be modified by the silanization reaction,[22] yet leaving sufficient shielded silanol sites to allow a high EOF to be generated.

Coupling of CEC with mass spectrometry provides high sensitivity and enhanced selectivity and also offers molecular structure information, especially when a mass spectrometer that has tandem mass spectrometric capability is employed.[23,24] In this regard, packed columns have been utilized for most CEC/MS studies.[5,23,24] Compared to the packed CEC/MS systems, the coupling of OT-CEC columns with commercial and "custom-built" mass spectrometry systems is much easier and straightforward.[13,25] Surprisingly, only a few studies so far have been conducted with OT-CEC/MS systems.[13,15,25] In this study, the migration behavior of a group of peptide hormones and a protein digest as a function of several experimental variables has been investigated by using etched chemically modified capillaries coupled to an ion trap electrospray ionization mass spectrometer. The organic moieties bonded onto the inner capillary wall were either octadecyl (C_{18}) or cholesterol-10-undecenaoate. These investigations thus provide critical information about the effectiveness of OT-CEC/MS in peptide analysis as well as insight into the mechanism of differential migration and the retention processes underlying the separation of these biomolecules by OT-CEC/MS.

EXPERIMENTAL SECTION

Chemicals and Materials. Acetonitrile (ACN) and methanol (HPLC grade) were obtained from Biolab Scientific Pty Ltd. (Sydney, Australia). Water was distilled and deionized in a Milli-Q system (Millipore, Bedford, MA). Ammonium formate, ammonium acetate, and 100% (v/v) acetic acid were purchased from BDH Chemicals Australia Pty. Ltd. (Kilsyth, Australia). NaOH and 99% (v/v) formic acid were obtained from Ajax Chemicals (Sydney, Australia). The peptides (bombesin, bradykinin, bradykinin fragment 1−5, leucine enkephalin, luteinizing hormone releasing hormone, methionine enkephalin, oxytocin, substance P, and [Arg^8]-vasopressin) as well as cytochrome *c* (from horse heart) and TPCK (tosyl-phenylalanyl-chloromethyl-ketone)-treated trypsin (bovine) were obtained from Sigma Aldrich (St. Louis, MO) and used without any further purification. The untreated fused-silica capillaries were purchased from Agilent Technologies.

The stock solutions of 100 mM ammonium formate, pH 2.0 and pH 3.0, were made by titrating 100 mM ammonium formate with formic acid, while the stock solutions of 100 mM ammonium acetate buffer, pH 4.0 and pH 5.0, were made by titrating 100 mM ammonium acetate with acetic acid. The stock solution of 100 mM ammonium acetate was made without any pH adjustment. All the above stock solutions were filtered through a 0.22-μm-pore sized filter (Millipore, Bedford, MA). The OT-CEC, CE running electrolytes and sheath liquids were prepared by mixing appropriate proportions of the stock solution, Milli-Q water, and the organic solvent and were degassed by ultrasonication for 10 min before use.

For the protein cleavage, a total amount of 1 mg of cytochrome *c* was dissolved in 1 mL of 50 mM NH_4HCO_3, and the resultant mixture was digested with trypsin overnight at 37 °C, at a protein/trypsin ratio of 50:1 (m/m). The digestion was stopped by adding formic acid to a final volume fraction of 1%.

Preparation of the OT-CEC Capillaries. The OT-CEC capillaries used in this study (with *n*-octadecyl- and cholesterol-bonded surfaces) were fabricated by etching and chemically modifying fused-silica capillaries (50-μm i.d.) obtained from

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Polymicro Technologies (Phoenix, AZ). Detailed procedures for the etching of the surface of fused-silica capillaries and bonding of organic moieties onto the etched inner wall via the silanization/hydrosilation process have been described elsewhere.[18,22,26,27]

OT-CEC/ESI-MS Instrumentation. All the OT-CEC/MS and CE/MS experiments were carried out with an Agilent CE capillary electrophoresis system coupled to an Agilent 1100 series LC/MSD-SL ion trap mass spectrometer through an Agilent G1607A orthogonal electrospray interface (Agilent Technologies, Waldbronn, Germany). Electrical contact at the electrospray needle tip was established via a liquid sheath flow delivered by an Agilent 1100 series isocratic LC pump. All system control and data acquisition were conducted with Agilent ChemStation and MSD Trap Control software.

