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An Etched Porous Interface for On-Line Capillary Electrophoresis-Based Two-Dimensional Separation System

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The construction and evaluation of an on-column etched fused-silica porous junction for on-line coupling of capillary isoelectric focusing (CIEF) with capillary zone electrophoresis (CZE) are described. Where two separation columns were integrated on a single piece of fused-silica capillary through the etched ∼4 to 5-mm length porous junction along the capillary. The junction is easily prepared by etching a short section of the capillary wall with HF after removing the polyimide coating. The etched section becomes a porous glass membrane that allows only small ions related to the background electrolyte to pass through when high voltage is applied across the separation capillary. The primary advantages of this novel porous junction interface over previous designs (in which the interface is usually formed by fracturing the capillary followed by connecting the two capillaries with a section of microdialysis hollow fiber membrane) are no dead volume, simplicity, and ruggedness, which is particularly well suited for an on-line coupling capillary electrophoresis-based multiple dimensional separation system. The performance of the 2D CIEF−CZE system constructed by such an etched porous junction was evaluated by the analyses of protein mixtures.

The separation of proteins and peptides in biological origin has been of significance and interest in the study of proteomics. Most single-dimension separation modes of chromatography or electrophoresis could not well resolve complex biological matrixes or cellular extracts due to their insufficient resolving power and peak capacities.[1] Separations employing multiple dimensions offer better promise for such applications by combining different separation modes. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been widely used and proven the most powerful tool in analysis of complex protein mixtures; however, this technique in practice is time-consuming and labor-intensive.[2] Moreover, the planar 2D gel electrophoresis could not be easily, directly coupled with mass spectrometry (MS) for on-line identification.

Therefore, various column-based 2D separation alternatives that combine different separation modes in series have been developed to help reduce the analysis time and labor.[3] These include the use of an automated switching valve to couple reversed-phase liquid chromatography (RPLC) to capillary electrophoresis (CE),[4] size-exclusion chromatography (SEC) coupled to parallel RPLC columns rather than storage loops,[5] microcolumn LC coupled to capillary zone electrophoresis (CZE) by flow gating,[6,7] optical gating,[8] and pulse-contacting interface,[9] CE coupled to channel gel electrophoresis by moving the outlet end of the capillary across the entrance of the channel,[10] on-line integration of capillary isoelectric focusing (CIEF) with capillary RPLC using a microinjection valve,[11] and even the zero dead volume connection of two electrophoresis separations on chips.[12−14]

The key problem in constructing a 2D separation system lies in the construction of a reliable interface to switch the effluents from the first-dimension column to the second one. Although various switching valves and storage loops employed as interfaces for LC/LC and LC−CE have been developed,[4,6−9,11] it is still a hard nut to fabricate a satisfactory interface for a 2D-CE system.

To date, only a few 2D-CE systems have been reported due to the difficulties in interfacing to capillaries.[15−20] Michels et al.[15] first reported the development of a fully automated 2D-CZE/CZE

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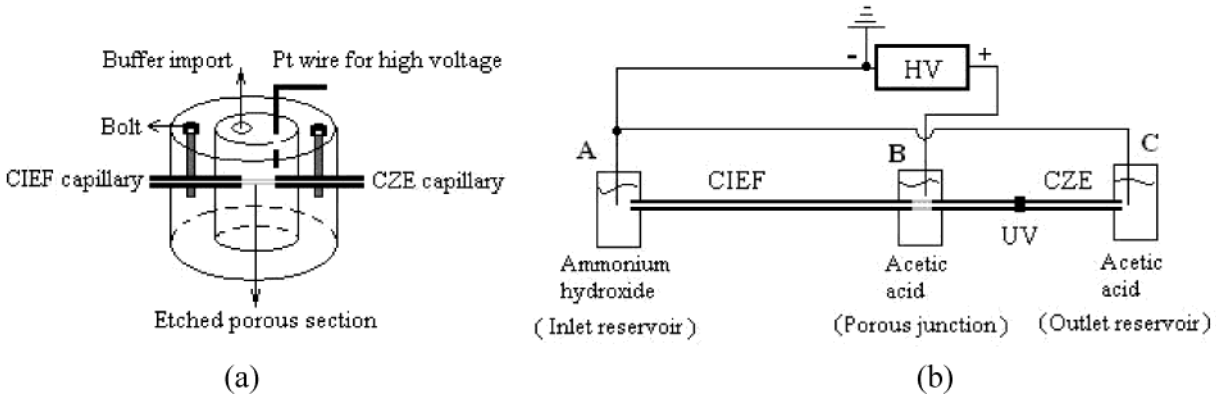

