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Short communication

On-line combination of monolithic immobilized pH gradient-based capillary isoelectric focusing and capillary zone electrophoresis via a partially etched porous interface for protein analysis

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ABSTRACT

An integrated platform consisting of monolithic immobilized pH gradient-based capillary isoelectric focusing (M-IPG CIEF) and capillary zone electrophoresis (CZE) coupled by a partially etched porous interface was established. Since carrier ampholytes (CAs) were immobilized on monolith in M-IPG CIEF to form a stable pH gradient, subsequent depletion of CAs at the interface to prevent the interference on CZE separation and detection were avoided. Moreover, a partially etched porous capillary column, which was facile for fabrication and durable for operation, was exploited as the interface to combine M-IPG CIEF and CZE. The RSD values in terms of the migration time for M-IPG CIEF separation, transfer protein from the first dimension to the second dimension, and CZE separation, were 2.4%, 3.9% and 2.3%, respectively. With a 6-protein mixture as the sample, two-dimensional capillary electrophoresis (2D-CE) separation was successfully completed within 116 min, yielding a peak capacity of ~200 even with minute sample amount down to 5.0 μg/mL. The limit of detection was 0.2 μg/mL. In addition, proteins extracted from milk were used to test the performance of such a 2D-CE separation platform. We expect that such a novel 2D-CE system would provide a promising tool for protein separation with high throughput and high peak capacity.

1. Introduction

Efficient separation is a prerequisite step for the characterization of proteins, especially the complex protein mixtures. Although two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is still a widely recognized approach, it suffers from unavoidable disadvantages, such as time and labor consuming, poor capacity to separate large and hydrophobic proteins, and extremely acidic and basic proteins [1,2]. However, many of the disadvantages of 2D-PAGE are not shared by two-dimensional (2D) separation methods based on capillary electrophoresis (CE). Moreover, high column efficiency, high resolution, and ease in coupling with a variety of detectors (e.g. laser-induced fluorescence and mass spectrometry) are often demonstrated, thus 2D-CE separation systems have drawn much attention recently [3–5].

Developing a reliable and robust interface is a key issue to construct an efficient 2D-CE separation system. Over the past years, a variety of 2D-CE interfaces have been introduced, including cross interface [6], valve [7], microdialysis device [8–10] and whole etched porous interface [11]. Dovichi's group [6] first reported a system for automated protein analysis using an interface that aligned two separating capillaries and two waste capillaries which were held in place by tightening the ferrules in the Valco cross. Rassi's group [7] coupled capillary isoelectric focusing (CIEF) to capillary electrochromatography via a nanoinjector valve to perform 2D separation. Mohan et al. [8] employed microdialysis junction as the interface for on-line coupling of CIEF with transient capillary isotachophoresis/zone electrophoresis (CITP-CZE) for the separation of tryptic digests of proteins. In our group, microdialysis junction was also developed as 2D-CE interface to achieve the buffer exchanging and necessary electrical connection [9,10]. With such interfaces, CIEF-capillary gel electrophoresis (CIEF-CGE) [9] and CIEF-capillary non-gel sieving electrophoresis (CIEF-CNGSE) [10] were successfully established. Besides the microdialysis devices, a

Abbreviations: M-IPG CIEF, monolithic immobilized pH gradient-based capillary isoelectric focusing; CZE, capillary zone electrophoresis; CAs, carrier ampholytes; LPAA, linear polyacrylamide; γ-MAPS, 3-(trimethoxysilyl)propyl methacrylate; PEG, poly(ethylene glycol); 2D-CE, two-dimensional capillary electrophoresis; HF, hydrofluoric acid.

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whole etched porous capillary was developed as interface as well, though some fragileness was observed [11].

CIEF is preferably used as the first dimension in the construction of 2D-CE systems [7–11], due to its high separation resolution, high peak capacity, and excellent concentration capability. Carrier ampholytes (CAs) are indispensable to establish a stable pH gradient and usually reduce UV detection sensitivity due to the high absorbance at low wavelengths. To solve this problem, monolithic immobilized pH gradient (M-IPG) columns, by which CAs were bonded to the surface of the monolith in a capillary, were developed in our group [12,13]. The M-IPG columns showed several advantages including the elimination of interference of CAs for separation and detection, reduced diffusion of focused zones and easily coupled with separation and/or detection systems.

Based on our previous work on the preparation of a whole etched porous interface [11] and the construction of CIEF based separation systems [9–13], a partially etched porous capillary interface and a novel 2D-CE platform were developed herein. In this platform, with the partially etched porous capillary column as the interface, for the first time, M-IPG CIEF was successfully hyphenated with CZE for protein separation. The partially etched porous interface, which was prepared in a simple manner, illustrated effective electrical contact and improved robustness. The performance of the 2D-M-IPG CIEF-CZE platform was demonstrated by the separation of 6-standard protein mixture and proteins extracted from milk.