OT-CEC/ESI-MS Conditions. All the OT-CEC and CE capillaries employed in this study had a total length of 68 cm and were thermostated at 25 °C during all experiments, with the exception of the capillary section that joins CE and MS (36 cm). When a new OT-CEC capillary was first used, a preconditioning step with water for 10 min and 90% MeOH (v/v) for 1 h was performed. All the samples were injected hydrodynamically at 50 mbar. Between runs, the OT-CEC capillaries were regenerated by flushing with water, 90% MeOH (v/v), water, and the fresh separation buffer electrolyte for 5 min, respectively. During all these flushing steps, the mass spectrometer was set to the standby mode. All the experiments were conducted under positive polarity mode at 20 kV (with the anode at the CE inlet and cathode at the MS side) with typical currents of 10−30 μA. The sheath liquid consisted of 1 mM ammonium acetate in 50% MeOH (v/v) and was delivered at a flow rate of 3 μL/min.

ESI-MS analysis was carried out in the positive ion mode. The electrospray voltage was 3.5 kV, and the ion trap mass spectrometer was operated in full-scan mode (partially varied in the range of 50−1200 m/z). The nebulizing gas (N_2) pressure, the drying gas (N_2) flow rate, and the drying gas temperature were set at 41.3 kPa, 5 L/min, and 150 °C, respectively. The ion accumulation time was automatically adjusted using the ion charge control (ICC) feature of the instrument. The maximal accumulation time was set at 300 ms, and the ICC target was set at 30 000. A smart parameter setting of the Agilent MSD Trap Control software was utilized to optimize the ion trap operation. The target mass was set at 600 m/z, the compound stability was set at 50%, and the trap drive level was set at 100%. For the tandem mass spectrometry experiments, the instrument was automatically switched from MS to MS/MS mode when the intensity of a particular ion exceeded the preset threshold. Automatic data analysis was achieved by postprocessing with Agilent data analysis software (version 5.2).

RESULTS AND DISCUSSION

The distinctive features of the etched chemically modified OT-CEC capillaries have been discussed in our previous papers,[14,19,21,26−29] with these studies performed with nonvolatile buffer electrolytes and UV detection. For the coupling of OT-CEC with electrospray ionization mass spectrometry, volatile buffer electrolytes are required in order to achieve improved performance and detection.[24,30] In this investigation, a group of peptides, which have diverse physicochemical properties and structures, were employed to characterize the performance of C_{18}- and cholesterol-bonded OT-CEC capillaries with ESI-MS-compatible buffers. In addition, the separation of a protein digest was also carried out to explore the potential of these OT-CEC capillaries for proteomic applications.

Stability and Reproducibility of the C_{18}- and Cholesterol-Modified OT-CEC Capillaries. In OT-CEC, the properties of the stationary phase immobilized onto the capillary wall not only influence the direction and magnitude of the EOF[16,17,25] but also have an effect on the chromatographic interaction of analytes.[13,27−29] Moreover, the stability of the bonded stationary phase is also very important for reproducible separations. Stationary phases fabricated by methods such as physical adsorption usually are not as stable as covalent bonded phases because the electrostatic interactions and hydrogen bonding that may be involved in physical adsorption are energetically weaker than covalent bonds.[13,17]

Our previous studies have shown that the stability and reproducibility of the etched C_{18}- and cholesterol-modified OT-CEC capillaries were excellent, with very high efficiencies obtained for all capillaries well after more than 200 consecutive injections had been performed.[26,27] Nevertheless, when the OT-CEC capillaries were coupled to the electrospray ionization mass spectrometry through a sheath liquid flow configuration, a special daily postconditioning procedure had to be adopted. This consisted of three flushing steps, i.e., flushing the capillary consecutively with fresh water, 90% MeOH, and air for several minutes, respectively. The application of this daily postconditioning procedure not only allowed the removal of the strongly retained analytes but also freed the capillary of buffer electrolytes. This was an important capillary column maintenance procedure, when the exit of the capillary is connected to electrospray ionization source of the mass spectrometer, since residual buffer electrolytes inside the capillary can crystallize and block the capillary if the capillary is not flushed dry with air when the OT-CEC/MS system is left in standby mode.

Unlike the capillaries used in a traditional OT-CEC system, where the capillaries can be stored with buffer electrolytes, this frequent drying and wetting procedure with an OT-CEC capillary potentially could have adverse effects on the stability of the bonded stationary phase. In order to test this possibility, the stability and reproducibility of the C_{18}- and cholesterol-bonded OT-CEC capillaries was periodically validated under identical experimental conditions. Figure 1 shows two separations of the same peptide mixture with an etched C_{18}-bonded OT-CEC capillary after 50 repetitive use cycles. During this time, 50 different experiments were carried out at different pH, buffers, and organic solvent contents, and the repetitive postconditioning procedure mentioned above was followed to regenerate and clean the capillary.