Figure 1. Schematic diagrams of (a) the etched fused-silica porous junction interface and (b) on-line coupling of the CIEF with CZE systems using an etched porous junction interface. A and C are grounded, and B is connected to a high-voltage power supply.

system for protein analysis using a modified version of Jorgenson's interface. In the system, proteins were separated in the first dimension by submicellar CE at pH 7.5, and then the fractions were subsequently transferred to the second dimension, where electrophoresis was performed at pH 11.1 to further separate proteins. Sheng et al.[16] have coupled micellar electrokinetic chromatography (MEKC) to CIEF by a 10-port valve interface with two conditioning loops. In the loops, salt and other unwanted first-dimensional effluent components were eliminated by dialysis, and carrier ampholytes for the second-dimension separation were added. Recently, Mohan et al.[17] have employed microdialysis as the interface for on-line coupling of CIEF with transient capillary isotachophoresis (CITP)−CZE for the separation of tryptic digests of proteins. In our group, a similar approach was developed for on-line hyphenation of CIEF with capillary gel electrophoresis (CGE),[18,19] and CIEF with CZE,[20] to achieve the buffer exchanging and necessary electrical connection before the second dimensional electrophoresis. However, the significant dead volume could not be avoided in all the present 2D-CE systems, whether by an interface of cross, valve/loop or dialysis hollow fiber, which definitely decreases the resolution of 2D separation.

The objective of our study is to develop a simple and useful interface with zero dead volume for 2D-CE separation. Here, we present the construction and evaluation of a simple porous junction with no dead volume by simply etching a short segment on a capillary. We used this novel junction to develop a 2D-CIEF−CZE method for separation of protein mixtures.

EXPERIMENTAL SECTION

Chemicals and Equipment. Fused-silica capillaries (50-μm i.d. × 375-μm o.d.) were purchased from Yongnian Optical Fiber Factory (Hebei, China). *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED, 99%) was obtained from Acros Organics (U.S.A.). Ampholyte (pH 3.0−10.0) was purchased from Pharmacia (Uppsala, Sweden). Lysozyme (from chicken egg white), hemoglobin (from bovine blood), myoglobin (from horse heart), and cytochrome *c* (from horse heart) were purchased from Shanghai Institute of Biochemistry (Shanghai, China). Hydrofluoric acid (40%) was obtained from Dalian Chemical Reagents Corp. (Dalian, China). Other reagents were of analytical grade unless otherwise stated. All solutions were prepared using MilliQ water (Bedford, MA) and further filtered through a 0.20-μm pore size membrane (Elite Instrument Inc., Dalian, China) prior to use.

Electrophoresis was performed on the TriSep-2010GV (Unimicro Technologies, Inc, Pleasanton, CA) equipped with a Data Module UV−visible detector and a high-voltage power supply. A Workstation Echrom98 of Elite Instrument Inc. (Dalian, China) was used for data acquisition.

Procedure for Fabrication of a Porous Junction. Fused-silica capillaries of 50-μm i.d. and 375-μm o.d. were used in this study. A schematic diagram of the porous junction interface is shown in Figure 1a. The porous junction was fabricated according to the following procedures: A length of ~4 to 5 mm of polyimide coating of a fused-silica capillary (33 cm from the inlet end and 17 cm from the outlet end) was first removed by a blade. On a Plexiglas reservoir (machined by Weida Analytical Instrumental Assembly Factory, Dalian, China), two holes were initially drilled straight in a line with inner diameter slightly smaller than the outside diameter of the capillary. The reservoir was then dissected into two parts through the centers of the holes; thus, the upper part and the lower part of the reservoir possessed a semicircular groove, serving as a slide. The fused-silica capillary was placed in the groove, and the section of the capillary without coating was positioned in the center of the lower part of the reservoir. Then the upper and the lower parts of the reservoir were fastened together by two bolts. Epoxy was used for sealing the boundaries between the two parts of the reservoir to prevent leakage. HF was used to etch the exposed fused-silica section according to the procedure first reported by Hu et al., but with modification.[21] The exposed fused-silica section was first immersed in 40% HF for 2.5 h at room temperature in a well-ventilated hood and then in 20% HF for another ~2 h. The etching procedure was continuously monitored by an optical microscope (Shanghai Optical Instrument Corp., Shanghai, China) and terminated until electrical conduction was established through the etched section wall and the thickness of the porous wall was observed thinner than 20 μm.

