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Research Article

Two-dimensional separation system by on-line hyphenation of capillary isoelectric focusing with pressurized capillary electrochromatography for peptide and protein mapping

A novel on-line 2-D system was developed for peptide and protein mapping. The system combines capillary IEF (cIEF) with pressurized CEC (pCEC) using a micro-injection valve as the interface. Sample fractions, which were focused and separated in the first-dimension cIEF based on their differences in p*I*s, were electrically mobilized and further successively resolved by their differences in size, hydrophobicity, and electrophoretic mobility in the second-dimension pCEC. In the presented system, the valve interface was free of the external electric field in two dimensions for the purpose of stabilization, safety, and facilitating manipulation. In the first dimension, cIEF separation was executed by a one-step method to simplify the operation procedure. Moreover, a home-made electrical decoupler was introduced to isolate the micro-injection valve from the cIEF electric field. For the second dimension, taking advantage of the combination of hydrodynamic flow with EOF, reversed-phase pCEC not only offers on-column refocusing the effluent fractions, but also brings enhanced separation resolution and elution speed. Separation effectiveness of this 2-D system was demonstrated by the analysis of tryptic digest of BSA and human red blood cell lysate. A theoretical peak capacity of approximately 24 000 has been achieved for BSA digest, which proves its promising potential for the application in proteomics.

Keywords:
2-D separation / Capillary IEF / Pressurized CEC DOI 10.1002/elps.201000419

1 Introduction

Multi-dimensional chromatography methods for analyzing the components of complex peptide/protein mixtures have provided a promising alternative approach to 2-D gel for proteome analysis [1]. Based on chromatographic and electrokinetic separation techniques, various separation schemes have been developed to analyze complex proteome mixtures for improvement of resolution and peak capacity, such as LC-LC [2–4], LC-CE [5–8], CE-LC [9–12], and CE-CE [13–27].

Among these non-gel-based 2-D separation methods, an electrokinetic separation candidate, namely capillary IEF (cIEF), has drawn much attention due to its unique characteristics. By transferring IEF separation from gel to capillary format, cIEF not only contributes to high-resolution separation, but also provides in-capillary concentration of protein/peptide samples. Using micro-injector interface, cIEF has been successfully on-line coupled to capillary RPLC for performing high-resolution separation of protein and peptide mixtures [9–12]. In these developed systems, focused proteins/peptides were hydrodynamically mobilized into the injection loop of the micro-valve under the loading position, followed by subsequent injection into capillary RPLC column. The attempts of hyphenation cIEF with other electrokinetic separation modes were also reported. Sheng and Pawliszyn coupled MEKC to cIEF by a 10-port valve interface with two conditioning loops [14]. Zhang's group and Mohan *et al.* designed a microdialysis interface for hyphenation of cIEF with CZE [15], capillary sieving electrophoresis [16, 17], and transient capillary ITP-zone electrophoresis (tITP-CZE) for the analysis of proteins or digests [18, 19]. Thereafter, an etched fused silica porous junction was designed to couple cIEF with CZE for the

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Abbreviations: cIEF, capillary IEF**; HPC,** hydroxypropylcellulose**; HRBC,** human red blood cell**; pCEC,** pressurized CEC

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analysis of protein mixtures [20]. Dovichi's group also reported cIEF-CZE coupling [22] by using a buffer-filled cross-interface for 2-D protein separation based on their previous study [13]. In addition, 2-D separation in microfluidic devices by integrating cIEF with following SDS PAGE [23] and cIEF-CZE [24] was also proposed to improve the peak capacity.

Recently, Zhang and El Rassi used a nano-injection valve to couple cIEF to capillary CEC for the separation of proteomic mixtures [28]. CEC offers the advantages of both CE and LC with regard to separation efficiency as well as compatibility with MS. The relatively simple setup, *i.e.* two capillaries interfaced with a valve, resulted in a theoretical peak capacity of approximately 55 000. Although such a system has been developed and verified to be a prototype system with high peak capacity for proteome analysis, with isocratic elution in the secondary dimension and elaborate system connections, its potential for application has not yet to be fully exploited.