2. Materials and methods

2.1. Materials

N,*N*,*N*′,*N*′-Tetramethylethylenediamine, ammonium persulfate, acrylamide and *N*,*N*′-methylenebisacrylamide were purchased from Acros Organics (Geel, Belgium). Glycidyl methacrylate was purchased from Fluka (St. Gallen, Switzerland). Ampholine (A5174, pH 3.5–10.0), 3-(trimethoxysilyl)propyl methacrylate (γ-MAPS), myoglobin from equine skeletal muscle (M1882, p*I* 7.3, 17.6 kDa), trypsin inhibitor from soybean (T9003, p*I* 4.5, 23.0 kDa), β-lactoglobulin A and B from bovine milk (L3908, p*I* 5.2, the molecular weight of the dimer, 35.0 kDa) and ribonuclease A from pancreas (R5500, p*I* 9.5, 13.5 kDa) were purchased from Sigma (Steinheim, Germany). Urease from sword bean (135143, p*I* 5.1, 480.0 kDa) was purchased from Merck (Darmstadt, Germany). Formamide was purchased from Tianjin Bodi Chemical Corporation (Tianjin, China). Azobisisobutyronitrile was purchased from The Fourth Shanghai Regent Plant (Shanghai, China). Poly(ethylene glycol) (PEG, MW 8000, 10,000) and albumin from bovine serum (RCCH735108, p*I* 4.9, 66.0 kDa) were from Sino-American Biotechnology Company (Shanghai, China). Skimmed milk was purchased from Mengniu Co. Ltd. (Huhehaote, China).

2.2. Sample preparation

Skimmed milk was centrifuged (Allegra^{TM} 64R Centrifuge, Beckman Coulter, Brea, CA, USA) at 15,000 × *g* for 30 min at 4 °C and filtered by 0.45 μm filter to remove the solid. Then it was dried by vacuum rotary evaporators (SPD131DDA-230, Thermo Fisher Scientific, San Jose, CA, USA), and redissolved in 10 mmol/L Tris–HCl (pH 8.0) with the final concentration of 0.046 mg/mL.

2.3. Preparation of M-IPG column

The inner surface of a silica capillary (Sino Sumtech, Handan, China) was pretreated as described previously [13] with minor modifications. Briefly, the capillary was washed by 0.5 mol/L HCl, water, 0.5 mol/L NaOH, water, and methanol, respectively, for

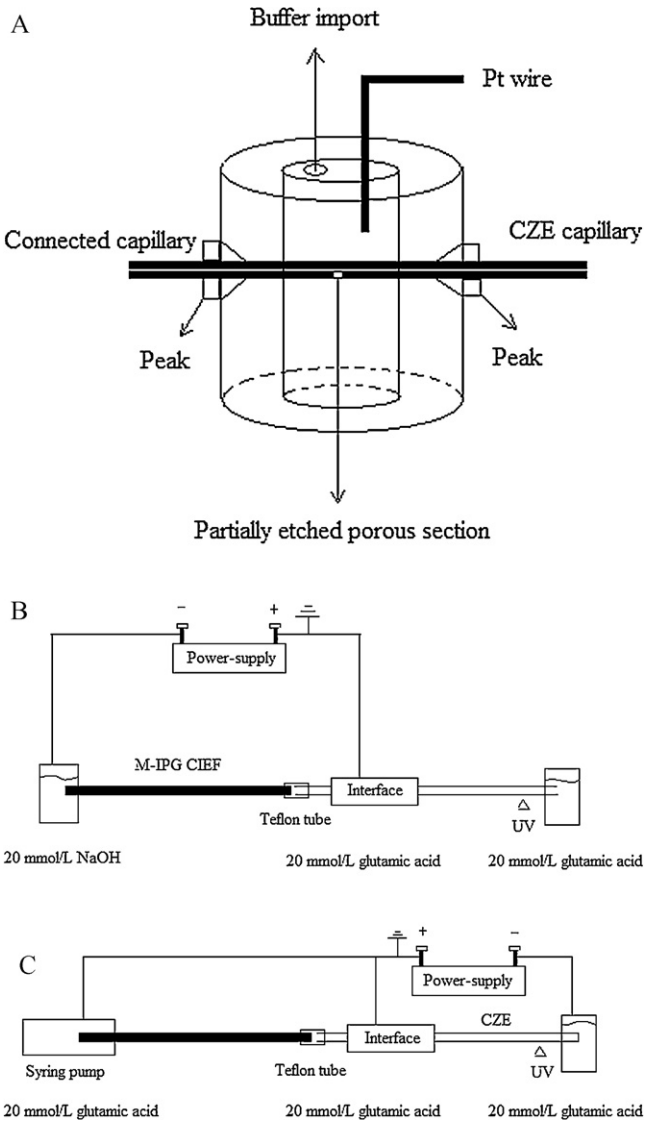

Fig. 1. Schematic diagrams of the partially etched porous interface (A), first dimensional separation (B) and second dimensional separation (C) of 2D-M-IPG CIEF-CZE system.