Construction of the 2D-CE System. As shown in Figure 1b, the above constructed junction device was employed as an interface for on-line combination of the CIEF and CZE separation

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system. CIEF with a channel length of 33 cm acted as the first dimension, the etched porous junction, and the CZE capillary with a channel length of 17 cm acted as the second dimension. The two were integrated on a single piece of a capillary. A detection window was made on the CZE capillary (10 cm from the porous junction) by removing another section of the polyimide coating. The capillary was then installed onto the UV detector. The inlet of the CIEF capillary was inserted into a reservoir. The outlet of the CZE capillary was inserted into another reservoir. A platinum wire was inserted into the interface reservoir and served as the common anode, and another two platinum wires were grounded and inserted into the inlet reservoir and outlet reservoir to provide the necessary electrical connection.

2D-CIEF−CZE Separation of Proteins. Prior to performing the 2D-CE separation, the inner wall of the bare fused-silica capillary was covalently coated with linear polyacrylamide (LPAA) by a modification of Hjérten's method[22] to eliminate electroosmotic flow (EOF) and prevent the adsorption of protein onto the capillary wall. Both the CIEF and CZE capillaries were initially filled with 1.0% acetic acid (HAc, pH 2.8)). The protein samples dissolved in 25 mM phosphate buffer (pH 6.8) containing 2.0% ampholyte (pH 3−10) were hydrodynamically introduced into the CIEF capillary up to the etched porous junction by a syringe pump with 1.0 psi pressure at the inlet capillary. The inlet reservoir was filled with 1.0% (v/v) ammonium hydroxide ($NH_3 \cdot H_2O$, pH 10.5). The interface reservoir housing the porous junction and the outlet reservoir were filled with 1.0% HAc. $NH_3 \cdot H_2O$ and HAc solutions were used as the catholyte and the anolyte in CIEF, the first dimension, respectively. HAc solution in both the CZE channel and the outlet reservoir served as the background electrolyte for performing CZE, the second dimension. All the platinum wires were placed in their appropriate reservoirs, as illustrated in Figure 1b. Focusing was performed by applying a constant voltage of +13.2 kV at the junction reservoir. The current decreased continuously during the protein focusing. When the current reduced to ~10% of the original value and remained nearly constant, the focusing was considered to be complete. Then hydrodynamic gravity was used to mobilize the focused protein zones into the second dimension by elevating the inlet reservoir 8 cm above the outlet reservoir for 1.0 min (the mobilization velocity about ~0.6 to 0.7 cm/min). The platinum wire for the outlet reservoir was then connected, and an electric voltage of +10.2 kV was applied at the interface reservoir to achieve CZE separation. At the same time, a data acquisition procedure for the proteins resolved in CZE was started using a UV detector at 280 nm. Repeated focused zone gravity-induced mobilization followed by CZE separations was performed until the entire CIEF capillary content was completely transferred and analyzed.

RESULTS AND DISCUSSION

A simple interface device was developed for the direct on-line coupling of CIEE with CZE that provides a higher resolving power 2D separation platform for the analysis of proteins. The device offers advantages over existing CE-CE interfaces, including ease of fabrication, ruggedness, and a true zero dead volume.

Construction of the Porous Junction. The present design of the 2D-CE interface is illustrated in Figure 1a. The key point is the construction of a porous junction by etching an exposed capillary segment with HF. HF etching of the exposed capillary was first used by Hu et al.[21] in CE with electrochemical detection for isolating the detector from the CE electric field, later utilized by Wei et al.[23] for on-line concentration of proteins and peptides in CE and recently employed by Janini et al.[24] as well as Whitt et al.[25] to couple CE on-line with MS with modifications. During the etching process, the etched section was continuously monitored by an optical microscope. When the thickness of the etched capillary wall was observed near to 20 μm, the current between the junction and the outlet of the capillary filled with 25 mM $NaH_2PO_4-Na_2HPO_4$ (pH 6.8) was measured to further monitor the thickness of the etched capillary wall. The current increased as the etching time was prolonged. When the current measured in the system with the etched junction is found to have no obvious difference from that observed in the system without the etched junction in comparing equal capillary lengths and the same separation voltages and buffers, the thickness of the etched section is considered to be thin enough and suitable. The etching process can then be terminated. For capillaries of the same dimensions and at the same ambient temperatures, the required etching time is reproducible to within 10 min. Although the porous junction is fragile, it has been proved to be durable when it is firmly held inside the reservoir and can be used for several weeks without any observable deterioration.