Pressurized CEC (pCEC), as a novel microcolumn separation technology, has been proved to be a powerful tool in the analysis of a wide range of compounds, especially including proteins and peptides [29–31]. By coupling of hydraulic pressure with high voltage, pCEC combines the benefit of high selectivity in HPLC and high efficiency in CE. Furthermore, the combination of EOF and the supplementary pressure also serves to increase peak capacity, shorten analysis time, and suppress the bubble formation. Though the pCEC mode has a disadvantage in the theoretical plate height, the major advantages of the method over pure CEC is that it could be amenable for a solvent gradient mode and increase the speed of separation and avoid bubble formation with the application of pressure. These characteristics make pCEC an attractive and compatible separation technique coupling to other separation mode to offer greater resolving power for the separation of complex mixtures. However, although some pCEC-based 2-D coupling systems have been proposed, including capillary size-exclusion chromatography-pCEC developed by Stahl [32] and strong cation exchange-pCEC [33] from our group, till now, there is no report for the hyphenation of pCEC with other electrokinetic separation techniques.

The aim of this study is to develop a comprehensive 2-D separation system coupling pCEC with cIEF for peptide and protein mapping. cIEF, having an orthogonal mechanism to pCEC, is used as the first dimension, whereas reversed-phase pCEC acting as the second dimension to perform high-resolution separation. In this presented system, focused zones from the cIEF separation part were electrically driven to a micro-injection valve, which was isolated from the electric field, and then successively transferred to the pCEC column for further separation. With simple and robust system design, this system allows the peptide/protein samples focusing and mobilization in one-step, and enables gradient elution fractions transferred from the first dimension with the aid of pressurized flow and EOF. Separation effectiveness of this platform was demonstrated using complex protein and peptide samples.

2 Materials and methods

2.1 Materials and chemicals

Fused silica capillaries with 100 μm id/375 μm od for cIEF were purchased from Yongnian Optical Fiber Factory (Yongnian, Hebei, China). HPLC-grade ACN and TFA were provided by Merck (Darmstadt, Germany). Hydroxypropylcellulose (HPC) was purchased from Aldrich Chemical (Milwaukee, WI). DTT and iodoacetamide were purchased from Amresco (Solon, OH). Pharmalyte (pH range of 3–10) was purchased from Pharmacia Biotech (Uppsala, Sweden). All protein standards were all purchased from Sigma Chemical (St. Louis, MO). Sequencing-grade modified porcine trypsin was purchased from Promega (Madison, WI, USA). All chemicals used in making buffer solutions were analytical-grade reagents. The water used in this study was purified using a Milli-Q water purification system from Millipore (Bedford, MA, USA).

2.2 Sample preparation

2.2.1 BSA trypic digest

BSA was dissolved in 100 mmol/L NH_4HCO_3 buffer at a concentration of 25 μg/μL and boiling for 15 min. The protein solution was digested overnight at 37°C with trypsin at a ratio of 25:1 w/w [21].

2.2.2 Proteins and trypic peptides from human red blood cells

Human whole blood containing heparin anti-coagulant was centrifuged at 1200 rpm for 10 min at 4°C. Then the supernatant was removed and the red blood cells were lysed by cold 0.8% w/v hypotonic NH_4Cl buffer according to the method previously described by Rambaldi *et al.* [34]. Afterwards, the lysate was centrifuged at $18\,000 \times g$ for 30 min (4°C), and the supernatant representing the erythrocyte soluble proteins was stored at −20°C for future use. Protein concentration of the erythrocytes samples measured by Bradford method is about 67.5 mg/mL. The proteins were reduced with 10 mmol/L DTT at 37°C for 1 h and then alkylated with 25 mmol/L iodoacetamide for an additional 30 min at room temperature in the dark. After diluting fivefold with 50 mmol/L NH_4HCO_3 buffer, the human red blood cell (HRBC) proteins were digested by adding sequencing-grade modified trypsin with trypsin-to-protein ratio of 1: 50 w/w, followed by incubation overnight at 37°C [35]. The samples of proteins and tryptic digests were desalted before

analysis with Sep-Pak-plus C_{18} cartridge (Waters, Milford, MA, USA).