As can be seen from Figure 1, the reproducibility with regard to the migration times of the peptides was excellent, indicating the ruggedness of the bonded stationary phases and the general applicability of the described postconditioning method. Although slight variations in peak areas and ion current peak intensities

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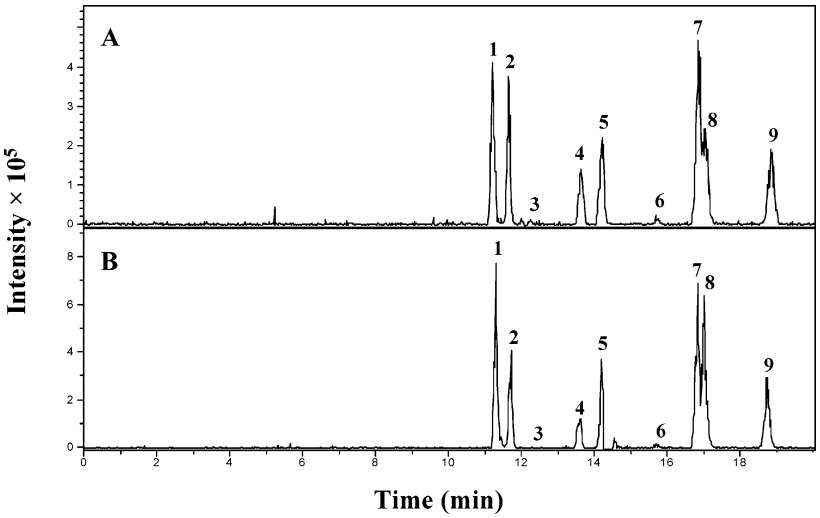

Figure 1. BPC of a peptide mixture separated with an etched C_{18} bonded OT-CEC capillary over a period of three weeks. Total length of the capillary, 68 cm; separation buffer, 20/20/60 ACN/100 mM $HCOONH_4$, pH 2.0/water (v/v/v); separation voltage, 20.0 kV; injection, 50 mbar × 5 s; sample concentration, 0.01 mg/mL. Nine peptides: (**1**) bradykinin; (**2**) bradykinin fragment 1−5; (**3**) substance P; (**4**) [Arg^8]-vasopressin; (**5**) luteinizing hormone releasing hormone; (**6**) bombesin; (**7**) leucine enkephalin; (**8**) methionine enkephalin; and (**9**) oxytocin.

were evident, these differences are thought to be related to minor changes in ionization conditions of the mass spectrometer, although great efforts were made to keep these operating parameters constant.

In addition to the long-term reproducibility, the run-to-run repeatability of the capillary separation was also tested, and the migration times found to be within 2% RSD for five consecutive separations. Considering that the sample tray of the CEC instrument and part of the capillary (from the outlet of the CEC instrument to the electrospray ionization chamber of the mass spectrometer) were not thermostated to the designated operational temperature, this result reflects the significant robustness of the described procedures, since significant changes in temperature are expected to lead to the variation of buffer electrolyte viscosity and, thus, the migration times of analytes.

Retention and Selectivity. Similar to the analysis of peptides with packed CEC capillaries, the retention and selectivity of peptides in OT-CEC can be modulated by changing the electrolyte composition. The etched C_{18}-modified OT-CEC capillaries were first employed to investigate the effects of buffer pH and organic solvent on the retention and migration of the peptide mixture. As demonstrated in the previous studies,[19,21] when buffer electrolytes of low pH values were utilized, a weak anodic EOF was generated with the application of positive voltage, due to the presence of nitrogen-containing functional groups as part of the capillary surface matrix as a consequence of the etching process with NH_4-HF_2. However, all the peptides were substantially protonated under these conditions and, thus, had very high electrophoretic mobilities relative to the EOF. Superimposed on this anodic EOF and high electrophoretic migration were the hydrophobic interactions between the peptides and the nonpolar ligands immobilized onto the inner surface of the OT-CEC capillary. The magnitude of these hydrophobic interactions could be controlled by adjusting the volume fraction of organic solvent in the buffer electrolyte.