30 min at the flow rate of 2.0 μL/min, and dried with N_2 at 70 °C for 1 h. A solution of γ-MAPS (50%, v/v in methanol) was injected into the capillary for 10 min at the flow rate of 2.0 μL/min and kept at room temperature for 24 h. Unreacted γ-MAPS was washed with methanol, and the capillary was purged with N_2 at 70 °C for 1 h. 20.6 mg glycidyl methacrylate and 34.3 mg CAs were dissolved in 679.4 mg formamide. After vortexed for a few minutes, the mixture was placed in a water bath at 40 °C for 1 h, and then put in a refrigerator at 4 °C for 10 min. Subsequently, 45.0 mg PEG-8000, 25.0 mg PEG-10,000, 20.0 mg *N*,*N*′-methylenebisacrylamide, 10.0 mg acrylamide and 0.5 mg azobisisobutyronitrile were added into the mixture. The mixture was vortexed and degassed with N_2 for 5 min, followed by injection into the γ-MAPS coated capillary (100-μm i.d. × 375-μm o.d.) at the flow rate of 2.0 μL/min. After that, with 20 mmol/L glutamic acid as anolyte buffer and 20 mmol/L NaOH as catholyte buffer, 400 V/cm was applied on the capillary for focusing the polymerization solution for 6 min. With both ends sealed, the capillary was put in an oven at 70 °C for 20 h to immobilize pH gradient onto the monolith. Finally, the monolithic column was washed with methanol for 2 h, followed by washing with water for 1 h.