2.3 cIEF separation with an electrical decoupler

2.3.1 Capillary coating and preparation of the electrical decoupler

In order to suppress the EOF and protect sample from adsorption on the capillary inner wall, cIEF capillaries were coated with HPC according to the method that given in [36] with a little modification. Briefly, a 50-cm capillary (100 μm id × 375 μm od) was filled with 5% w/v HPC aqueous solution and the polymer solution was then purged using N_2 at 1.5 MPa. After that, the capillary was heated in the oil bath from 60 to 140°C and held at 140°C for 20 min with N_2 (1.5 MPa) purging.

An electrical decoupler, which isolates the micro-injector interface from the electrical field of cIEF in our 2-D system, was prepared on the coated capillary at a distance of 0.7 cm from the cIEF capillary outlet-end, as described in our previous publication [37].

2.3.2 cIEF procedure

cIEF separation was performed using a TriSep-2100 CE system (Unimicro Technologies, Pleasanton, CA, USA) equipped with a ±30 kV high-voltage power supply, and a variable-wavelength UV/Vis absorbance detector.

The inlet-end of the 50 cm-long coated cIEF capillary was immersed in the anodic reservoir, whereas another end was connected to sample port of the micro-injector through the electrical decoupler-based cathodic cell. The solutions of 20 mmol/L H_3PO_4 containing 0.1% HPC and 20 mmol/L NaOH were employed as the catholyte and the anolyte, respectively. The cIEF capillary was initially filled with the sample containing 2% carrier ampholytes (pH 3–10). During cIEF running, the electrical decoupler was set to a common ground and focusing was performed by applying a constant voltage of +20 kV (~400 V/cm) between the anodic cell and the electrical decoupler. EOF mobilization was used to drive the formed pH gradient and focused protein/peptide zones through electricity-conductive joint, subsequently into the micro-injector. The absorbance of focused bands was on-column monitored at 280 nm by the UV detector which was positioned 6 cm from the outlet-end of cIEF capillary.

2.4 Gradient pCEC separation

All pCEC experiments were performed on a TriSep-2100 pCEC system (Unimicro Technologies), which comprised a solvent gradient delivery module, a high-voltage power supply (±30 kV), a variable wavelength UV–Vis detector, a microfluid manipulation module with the micro-valve injector, and a data acquisition module.

The solvent delivery pumping system was used to provide supplementary pressure and deliver solvent gradient at a flow rate of 0.06 mL/min. A splitting-cross was used to split solvent flow after the micro-injector valve (splitting ratio 100:1). A micro-splitter valve (Upchurch, Sulpecol) was placed at the outlet-end of the splitting capillary, acting as on–off switch of the effluent splitting. The inlet end of a capillary reversed-phase column (150 μm id × 20 cm, packed with 3 μm C_{18} silica particles, Global Chromatography, Zuzhou, China) was connected to the splitting-cross, which was set to ground potential. A negative high voltage was applied to the outlet end of the column.

Binary solvents of A (5% ACN/0.1% TFA) and B (95% ACN/0.1% TFA) were used in gradient elution. Elution for BSA digest sample was as follows: 0–25% B in 25 min, and further increased to 100% B in 5 min. Elution for HRBC tryptic digests was 0% solvent B to 50% solvent B in 30 min. The solvent gradient used HRBC proteins was 0–40% B within first 10 min, then increased up to 100% B in 20 min. A 6 kV voltage was applied across the capillary during the pCEC gradient elution all along. On-column UV detection was carried out at 214 nm. Data acquisition and processing was carried out by a Unimicro Trisep Workstation 2003 (Unimicro Technologies).