Figure 2A shows the separation of the peptide mixture with pure aqueous ammonium formate buffer at pH 2.0. As evident from this result, not all of the peptides were eluted, and several peptides were only partially resolved under this condition. However, with an increase in the content of acetonitrile as organic modifier in the buffer electrolyte, more peptides were eluted. When the volume fraction of acetonitrile was increased to 20% (v/v), as demonstrated in Figure 2B, all peptides were eluted from the OT-CEC capillary. The previously coeluting peptides **1** and **2** were baseline resolved, while better separation of peptides **7** and **8** was achieved at the same time. The migration order of the peptides, i.e., **1** < **2** < **3** < **4** < **5** < **6** < **7** < **8** < **9**, was identical to that obtained in the corresponding separation carried out solely in the CE mode (data not shown). Further increases in the volume fraction of acetonitrile in the buffer electrolyte only led to the variations of peptide migration times, and no change in the elution order of the peptides. These results clearly indicated the occurrence of significant chromatographic retention effects, although the overall separation process of the peptides with buffer electrolytes of low pH was still dominated by electrophoretic migrations. In addition, it should be noted that, due to their unfavorable ionization behavior in the ESI-MS interface, the peak intensity of peptides **3** and **6** under most experimental conditions was very weak.

An increase in the pH value of the buffer electrolyte led to a decrease of peptide net charge and, thus, weakened electrophoretic mobility. At around pH 4−5, depending on the property of the bonded phase, a reversal of the EOF from anodic to cathodic occurred.[14,19,27] The overall electrochromatographic process of the peptides in this pH range still reflected the combination of electrophoretic migration and hydrophobic interactions with the bonded phase, and this could be tuned by the acetonitrile content in the buffer electrolyte. At neutral pH, some peptides were negatively charged; nevertheless, they were still driven to the mass spectrometer by the relatively strong cathodic EOF. The effects of acetonitrile content in the range from 0 to 20% (v/v) on the retention and separation selectivity of the peptides with a 20 mM ammonium acetate buffer, pH 7.0, are shown in Figure 3. It can be seen that the increase in acetonitrile concentration resulted in the elution of some retained peptides, with significant changes in the separation selectivity and elution order also taking place at the same time. It is noteworthy that peptides **7** and **8** coeluted as

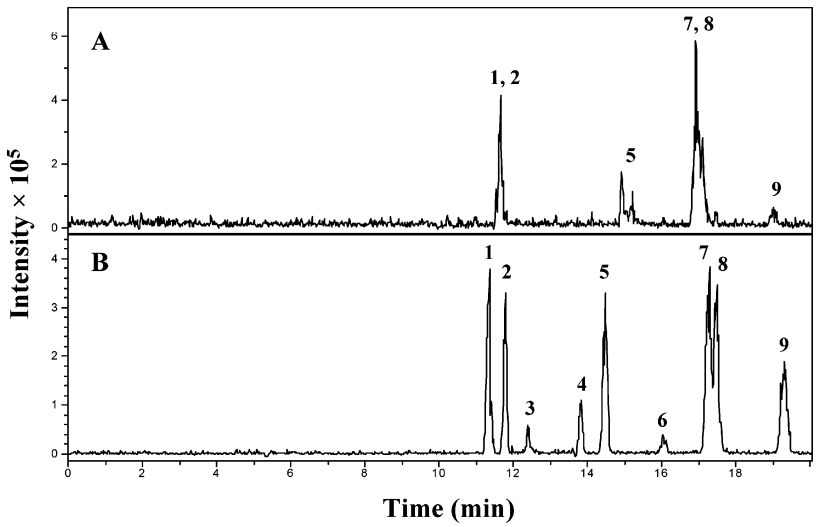

Figure 2. BPC of the peptides illustrating the effects of acetonitrile content in the buffer electrolyte on the retention of the peptides at pH 2. Separation buffers: 20 mM $HCOONH_4$, pH 2.0 in (A) 0% ACN and (B) 20% ACN; other conditions as in Figure 1.