Characterization of the Porous Junction. Visualization of the microstructure of the etched junction was accomplished through scanning electron microscope (SEM). All scanning electron micrographic images were obtained using a JEOL JSM-6360LV SEM. Figure 2A represents the cross-sectional view of an etched segment of the junction at a magnification of 850×. Observation at this magnification reveals that the thickness of the etched capillary wall is in the range of ~10 to 20-μm. Figure 2B, a SEM partial view of the outer surface of the etched segment at a magnification of 100 000× roughly reveals the porous microstructure of the etched glass matrix. The current in the system with a junction measured at 25.8 μA was quite near to 26.3 μA measured in the normal CZE system without a junction using 25 mM phosphate buffers, equal capillary lengths of 20 cm, and the same separation voltages of 9.6 kV for the two systems. Due to the limitation of the resolving power, the SEM image does not clearly show the microstructure of the etched section; however, the measured currents indicate that the pores of this porous structure are large enough to allow the permeation of small electrolyte ions upon application of a potential to the system. Since no liquid flows through the wall of the etched segment, the pores of the porous junction should be smaller than large molecules, such as proteins or polypeptides, thereby preventing them from permeating the porous junction.

Band-Broadening Considerations. To evaluate the broadened peaks resulting from the focused proteins adsorbed on the capillary inner wall at the porous interface, where the coating is damaged by HF etching, CZE should be performed both in a capillary coated with LPAA prior to etching and in a capillary coated with LPAA after etching, respectively. To perform these

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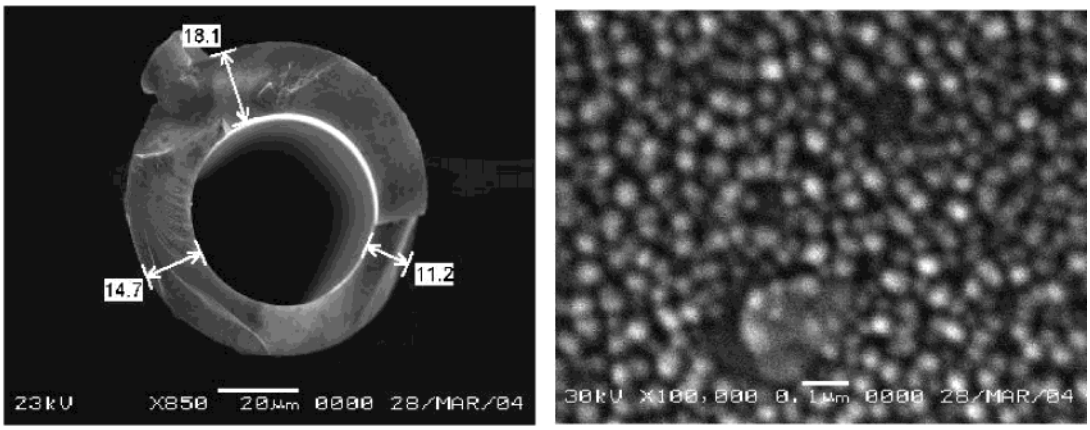

Figure 2. Scanning electron micrograph of an etched fused-silica capillary with inner diameter of 50 μm. (A) Cross-sectional view; magnification, 850×; (B) partial view of the outer surface of the etched porous section; magnification, 100 000×.

comparisons, on-column, the UV detector was located behind the etched junction. Since electrostatic interacting likely occurred between the positive charged analytes and the negative charged silanol, the highly basic protein lysozyme was chosen to perform CZE in 25 mM phosphate buffer (pH 6.8). Through the control experiments, it was found that some tailing was encountered in the capillary coated with LPAA prior to etching, which demonstrated that the LPAA coating was damaged by HF at the etched section. To overcome this problem, the inner wall of the capillary should be modified with LPAA after etching. An improved peak shape was obtained in this case (Figures not shown).