2.5 Integrated 2-D separation system

A diagram of the cIEF-pCEC system is shown in Fig. 1, the micro-injector with a 500 nL external injection loop was employed for on-line hyphenation of cIEF with pCEC to make an integrated 2-D system. Samples were injected and separated by cIEF as described above. The focused bands were then electrically mobilized into the loop attached to the micro-injector under the loading position. The mobilization velocity was measured to be around 0.8 cm/min, corresponding to a volume flow rate of 0.06 μL/min inside a 100-μm-id. cIEF capillary. After that, the high voltage applied upon the cIEF column was temporarily shut off, and the micro-injector was switched. Peptides/proteins fractioned by loop were flushed past the splitting-cross onto the top inlet of the C_{18} reversed-phase capillary column for trapping and refocusing, whereas the carrier ampholytes were directed to waste prior to pCEC separation. During this loading process, all of the fractions introduced into the injector completely went to the column without splitting by closing the splitting vent. After loading, the splitting vent was reopened, and the second-dimensional analysis of the trapped sample was initiated on-line. Repeated electroosmosis-driven mobilization followed by pCEC separations was performed until the entire cIEF capillary content was completely transferred and analyzed.

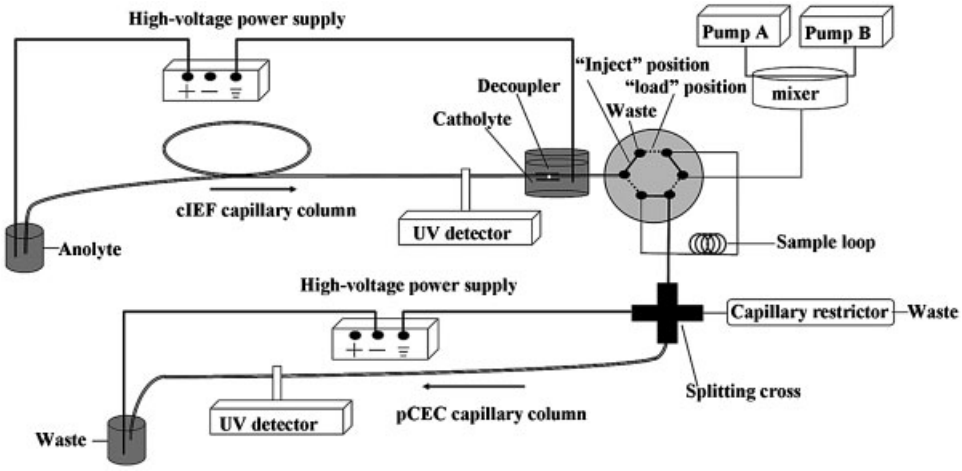

Figure 1. Schematic diagram of the on-line cIEF-pCEC 2-D system.

3 Results and discussion

3.1 cIEF fractionation

In most of the reported cIEF-based multi-dimensional systems, two-step cIEF separation was popularly performed, in which focusing and pressure-mediated mobilization took place sequentially and independently. The focused bands were often hydrodynamically transferred to the second dimension, thereby may leading to prolonged runtime and increased risk of zone broadening. In this 2-D system, the cIEF separation was executed by a one-step method, which combined the focusing and mobilization steps, thereby simplifying the operation of cIEF and reduced time [17]. With moderate EOF toward the cathode, the formed pH gradient and focused zones were driven to the valve interface, thus eliminating the need of mobilization by hydrodynamic force or adding salts. In order to obtain the reduced EOF and controlled mobilization speed, the cIEF capillary was internally coated with a very thin film of HPC by purging with N_2 in high-pressure mode prior to use, thereafter, 0.1% HPC was added into sample buffer and anolyte solution, respectively, during the separation process.