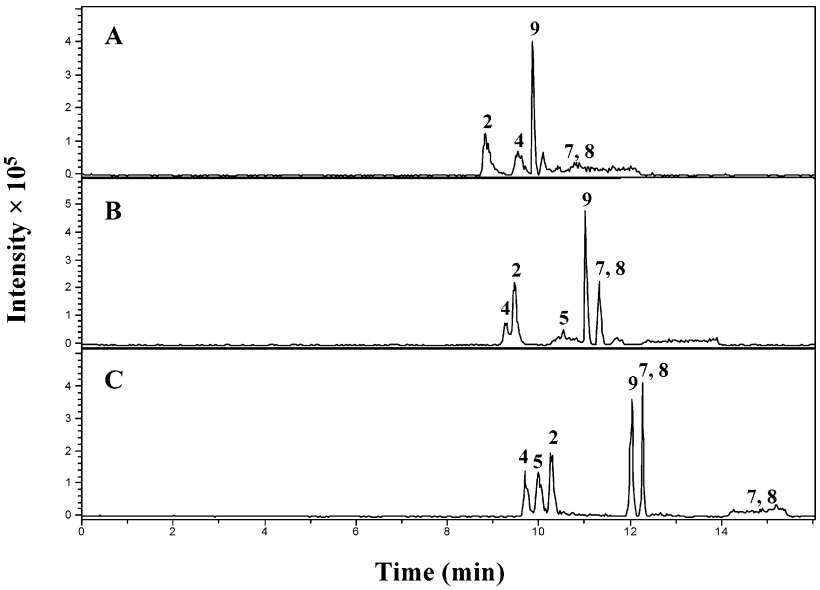

Figure 3. BPC of the peptides illustrating the effects of acetonitrile content in the buffer electrolyte on the retention of the peptides at neutral pH. Separation buffers: 20 mM NH_4Ac in (A) 0, (B) 10, and (C) 20% ACN; other conditions as in Figure 1.

a broad peak with 0% (v/v) acetonitrile, but they coeluted as a sharp and a broader peak with 20% (v/v) acetonitrile in the buffer electrolyte (see below for discussion of the origin of this phenomenon). Further increase in the acetonitrile concentration had no effect on the separation of these peptides. Once again the observed elution order was identical to that obtained in the corresponding separation by CE, although the selectivity was not exactly the same (data not shown). These results clearly indicated that the chromatographic retention could be fine-tuned by the content of acetonitrile to achieve the desired selectivity over a relatively narrow experimental range. Although the chromatographic retention was the dominant process under these conditions, it could be significantly suppressed by acetonitrile concentrations of >20% (v/v).

As in HPLC, the separation selectivity and resolution of the peptide samples can be affected by the type of organic solvents employed. However, in CEC, a change in the type of organic solvent causes alteration of not only the buffer elution strength but also the magnitude of EOF and the electrophoretic mobilities of the peptides.[31−33] The effects of acetonitrile and methanol on the separation of the peptide mixture at pH 7.0 with a 20 mM ammonium acetate electrolyte are illustrated in Figure 4. As evident from these results, a decrease in the EOF occurred when acetonitrile was replaced by methanol, which in turn led to increased migration times of all peptides. A change in the separation selectivity between several peptides, for instance, peptides **2** and **5**, was also obvious. However, the most significant change was the occurrence of the peptide-specific multizoning effects. For example, peptides **7** and **8** coeluted as one sharp peak

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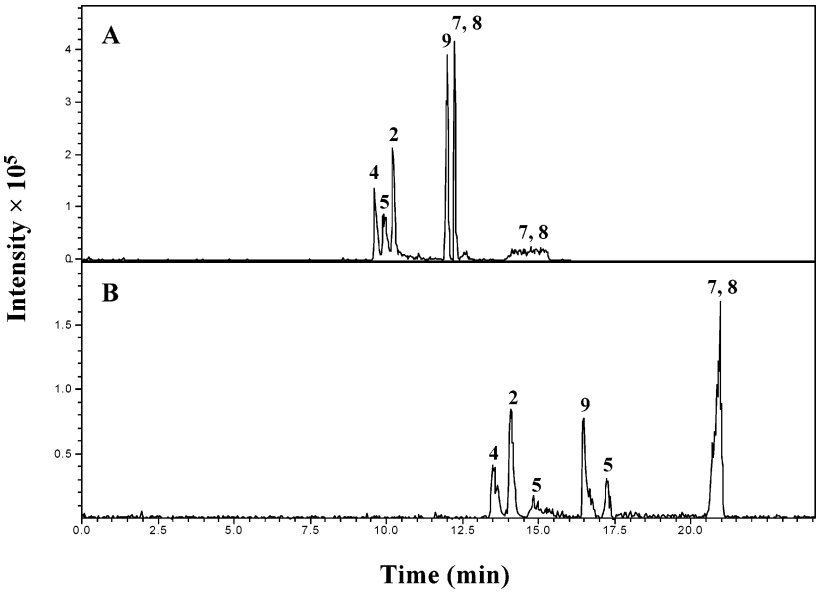

Figure 4. BPC of the peptides showing the effects of organic solvents in the buffer electrolyte on the separation selectivity and resolution of the peptides. Separation buffer: 20 mM NH_4Ac in (A) 20% ACN and (B) 20% MeOH; other conditions as in Figure 1.

and one broad low-intensity peak with 20% (v/v) acetonitrile, but were observed as a single peak of two coeluting peptides with 20% (v/v) methanol while peptide **5** was observed as a single peak in 20% (v/v) acetonitrile, but was detected as two separated peaks in 20% (v/v) methanol in the running electrolyte.