Carrier Ampholytes in 2D System. Carrier ampholytes were employed to form a pH gradient inside the CIEF capillary. After focusing in CIEF, the focused zones containing a blend of carrier ampholytes and proteins were hydrodynamically injected into the second dimension to perform CZE. Ampholytes are composed of large numbers of amphoteric molecules, which are composed of small and highly charged polyamino acids. During the CZE process, the higher mobility ampholyte ions rushed to the front of the focused zone. The mobility of the background electrolyte, acetic acid, must be low during the initial CZE step. Thus, large proteins exhibiting small electrophoretic mobility stacked between the higher mobility ampholytes and the low mobility acetic acid. Therefore, there might exist a similar transient capillary isotachophoresis (CITP) step during the initial CZE process. The similar transient CITP step could, to a certain extent, play a role in concentrating the protein zone.

During the second dimension CZE separation, most carrier ampholytes with higher mobility rushed to the front of the zone, and large proteins with small mobility eluted later. Therefore, early migration of most carrier ampholytes significantly decreased the potential interference with proteins in the second dimension separation. The mobility behaviors of the carrier ampholytes have been reported in a previous study.[17] In addition, wavelengths limited to 280 nm were used for proteins detection in this 2D separation, thereby eliminating the background absorbance from carrier ampholytes. On the basis of these facts, the presence of the carrier ampholytes in the second dimensional separations had little effect on the identification of proteins and did not increase the peak numbers of the 2D separation.

Evaluating the Overall Performance of the Porous Junction in the 2D-CE System. CIEF was on-line coupled with CZE by the on-column etched porous junction. CIEF, the first separation dimension in the 2D system, concentrates and resolves proteins based on their p*I*, whereas CZE, the second dimension, is based on their differences in electrophoretic mobility. Then the separation mechanisms of CIEF and CZE are based on unrelated characteristics of the sample, and the number of resolution elements should be greatly increased.

Cytochrome *c* is a relatively complex system since the sample from different origins usually contains different variants with different p*I*s; therefore, cytochrome *c* is a good candidate to evaluate the performance of the porous junction in 2D separation. Figure 3a shows the electropherogram of cytochrome *c* in a single CIEF. As shown in Figure 3a, there are at least two variants contained in this cytochrome *c*. The transferred focused band likely containing the same p*I*s was further separated according to their differences in mobility and completed within 3 min in CZE by using an effective column length of 10 cm and electric field strength 600 V/cm. As shown in Figure 3b, a comprehensive 2D separation of the variants of cytochrome *c* demonstrated the viability of the porous junction and an adequate performance of the 2D-CE system. Note that the allocation of the focused bands with a, b, and c in Figure 3a is not exactly accurate since the starting point of the first transferred band is unknown. The 2D electropherogram of cytochrome *c* indicates that the purposed 2D system obviously has great potential to provide high-resolving power for complex proteins, and these could be attributed to the following aspects: First, CIEF could provide a high-resolution separation based on p*I* with a typical concentration factor of 50−100 fold. Second, there was no loss in separation resolution across the etched porous junction due to no dead volume associated with the junction because the inner wall of the capillary remains intact. Moreover, during the CZE process, an electric voltage was continuously applied over the first dimensional capillary to maintain a pH gradient for protein zones focusing in CIEF capillary.

The reproducibility of migration time in such a 2D-CE system was investigated run-to-run by using gravity-induced transfer when the same capillary was used. Provided that the used capillary has