Moreover, among these developed cIEF-based 2-D systems, an external or internal injection loop attached to the switched valve was placed between the anodic and cathodic cells of cIEF separation section for transferring focused fractions into the second dimension. Therefore, the switching valve was integrated into the cIEF electric field. These connection designs obviously benefit to minimize void volume of the 2-D system; however, it may increase the possibility of bubble generation in the micro-valve, resulting in current breakage. In our present study, cIEF system was coupled to the micro-valve *via* an electrical decoupler, which separates the outlet-end of cIEF capillary from applied potential field and grounds the separation voltage prior to the micro-injector valve. Therefore, the micro-injector was free of the electric field in cIEF dimension. This connection arrangement permits a linear pH gradient to be maintained in the separation capillary and makes major contribution to the inhibition of bubble formation. In addition, the cathodic cell and micro-injector valve can be easily and safely hyphenated with the secondary dimension.

The electrical decoupler, which was made by using the method of on-column fracture coated simply with cellulose acetate membrane [37], proved to be excellent for electrolyte connections and prevention of leakage. However, the introduction of the electrical decoupler may increase the possibility of separation efficiency degradation due to laminar flow formed in the potential-free section between the electrical joint and the capillary outlet-end, thus, this segment should be as short as possible. In our experiment, a geometry-required minimum distance of 0.7 cm was chosen to minimize the risk of resolution loss. Although due to geometry-restriction, it is difficult to make another UV detection window behind the electrical joint to examine exactly the decoupling influence on the operation of cIEF system, the efficiency loss on such a short segment is obviously negligible when using a 50-cm-long separation capillary.

Finally, the single cIEF performance was evaluated by using six standard proteins and BSA digest as model samples. A typical electropherogram, obtained by one-step cIEF separation with the electrical decoupler-based cathodic cell, is shown in Fig. 2. During the separation, wavelength limited to 280 nm was used for detection due to the background absorbance of carrier ampholytes. Consequently, very well-resolved peaks of six standard proteins, having peak widths of ~12 s at the base, were reproducibly achieved with average RSD values for six replicate runs of 5.6 and 10% in migration time and peak height, respectively. In addition, an estimated capacity of 198 was projected for separation of BSA peptides (Rs = 1). Therefore, with high separation resolution and satisfactory reproducibility, the cIEF system is ready to be hyphenated with the second dimension to enhance the resolving power.