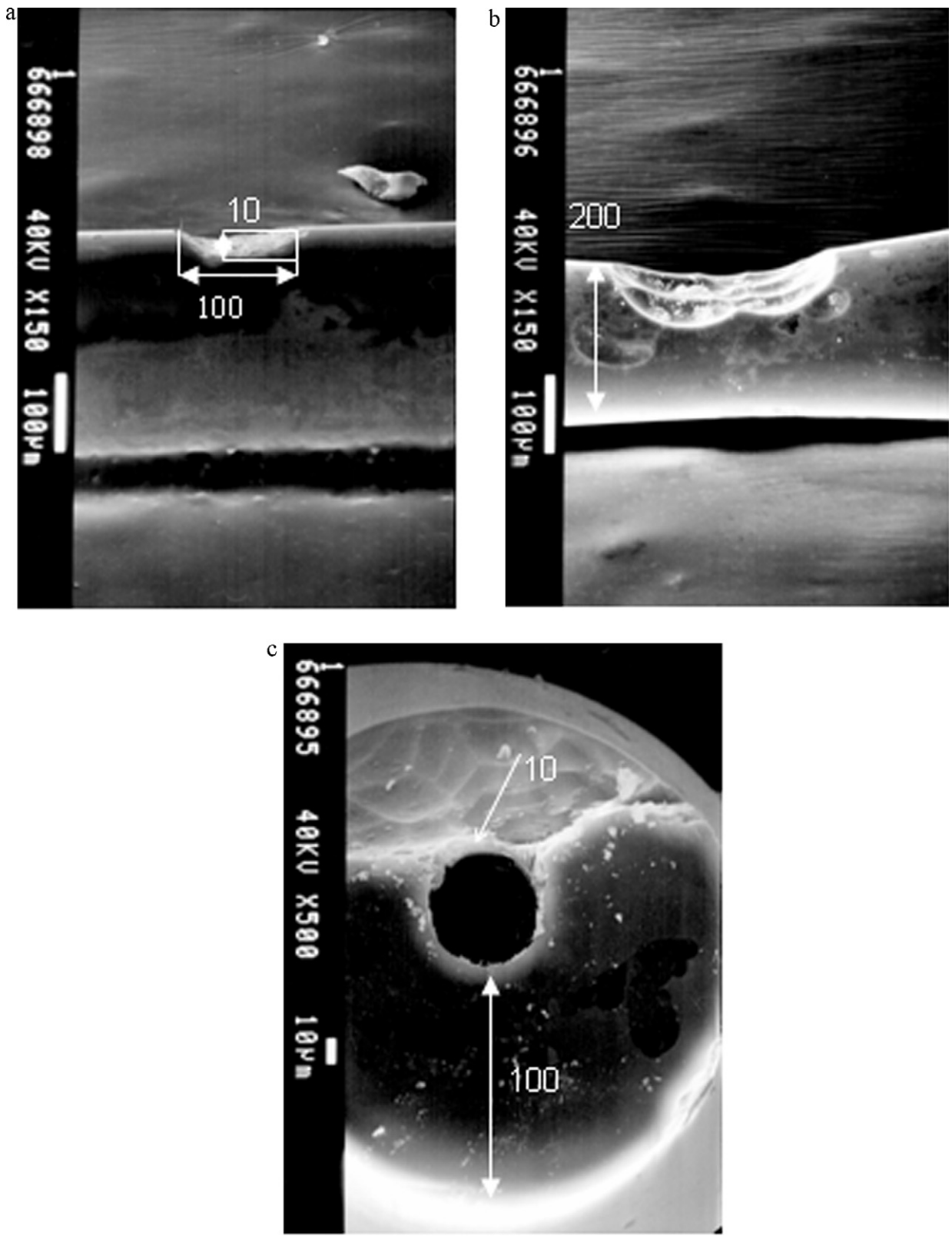

Fig. 2. Scanning electron micrographs of the partly etched fused-silica porous interface. (A) Outer surface of the fused-silica capillary after removing polyimide coating by a blade before etching (150×); (B) outer surface of the interface after etching (150×); (C) cross-section view of the interface after etching (500×).

2.4. M-IPG CIEF separation

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

M-IPG CIEF was performed in a 20 cm long M-IPG column. Cathode buffer was 20 mmol/L NaOH, and anode buffer was 20 mmol/L

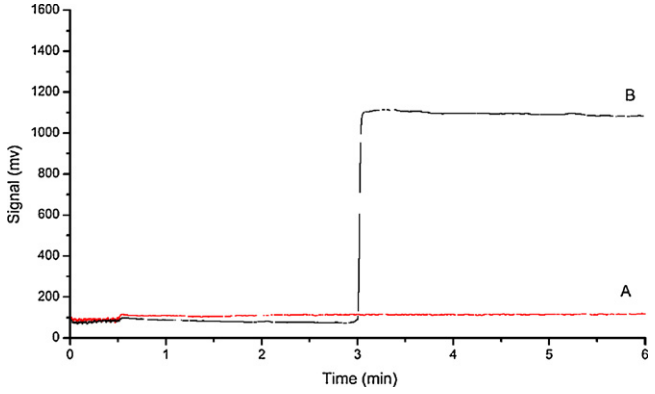

Fig. 3. Evaluation of the size of pores in the partially etched porous interface. (A) CZE coupled with the interface which was filled with sample; (B) CZE separation without the interface. Experimental conditions: sample, 2 mg/mL ribonuclease A dissolved in 100 mmol/L Na_2HPO_4 (pH 8.0); non-coated capillary, 50-μm i.d. × 375-μm o.d., 30 cm total length, 20 cm effective length; electric field strength, 333 V/cm; detection, 214 nm; buffer, 100 mmol/L Na_2HPO_4 (pH 8.0).

glutamic acid. The sample was injected until the whole capillary was full. Then the voltage of 14 kV was applied for focusing. Once the current reduced to ~10% of the original value, the focusing was considered to be complete. After that, the M-IPG column was coupled with a capillary which made a detection window (10.0 cm of total length, 2.5 cm of effective length, 50-μm i.d. × 375-μm o.d.), and then the zones were pumped through the detection window at a flow rate of 70 nL/min by syringe pump (Baoding Longer precision pump Co. Ltd., Baoding, China).

2.5. Preparation of linear polyacrylamide coating of CZE column

The inner wall of a CZE column was coated with linear polyacrylamide (LPAA) by the previously described method [14] with minor modifications. Briefly, the γ-MAPS coated capillary (50-μm i.d. × 375-μm o.d.) was flushed with degassed solution containing 25.0 mg acrylamide in 0.5 mL of water, 8 μL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (10%, v/v in water), and 8 μL of ammonium persulfate (10%, w/v in water) at the flow rate of 2.0 μL/min and kept at room temperature for 12 h. The capillary was ready to use after the residuals were rinsed out.

2.6. Fabrication of the partially etched porous interface

On a Plexiglas reservoir, two holes were drilled straight, as shown in Fig. 1A. One fused silica capillary, on which about 0.5–2 mm length and 1/6 to 1/2 of diameter width of polyimide coating was removed by a blade, went through the holes and was screwed to the reservoir. HF was used to etch the exposed fused-silica section according to our previous procedure [11], with slight modifications. The fused-silica section which was partially removed of polyimide coating was immersed in 40% HF for ~1 h at room temperature in a well-ventilated hood. The etching procedure was monitored by measuring the current of the capillary filled with 100 mmol/L NH_4HCO_3 buffer periodically, and terminated until constant electrical conduction was established through the etched section wall.