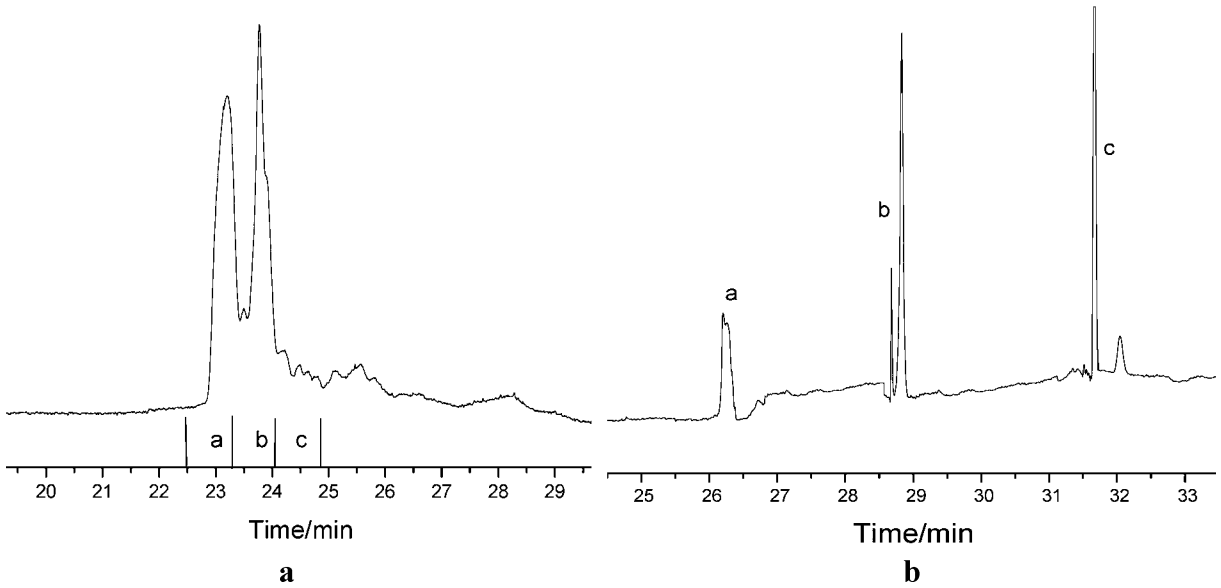

Figure 3. Separations of cytochrome *c* in (a) CIEF and (b) 2D-CIEF−CZE. Experimental conditions: (a) capillary covalently modified with linear polyacrylamide (LPAA), 50-μm i.d. × 375-μm o.d., 40 cm total length, 33 cm from the cathodic end to detection window; anolyte, 1.0% (v/v) HAc; catholyte, 1.0% (v/v) $NH_3 \cdot H_2O$; electric field strength, 400 V/cm; hydrodynamic mobilization by elevating the inlet reservoir 8 cm above the outlet reservoir; detection, UV 280 nm; sample, 1.0 mg/mL cytochrome *c* in buffers containing 25 mM $NaH_2PO_4-Na_2HPO_4$ (pH 6.8), 2.0% ampholyte (pH 3−10) and 0.25% TEMED. (b) CIEF capillary, 33 cm; CZE capillary, 17 cm total length, effective length 10 cm; CZE running buffer, 1.0% (v/v) HAc; mobilization by elevating the inlet reservoir 8 cm above the outlet reservoir for 1.0 min (~0.6 to 0.7 cm/min); Sequence of voltages applied during 2D separations shown in Table 1, Data acquisition at the beginning of CIEF. Other conditions were the same as in panel (a) (details shown in the Experimental Section).

Table 1. Sequence of Voltages Applied during 2D Separations

	reservoir		
	inlet (point A)	interface (point B)	outlet (point C)
step (CIEF)	ground	+13.2 kV	off
step (injection)	ground	off	off
step (CZE)	ground	+10.2 KV	ground

a sufficiently stable inner coating for several hours, the relative standard deviation (%RSD) of migration times of four consecutive runs was about 5−10% (data not shown). The application of computer control and pressure-induced injection might further improve the reproducibility of 2D separation in our future further studies.

Application of 2D-CIEF−CZE to the Separation of Protein Mixtures. A protein mixture, myoglobin and hemoglobin containing variants with p*I*s of 6.8, 7.05, 7.15, 7.2, 7.3, and 7.5, was used to demonstrate the utility and the resolving power of the proposed 2D-CE system. Figure 4 shows the electropherogram of the protein mixtures in a single CIEF. The focused bands were sequentially and hydrodynamically injected into the CZE capillary soon after the focusing was complete. The transfer time was 1 min, and the mobilization velocity was ~0.6 to 0.7 cm/min. A total of ~50 transfers were required to inject all fractions from the first capillary to the second capillary, but only five fractions contained proteins. In all of the experiments, the same height was set to perform hydrodynamic mobilization by elevating the inlet reservoir above the outlet reservoir either in a single CIEF or in 2D separation. It should be noted that the total length of the capillary used in a single CIEF was 40 cm, but the total length of the capillary in 2D separation was 50 cm. Thus, the mobilization