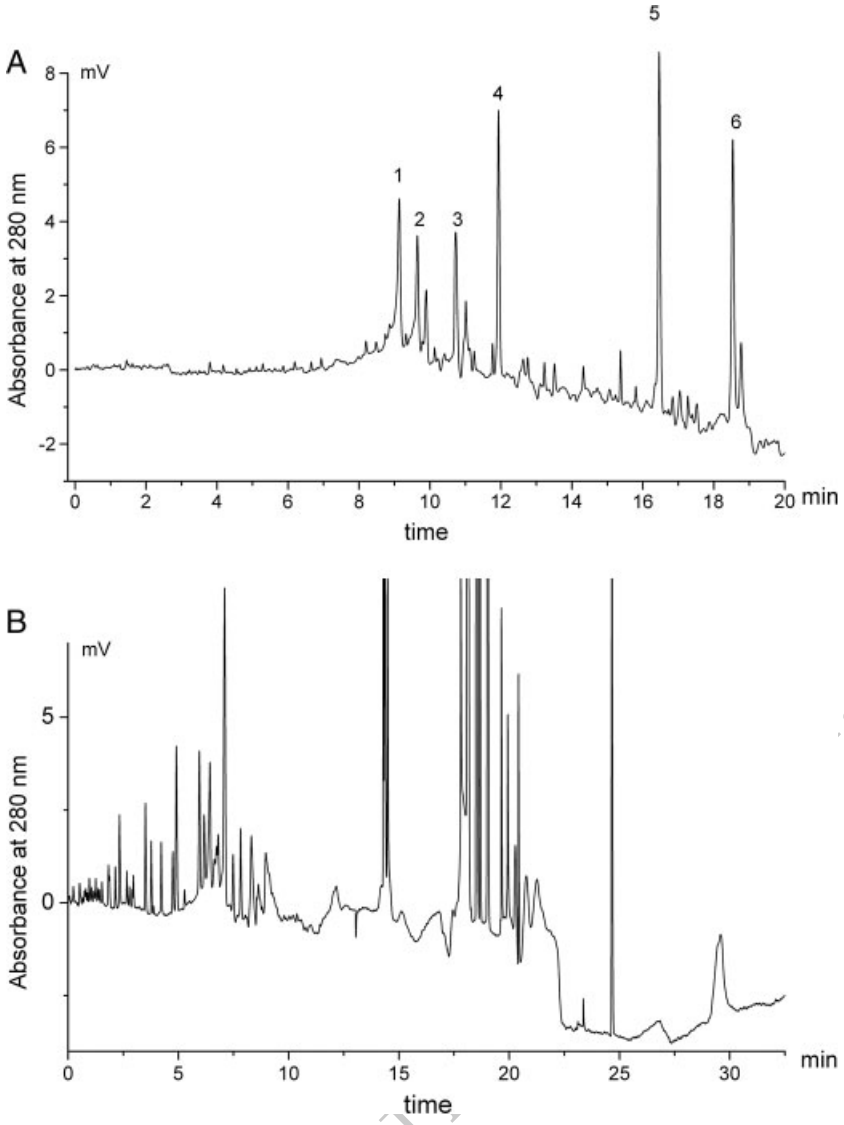

Figure 2. One-step cIEF electrophoregrams for six standard proteins (A) and BSA tryptic digest (B). Conditions: HPC-coated capillary, 100 μm id × 50 cm; anolyte, 20 mmol/L H_3PO_4 containing 0.1% HPC; catholyte, 20 mmol/L NaOH; Injection by filling the whole capillary column with sample (proteins at 1 μg/μL each and BSA peptides at 5 μg/μL) dissolved in 2% v/v Pharmalyte containing 0.1% HPC, focusing voltage 20 kV (400 V/cm); UV detection, 280 nm. For six standard proteins (A), peak identifications from left to right: 1, Lysozyme; 2, Cytochrome C; 3, RNAase A; 4, Myoglobin; 5, Carbonic Anhydrase; 6, β-lactoglobulin.

3.2 Gradient pCEC separation

For the second dimension of pCEC separation, EOF caused by the voltage is superimposed on a pressure-induced hydrodynamic flow; therefore, both pressure and electric field are applied across a capillary column simultaneously. This combination brings several advantages.

First, the combination of a pressure-driven flow with an electric field in pCEC instrumental setup was fulfilled in the manner that a negative voltage was applied to the outlet of the column, and the inlet of the column was connected to the splitting-cross and grounded. Therefore, the valve interface of the 2-D separation system for fraction transfer was also effectively isolated from the electrical field of pCEC dimension for the purpose of facilitating operation and safety.

Second, with solvent compatibility to cIEF mode, the second-dimension pCEC offers the capability of on-column sample enrichment for avoiding the effluent fraction diffusing and further refocusing the analyte zones. Additionally, early elution of the carrier ampholytes significantly decreased the potential interference with the detection of proteins/peptides; therefore, a wavelength of 214 nm with more sensitivity could be used for UV detection in the pCEC dimension.