The effects of buffer composition in terms of its pH value and acetonitrile content on the migration and retention behavior of the peptide mixture was also investigated with an etched cholesterol-bonded OT-CEC capillary, and surprisingly, similar findings were observed. Compared to the C_{18}-bonded OT-CEC capillary, no significant differences in the separation selectivity of this group of peptides were found. This is in contrast to our previous studies where the resolution and selectivity of many kinds of analytes, including peptide samples, were affected by changing the type of chemically bonded stationary phase.[19,26,27]

Multizoning Effects of Peptides. One interesting observation made in this work was the occurrence of the peptide-specific and solvent-dependent multizoning effects, noted above. Similar multizoning effects in the analysis of some synthetic peptides with capillary electrophoresis and open-tubular capillary electrochromatography have been reported previously.[27,34] Although several possible mechanisms have been put forward,[27,34] no conclusive explanation has yet been reached. Compared to monitoring such phenomena by UV spectrometry, the use of ion trap mass spectrometric detection offers a better chance to further elucidate the origin of this phenomenon, due to its specificity and tandem mass spectrometry capability.

First, the presence of contaminating compounds in the peptide sample as the cause of the multizoning behavior could be excluded based on the mass spectroscopic data. Considering the amino acid sequence of the peptides, other explanations, such as cis/trans isomerization of proline-containing peptides or hydrolytic cleavage of peptide bonds under the OT-CEC experimental conditions could also be excluded as the cause of this multiple peak behavior. In the case of peptides **7** and **8**, the two major peaks shown in Figure 3 had identical molecular masses, and the tandem mass spectra of the two peaks also demonstrated identical fragmentation patterns (data not shown). If the peptides underwent a peptide-specific, monomer−dimer self-association under the chosen OT-CEC conditions, and these monomer−dimer species were resolvable as a result of subtle difference in their effective charge or hydrophobicity in the OT-CEC environment, they should be identified by the mass spectrometer as discrete species, provided that the dimer structure was not disrupted by the soft electrospray ionization process. Since no m/z ion, corresponding to twice the molecular masses of these two peptides, was observed in our experiments, either dimer−monomer dissociation occurred within the ionization/ion migration stages of the mass spectrometer on a time scale too rapid to be detected or alternative self-association was not a significant process.

Moreover, similar multizoning effects with the peptides **7** and **8** were also found on the other hand in the corresponding capillary electrophoresis separation (Figure 5). Therefore, other possibilities need to be explored to account for the occurrence of the multiple peaks for the same peptide. In this context, the participation of multiple, reversible equilibrium binding processes that are associated with regions of the OT-CEC capillary surface that have micro-heterogeneities at the atomic scale,[19] due to the presence of regions of more densely clustered hydrophobic moieties adjacent to less densely populated regions of coverage, is one candidate possibility. The importance of such surface micro-heterogeneities in generating multizones requires further investigation by, for example, X-ray photoelectron spectroscopy, particularly since a similar multiple peak phenomenon also occurs with untreated fused-silica capillaries.

Another possibility that would account for these observations is that the peptides exists in different solvated charged states or in different conformational states that are resolvable under the specific OT-CEC conditions. One experimental result that supports this conclusion is illustrated in Figure 4, where the multiple peaks

(34) Yang, Y.; Boysen, R. I.; Chen, J. I. C.; Keah, H. H.; Hearn, M. T. W. *J. Chromatogr., A* **2003**, *1009*, 3−14.