Surface scanning and cross-sectional scanning electron images of capillaries before and after HF etching were photographed with a JEOL JSM-6360LV scanning electron microscope (JEOL, Tokyo, Japan) operated at 40 kV after coating the capillary segments with gold–palladium in a vacuum evaporator.

2.7. Construction of 2D-M-IPG CIEF-CZE platform

The partially etched porous interface was exploited for on-line combination of the M-IPG CIEF and CZE separation system. M-IPG column of 20 cm length acting as the first dimension was connected with the interface via a capillary of 2.5 cm length (50-μm i.d. × 375-μm o.d.) coated with LPAA, and a CZE capillary of 30 cm coated with LPAA was served as the second dimension (Fig. 1B). The sample was introduced into the M-IPG column by manually using a syringe. During M-IPG CIEF focusing, one platinum wire was inserted into inlet reservoir, serving as the cathode, and another one was inserted into the interface, serving as the anode. During the second dimensional separation, the outlet of CZE capillary was served as the cathode, and the interface was served as the anode (Fig. 1C). After completing M-IPG CIEF separation, the inlet of M-IPG column was removed from the inlet vial, and connected to a syringe pump. A fraction from the M-IPG column was pumped to the CZE capillary at the flow rate of 70 nL/min for 1 min. With the pump stopped, this fraction was further separated by CZE in a 10 min-run, and on-line detected with a window 20 cm from the partially etched porous junction. After that, the CZE separation was stopped, and the syringe pump connected to the M-IPG CIEF column was restarted for another 1 min fraction transfer, followed by CZE separation. Such procedure was repeated until no more peaks were observed in CZE with all fractions from M-IPG CIEF analyzed.

3. Results and discussion

3.1. Characterization of the partially etched porous interface

The HF etched porous interface had been used in 2D-CE system [11], on-line concentration of proteins and peptides in CE [15], the interface of CE-MS [16,17] and with electrochemical detection for isolating the electrochemical detector from the CE electric field [18]. Capillary interfaces made by above methods were easy to break from the etched section. Zhang et al. [19] used a hole-opened capillary for in-capillary SPE-CE concentration of chlorophenols. In this study, the idea of hole-opened capillary was applied with minor modifications, with the polyimide coating wall of capillary partially removed. Furthermore, such a capillary was partially etched by HF. With this approach, the robustness of the capillary was significantly enhanced.

The microstructure of the partially etched junction was accomplished by scanning electron microscope. It could be seen that before etching, the thickness of the removed polyimide coating wall was ~10 μm and the length of the removed polyimide coating wall was ~100 μm (Fig. 2A). After the treatment, the outer diameter of the capillary was distinctly decreased to ~200 μm (Fig. 2B). When the fused-silica capillary was etched by the completely etching method, as shown in our previous work [11], the thickness of the whole etched interface was about 11.0–18.0 μm, making the interface easy to break. However, when the fused-silica capillary was etched by the partially etching method, the thickness of etched part, less than 30% of the interface cross-section, as shown in Fig. 2C, was ~10 μm, while that of the majority of the capillary wall was ~100 μm, rendering enough robustness of the interface for operation. Moreover, it was found that the partially etched interface could be used several months without any observable deterioration.

It should be noted that the diameter of the etched pores was supposed to be in the low-nanometer range [11], below the resolution limit of the electron microscope. Under the same conditions, with 14 kV voltage, a 30 cm-length separation capillary and 20 mmol/L glutamic acid as buffer, the current for 2D-CE was 2.0–2.2 μA, quite close to that measured in CZE without the interface (2.2 μA), indi-