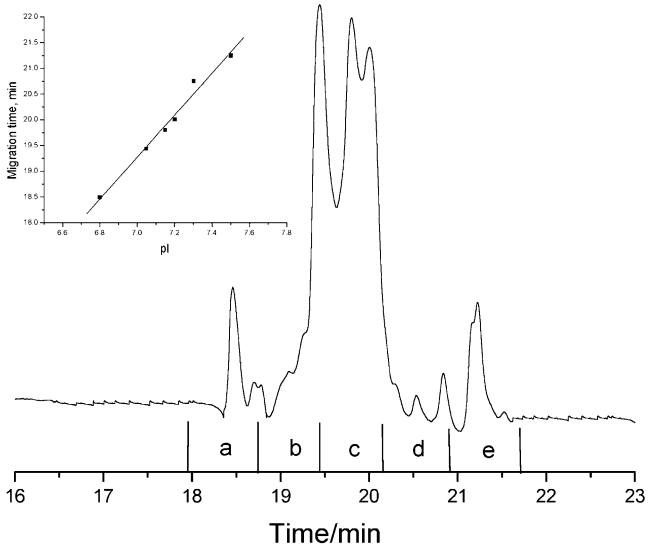

Figure 4. CIEF separation protein mixtures of myoglobin and hemoglobin. Conditions: sample, myoglobin and hemoglobin with 1.0 mg/mL, dissolved in buffers containing 25 mM $NaH_2PO_4-Na_2HPO_4$ (pH6.8) and 2.0% ampholyte (pH 3−10), respectively, without TEMED. Other conditions were the same as in Figure 3a. The inset illustrates the linearity of the protein's p*I* versus the migration time ($T = -9.27 + 4.08 \times pI$; $R = 0.9908$).

velocity in 2D was ~0.8 times that in a single CIEF. Therefore, a time window of 1 min in a single CIEF corresponded to a 0.8-min time window for the CIEF in the 2D-CIEF−CZE separation. Then an injected focused band for 1 min by elevating the inlet reservoir in 2D separation corresponded to a 0.8-min time window of the focused band in a single CIEF. According to the above analysis, five injected fractions contained proteins in 2D separation (shown in Figure 5), which corresponded to all bands of a−e distributed