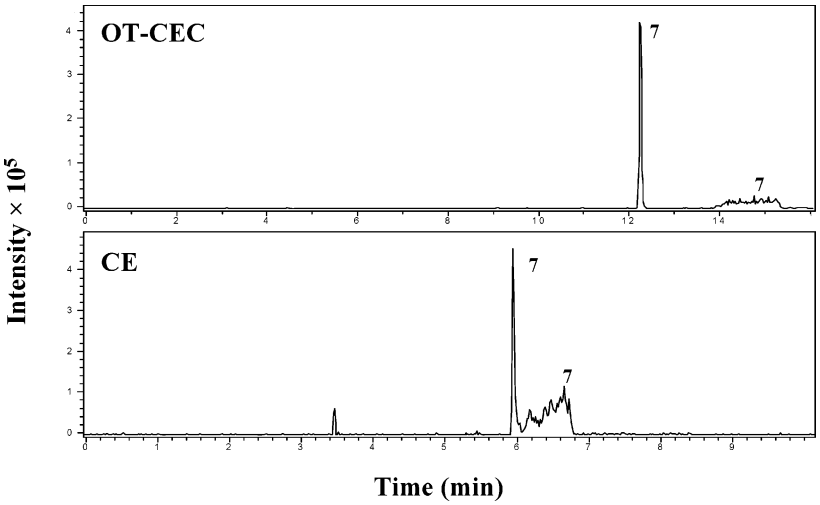

Figure 5. EIC of peptide **7** obtained in OT-CEC (upper trace) and CE (lower trace) experiments. The total length of both the C_{18}-bonded OT-CEC and the CE capillary were 68 cm, with an internal diameter of 50 μm. Separation buffer: 20 mM NH_4Ac in 20% ACN; other conditions as in Figure 1.

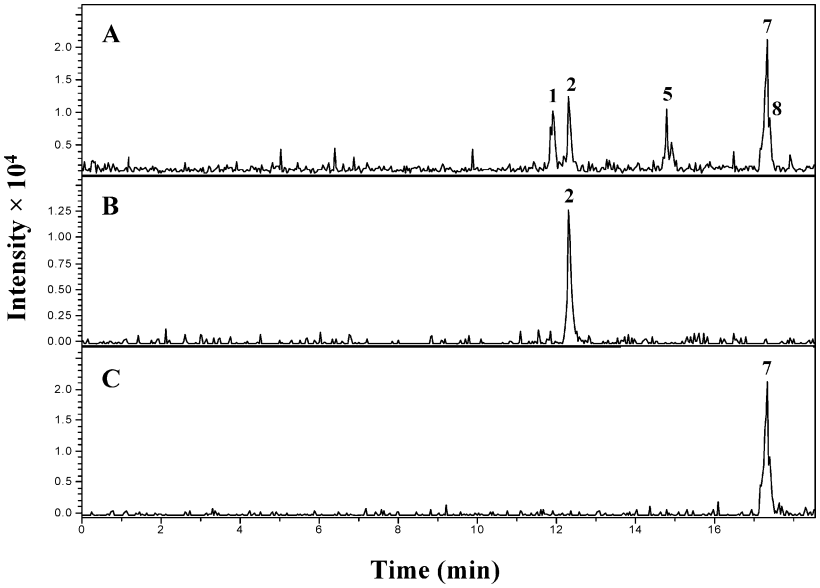

Figure 6. Sensitive analysis of the peptide mixture with an etched C_{18}-bonded OT-CEC capillary. (A) BPC of the peptide mixture; (B) EIC of peptide **2**; and (C) EIC of peptide **7**. The concentration of the peptide mixture was 0.2 μg/mL; hydrodynamic injection at 50 mbar for 20 s; other conditions as in Figure 1.

for peptides **7** and **8** disappeared when the organic solvent modifier was changed from acetonitrile to methanol, while peptide **5** eluted as one single peak in 20% (v/v) acetonitrile but changed to two peaks with 20% (v/V) methanol present in the buffer electrolyte. In this context, the solvation effects of these organic modifiers or some conformation changes of the peptides induced by a specific organic solvent may contribute to this multiple peak behavior. Elsewhere, we have shown that the clustering of solvent molecules with water−organic solvent mixtures occurs with peptides, and this can induce the selectivity pattern seen in these studies when acetonitrile is substituted with methanol.

Detection Sensitivity. As mentioned above, etching the capillary surface before the covalent bonding of the stationary phase increases the internal surface area by a factor of up to 1000.[18,19] As a result, more stationary phase can be bonded to the surface, which in turn, increased the loading capacity of the OT-CEC capillary. Compared to the CE system, higher sensitivity can thus be obtained in an OT-CEC system, because more sample can be injected and preconcentrated at the head of the OT-CEC capillary without deteriorating the separation performance. Figure 6 shows the sensitive analysis of the peptide mixture with an etched C_{18}-bonded OT-CEC capillary. The concentration of each peptide in the mixture was ~0.2 μg/mL, and the sample was injected hydrodynamically at 50 mbar for 20 s. The basic peak chromatography (BPC) of the peptide mixture, the extracted ion chromatography (EIC) of peptides **2** and **7** is demonstrated in Figure 6A−C, respectively. It can be seen that most components in the mixture have good signal-to-noise ratio, and this sensitivity of detection was ~10−100-fold higher than that obtained in a CE-ESI/MS system.