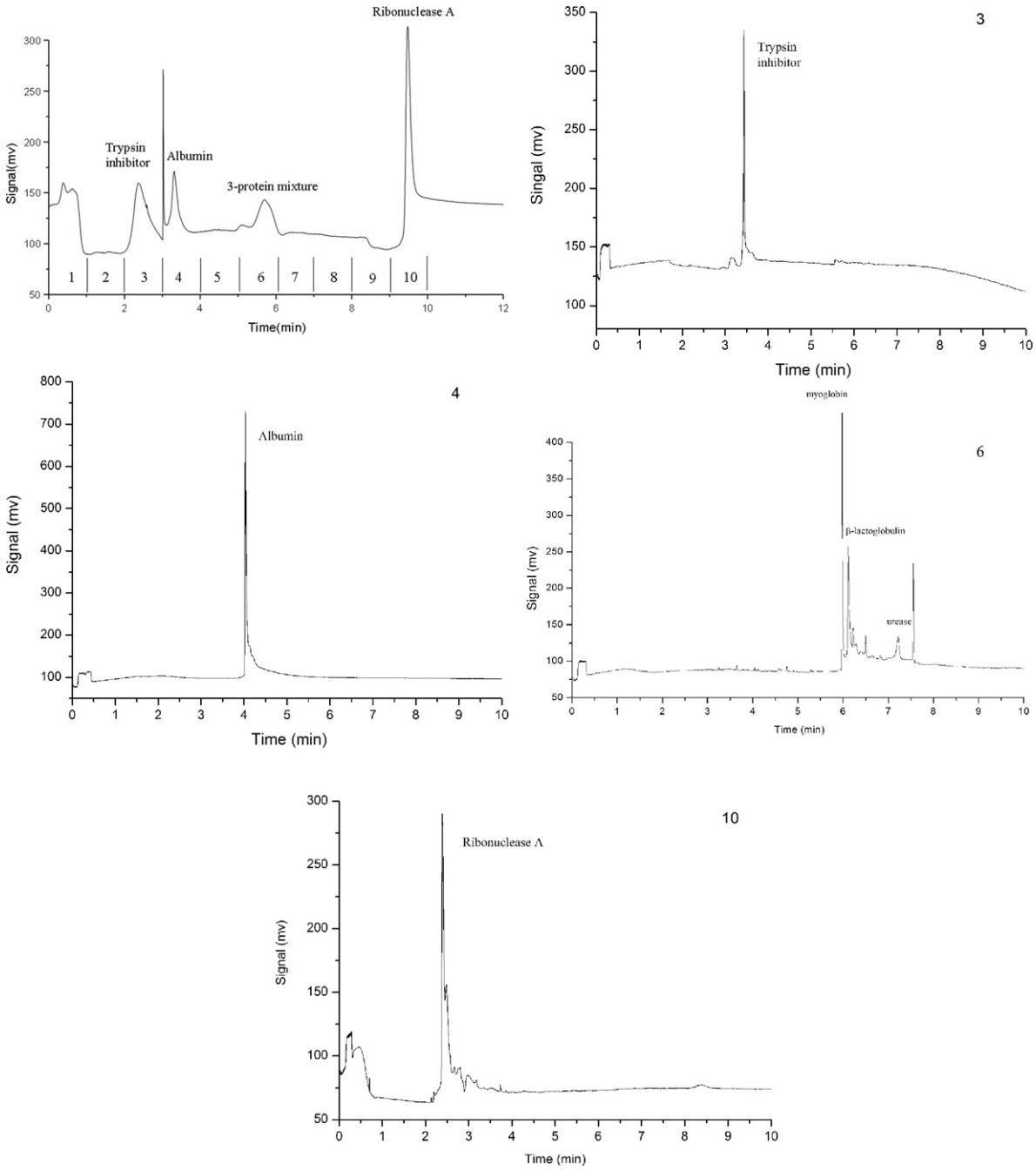

Fig. 4. M-IPG CIEF (A) and 2D-M-IPG CIEF-CZE (B) separation of 6-protein mixture. (*Note*: in (B) the remarked letters (3, 4, 6, 10) correspond to the M-IPG CIEF fractions resolved by CZE.) Experimental conditions: for M-IPG CIEF separation: M-IPG column, 20 cm column length, 100-μm i.d. × 375-μm o.d.; anolyte, 20 mmol/L glutamic acid, catholyte, 20 mmol/L NaOH; electric field strength, 622 V/cm; detection, UV, 214 nm; the flow rate of syringe pump, 70 nL/min; for CZE separation: capillary coated with LPAA, 50-μm i.d. × 375-μm o.d., 30 cm total length, 20 cm effective length; electric field strength, 467 V/cm; buffer, 20 mmol/L glutamic acid; for the 2D-M-IPG CIEF-CZE system: the separated proteins from first dimensional were injected to second dimensional by a syringe pump, 70 nL/min; the injected time of every fraction, 1 min; sample, dissolved in 10 mmol/L Tris–HCl (pH 8.0), concentration of each protein was 5.0 μg/mL. Identified peaks: trypsin inhibitor (p*I* 4.5, 23.0 kDa); albumin (p*I* 4.9, 66.0 kDa); urease (p*I* 5.1, 480.0 kDa); β-lactoglobulin (p*I* 5.2, the molecular weight of the dimmer, 35.0 kDa); myoglobin (p*I* 7.3, 17.6 kDa); ribonuclease A (p*I* 9.5, 13.5 kDa).