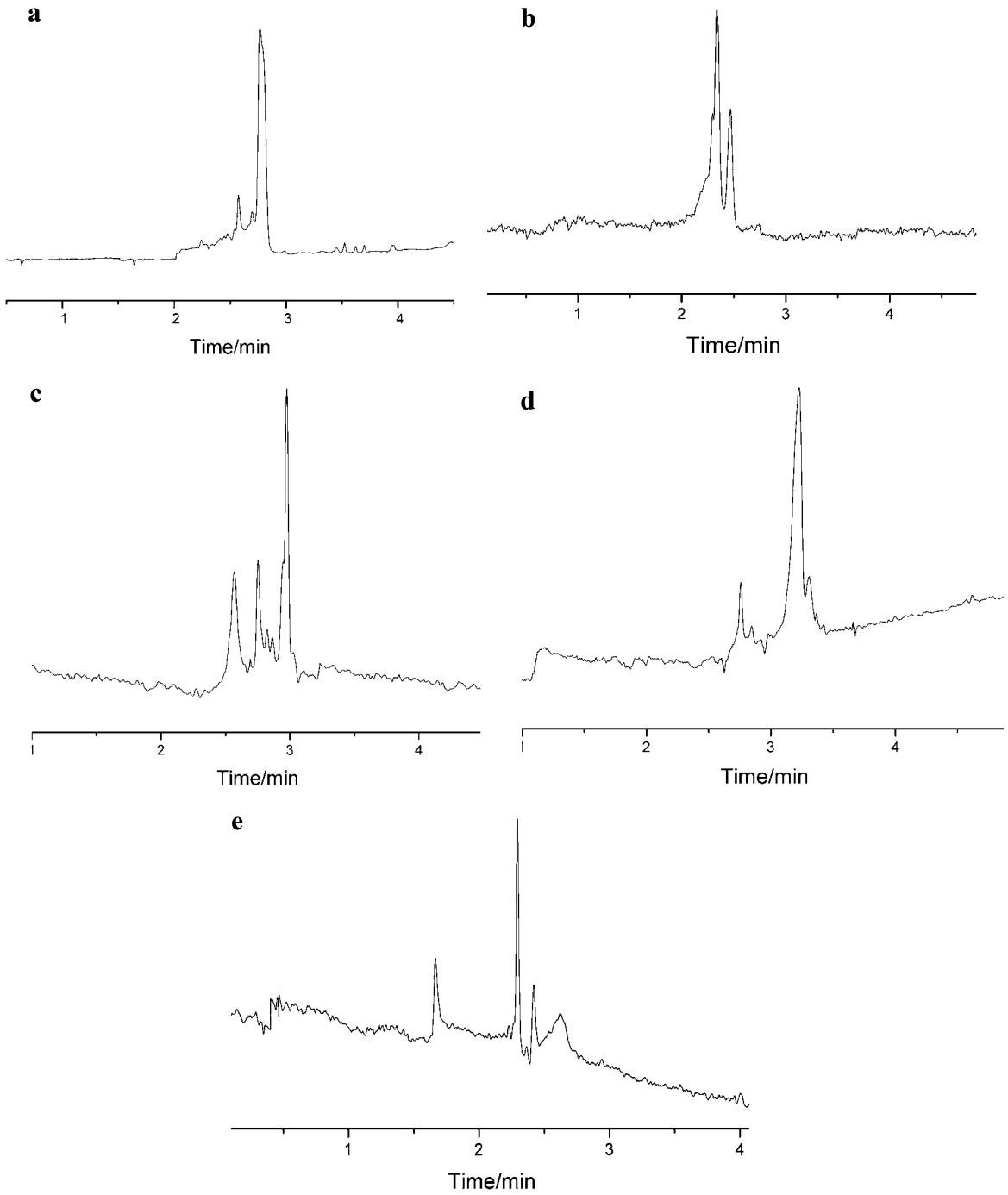

Figure 5. 2D-CIEF−CZE separation protein mixtures of myoglobin and hemoglobin. Conditions were the same as in Figures 3b and 4 (details shown in the Experimental Section.); a−e represent the sequence of CIEF fractions further resolved by CZE from acid to basic pHs.

in a period of around 4 min in a single CIEF (shown in Figure 4). Owing to the differences of hydrodynamic mobilization velocity both in a single CIEF and in 2D-CIEF−CZE, as well as the transfer of focused IEF zones to the second dimension carried out by manual elevation of the capillary inlet, the allocation of the focused bands with a−e in Figure 4 only approximately corresponds to the sequence of CIEF injected fractions in the 2D separation.

At the beginning of CZE, data acquisition was started for each injected fraction. In this study, proteins with lower p*I*s eluted ahead of proteins with higher p*I*s as a result of the use of a porous junction as the anode for the 2D separation. As shown in Figure 5, each electropherogram represents the sequence of CIEF fractions further resolved by CZE. A higher resolving power, improved peak shapes, and increased total peak numbers are all observed in the electropherograms of the 2D separation. All of these should be attributed to the orthogonal separation mechanisms of CIEF and CZE, the zero dead volume junction, CIEF concentrating properties, and a transient CITP−CZE step.

CONCLUSIONS

In this work, on-line coupling of CIEF with CZE has been developed by using an on-column etched fused-silica porous junction. The feasibility and performance of the proposed 2D system have been demonstrated by the analyses of proteins.

Compared with previously reported 2D-CE using either a cross, valve/loop, or microdialysis hollow fiber interface, the obvious advantage of the present junction is its completely zero dead volume to ensure a high resolution of the 2D system. In addition, the etched porous junction is really simple, effective, and durable. A multidimensional capillary electrophoresis-based system involving more than 2D can even be achieved by such a junction. Since the main obstacle to the construction of a 2D-CE system lies in the difficulties of preparing a good conductive interface, we are confident that the etched fused-silica porous junction can provide an easy way for researchers who are interested in the construction of two-dimensional or multidimensional capillary electrophoresis-based separation system.

Here, one should note one limitation of this 2D-CE system. The orthogonally coupled CIEF−CZE, while possessing higher resolving power and overall peak capacity than 1D, provides very little information concerning the identity of the separated components. An alternative system should employ MS to perform online identification of the separated effluents, which is underway in our further research.

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