Separation of a Tryptic Protein Digest. During the fabrication of the OT-CEC capillaries, ~95% of the silanol groups on the

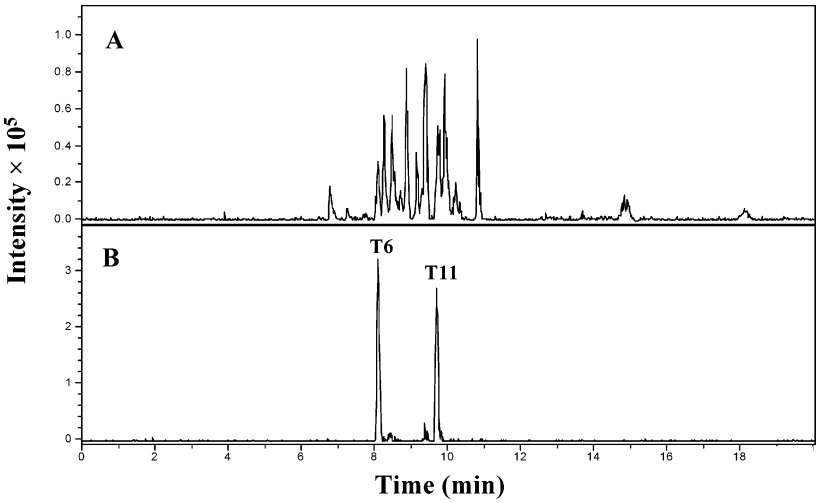

Figure 7. Separation of a cytochrome *c* tryptic digest with an etched cholesterol-modified OT-CEC capillary. (A) BPC of the tryptic peptides; (B) EIC of ions with a *m*/*z* of 261.2 that corresponded to the tryptic peptide T6 and T11. Separation buffer: 20 mM NH_4Ac in 20% ACN; other conditions as in Figure 1.

capillary wall are removed by the silanization reaction,[22,26] thereby significantly reducing the undesirable adsorption effects between the positively charged analytes and the ionized silanol groups. As a result, separation efficiencies of the peptide mixture with buffer electrolytes encompassing a broad pH range, as demonstrated in Figure 2B and Figure 3C, were generally very high. In fact, the peak efficiency of peptide **9** and coeluted peptides **7** and **8** in the lower panel of Figure 3 correspond to over 300 000 plates/m, although the separation was performed with a pH 7.0 buffer electrolyte.

This characteristic of the etched chemically modified OT-CEC capillaries, in terms of high efficiency and resolving power that arises from the interplay of multiple separation mechanisms, is essential for the analysis of complex biological samples, such as protein tryptic digests. In order to test the performance of these OT-CEC capillaries for such applications, a bovine cytochrome *c* sample was digested with trypsin and separated with a cholesterol-bonded OT-CEC capillary under pH 7.0 conditions. As shown in Figure 7A, good separation was achieved, with all the major peptide fragments easily and confidently identified by mass spectrometry. The separation of two isobaric peptides, T6 (GlyGlyLys) and T11 (AsnLys), as illustrated in Figure 7B, should be noted. These two peptides cannot be well resolved by high-performance reversed-phase liquid chromatography, due to the similarity in their hydrophobicities. However, an excellent separation of these two peptides, with regard to their selectivity and peak shapes, was achieved with the OT-CEC capillary. Although these two peptides have identical mass/charge ratios and were not distinguishable from their mass spectra, the difference in their tandem mass spectra allowed a straightforward identification.

CONCLUSIONS

This study documents the applicability of OT-CEC-MS for the analysis of peptides. The migration behavior of a group of peptides has been studied with two different types of etched chemically modified OT-CEC capillaries. As described in this paper, the overall electrochromatographic migration of the peptides could be readily modulated by the composition of the buffer electrolyte, such as its pH and the type and the content of organic solvent modifier. Due to the unique properties of the OT-CEC capillaries, highly efficient and reproducible separations of the peptides were achieved over a broad pH range. When compared to the corresponding capillary electrophoresis-mass spectrometry procedures, the OT-CEC-MS system also offered more sensitive analysis since the bioanalytes could be preconcentrated at the head of the capillary before separation. In addition, the employment of more specific mass spectrometric detection not only provided a better opportunity to structurally characterize the resolved peptides, but it was also useful to probe the origin of peptide-specific multizoning effects in complex biological samples. The excellent separation, detection, and characterization of a protein digest further demonstrate the potential of such OT-CEC-MS systems in proteomic applications.

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