cating that the pores of this porous structure were large enough to allow the permeation of small electrolyte ions upon application of a potential to the system. A linear relationship (R^2 = 0.999) between the current in the separation capillary and the applied voltage was obtained, suggesting that constant electric conductivity in the separation capillary could be achieved. The resistance of the partially etched joint was calculated to be 0.71×10^9 Ω, which was the half of whole etched joint [20]. To further investigate the effect of pores size on the penetration of proteins, a small protein ribonuclease A (13.5 kDa) was chosen as the sample. According to the previous method to evaluate pore size on etched capillaries [21], as it can be seen from Fig. 3, when ribonuclease A (13.5 kDa), the smallest protein applied in this study, was added in the inlet vial, and continuously injected by EOF, the protein signal was observed from 3 min (line (B)). While with ribonuclease A added in the interface buffer, and continuously injected by EOF under the same conditions (line (A)), no protein signal could be observed, which indicated that the pores in the wall could restrict ribonuclease A from passing through the interface, ensuring no protein loss during 2D separation. The adsorption of ribonuclease A on uncoated silica capillary

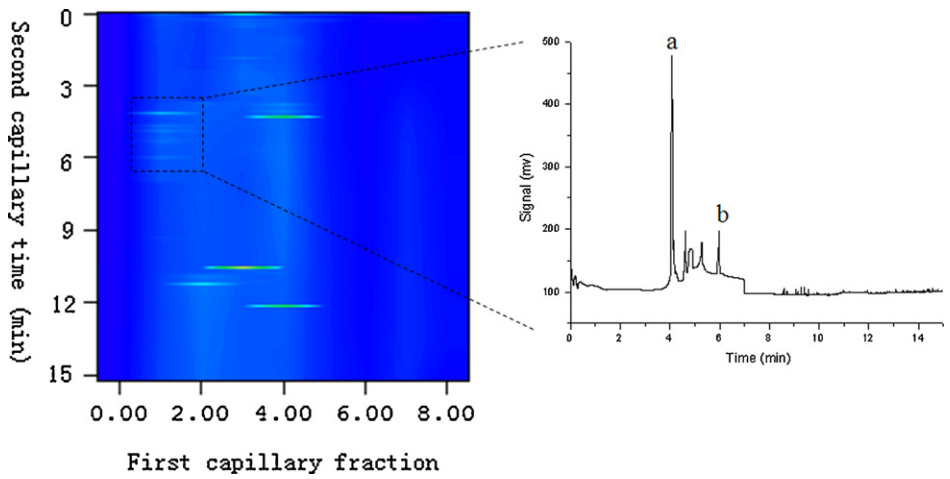

Fig. 5. 2D-M-IPG CIEF-CZE separation electropherogram of proteins extracted from milk. Experimental conditions: sample, 0.046 mg/mL; the injected time of every fraction, 1.5 min. Other experimental conditions were the same as in Fig. 4.

might occur. However, with high concentration protein, the adsorption could not affect the evaluation of pore size. Therefore, the pores of the porous junction could accommodate protein molecules, preventing them from permeating the porous junction.

3.2. Evaluation of 2D-M-IPG CIEF-CZE system

Due to the existence of back pressure, the migration time and the peak width of proteins upon pressure-driven mobilization was greatly influenced by the length of M-IPG column when the syringe pump was employed. With the mixture of trypsin inhibitor, myoglobin, and ribonuclease A as the sample, the effect of column length on the migration time and peak width of the last eluted protein, ribonuclease A, was studied. By comparison, taken 20 mmol/L NaOH as catholyte and 20 mmol/L glutamic acid as anolyte, with the column length, respectively, as 40, 30 and 20 cm, the migration time and the peak width of ribonuclease A were decreased from 100.2, 61.7 to 18.8 min, and 8.4, 8.1 to 1.1 min in the M-IPG CIEF separation, respectively. Therefore, a 20 cm long M-IPG column was applied to establish the 2D-M-IPG CIEF-CZE platform. Furthermore, to perform 2D separation, the buffer of CZE should be the same as the anode buffer of M-IPG CIEF. Although the traditional anode buffer of CIEF was composed of phosphoric acid and acetic acid, the high current generated in CZE separation might interrupt 2D separation with bubbles formed. Therefore, glutamic acid was chosen as the CZE separation buffer, beneficial to generate low current, even with high voltage applied. Moreover, to achieve high speed analysis in CZE, the optimal applied electric field strength was 467 V/cm.

The mixture of myoglobin and ribonuclease A was used to examine the reproducibility of M-IPG columns. Under manual sampling and mobilization by syringe pump, for one analysis performed in each of the three parallel columns, the RSD value in terms of the resolution of two proteins was 1.5% and the RSD values for the migration time of these two proteins were 2.4% and 0.8%, respectively, demonstrating the good reproducibility of such columns for M-IPG CIEF separation. The RSD values for the peak area of myoglobin and ribonuclease A were 23.5% and 6.0%, which should be further improved. However, if two consecutive runs were performed on the same M-IPG column, the RSD value for the resolution of these two proteins was 7.4%, and the RSD values for the migration time of two proteins were 14.6% and 13.5%, respectively which might be caused by the possible collapse of the monolithic matrix, indicating that it was better to use the M-IPG column only once. The RSD values for the migration time and peak area to transfer β-lactoglobulin from the first dimension to the second dimension in ten consecutive runs were 3.9% and 17.4%, respectively. In CZE separation, the RSD values for the migration time and peak area of ribonuclease A were 2.3% and 7.2% ($n = 21$), respectively, similar to that reported in the previous work [22]. The reproducibility of M-IPG CIEF separation, protein transferring and CZE separation were evaluated, respectively, and the acceptable RSD values could be obtained, indicating that the 2D-M-IPG CIEF-CZE system also had good reproducibility.

A 6-protein mixture with 5.0 μg/mL of each protein was used to demonstrate the utility and the resolving ability of the developed 2D-M-IPG CIEF-CZE system. Fig. 4A presents the M-IPG CIEF separation of the 6-protein mixture. Three proteins, trypsin inhibitor, albumin and ribonuclease A were baseline resolved. However, the other 3 proteins, urease, β-lactoglobulin, and myoglobin, were eluted together. A total of 10 fractions (1, 2, . . ., 10) from the M-IPG column were injected into CZE, and 4 fractions were recorded with obvious UV signals, as shown in Fig. 4B. Fractions of 3, 4 and 10 yielded one peak, respectively, corresponding to the three separated proteins by M-IPG CIEF. The co-eluted fraction containing 3-protein mixture from M-IPG CIEF yielded baseline separation by CZE (Fig. 4B6). The elution order of proteins was determined according to the charge/size ratio of proteins, as supposed by Sheng et al. [23]. Since the p*I* of myoglobin is the highest, and the molecular weight is the lowest among the three proteins, with the highest charge/size ratio in acidic buffer, it was the first protein to be detected in CZE, followed by β-lactoglobulin and urease, with decreased charge/size ratios. The limit of detection was 0.2 μg/mL, which was calculated at S/N > 3 by ribonuclease A, with the medium intensity of UV signal among the 6-protein mixture. Therefore, efficient analysis of minute samples could be anticipated with such a 2D-CE system.

The peak capacity was calculated according to the criteria proposed by 2D separation [24]. The peaks in CZE separation eluted over a range of 5.30 min, from 2.00 min to 7.30 min, and the peak width (4σ) in CZE separation was 4.0 s. Thus the peak capacity of CZE was ~20. Since 10 fractions were eluted from M-IPG CIEF onto CZE, the peak capacity was ~200.

3.3. Application

Proteins extracted from cow milk, which mainly composed of caseins (p*I* 4.5, 80%), β-lactoglobulin (p*I* 5.2, ~10%), α-lactalbumin (p*I* 4.2–4.5, ~4%), albumin (p*I* 4.9, ~1%) and other proteins (~5%) [25], were used to evaluate the performance of the 2D-M-IPG CIEF-CZE system. A total of 8 fractions from the M-IPG column were

injected into CZE, and 4 fractions yielded detectable UV signals (Fig. 5). In the representation, M-IPG CIEF cycle number was along the *x*-axis, and CZE separation time was along the *y*-axis, and the density at each point is proportional to the UV signal. Many spots were observed in the early 4 eluted fractions, which were in the position of acidic end of M-IPG column, in accordance with the composition of milk. As shown in the inset of Fig. 5, five main peaks were resolved in the first fraction, and the peaks a and b were identified as albumin and β-lactoglobulin, respectively, according to migration time of each protein in the standard proteins separation in 2D-M-IPG CIEF-CZE system, showing the excellent separation efficiency of 2D-CE.

In the 2D-M-IPG CIEF-CZE system, overall resolution was greatly improved, although might be not as impressive as some other platforms concerning 2D-CE separation followed by laser induced fluorescence detection [4]. It is anticipated that the constructed 2D-M-IPG CIEF-CZE platform might be applicable to the separation of more complex samples (e.g. proteomic samples from body fluids, cells and tissues).

4. Concluding remarks

In this work, the feasibility of an on-line combination of M-IPG CIEF and CZE system for protein analysis by a partially etched porous interface has been demonstrated. Compared with the previously reported 2D-CE using the whole etched porous interface, the partially etched interface showed better robustness and easier fabrication. Both standard proteins mixture and proteins extracted from milk were successfully separated. Therefore, the 2D-M-IPG CIEF-CZE system might provide a powerful tool for protein separation. Further work on coupling this system with mass spectrometry is undergoing for the top-down profiling of proteomic samples.

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