Finally, separation resolution and elution speed were all enhanced in pCEC compared to those in pressure-driven capillary LC, which is shown in Fig. 3. When tryptic digests of BSA were separated without application of voltage, about 55 peaks were observed with average RSD of 1.6% for six consecutive runs. When −6 kV was applied to the column outlet, the number of peaks increased to average 67 with RSD value of 2.1% ($n = 6$). The increase in resolution was due to reduced peak width as a result of the EOF and the improved selectivity of some peptides due to the different extent of their electrophoretic migration. Additionally, highly efficient separations were still performed on a short column (20 cm) in a short-time span (30 min) by properly

tuning the applied voltage and the supplementary pressure. Therefore, in pCEC, the electric field improves both analysis speed and column efficiency without significant compromise being made between resolution and analysis speed. This characteristic makes the pCEC mode more suitable being employed as the second dimension in an on-line 2-D system.

3.3 Integration of cIEF with pCEC for protein/peptide mapping

Tryptic digests of BSA and HRBC lysate were subjected to the 2-D platform in order to further validate its separation performance and practical use. In order to alleviate the resolving pressure of the pCEC dimension, cIEF fractionation was operated intermittently, namely in stop–go pattern. In the ''go'' mode, high voltage is applied to the cIEF system to push focused bands into the loop attached to the microvalve for fraction transfer. In the ''stop'' mode, the cIEF run is temporarily halted by turning off the high-voltage power supply, thus proteins/peptides samples are remaining in the capillary waiting for next sampling, whereas the pCEC separation is carried out. Finally, in one cIEF-pCEC run, samples were focused and mobilized for around 8 min, and then followed by subsequent pCEC separation in about 30 min. A total of eight fractions in average (ranging from 7 to 9) sampled from the cIEF capillary were further analyzed by pCEC in 5 h. The resulting plots using UV absorbance at 214 nm for BSA digests and HRBC lysate are shown in Figs. 4 and 5, respectively. Each electropherogram represents the sequence of cIEF fractions further resolved by pCEC. Some extraordinary high peaks and crowded bands in Fig. 5 may be ascribed to the high-abundant proteins in human red cell. A higher resolving power, improved peak shapes, and increased total peak numbers were all observed in the electropherograms of the 2-D separation.

The peak capacity of this 2-D system was evaluated. In the case of BSA peptides, the average peak width for fairly resolved peaks in pCEC separation was about 15 s over a span of 30 min separation time, resulting in an estimated peak capacity of around 120 (Rs∼1). From the single cIEF electropherogram, the peak capacity was estimated to be 198, as described in Section 3.1. Thus, the theoretical peak capacity that in principle can be reached by the system was about 24 000. However, eight cIEF fractions of BSA digest in total were injected into the pCEC column under the current scheme. The practical peak capacity value is about 1000 (eight cIEF fractions × 120), which is only a small fraction (4.2%) of the maximum peak capacity that in principle can be reached by the system. Significant enhancement in the

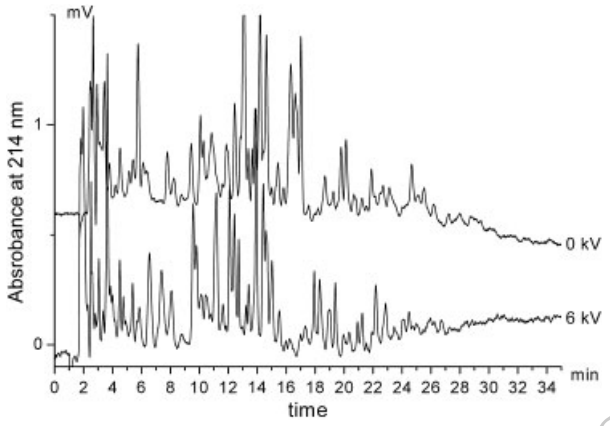

Figure 3. UV chromatograms of 8 μg/μL BSA tryptic peptides separated by pCEC (6 kV voltage applied) and capillary LC (no voltage applied) in single dimension. Conditions: capillary column packed with 3 μm C_{18} silica particles, 150 μm id × 20 cm; UV detection, 214 nm; sample loop, 500 nL; flow rate of pump, 0.06 mL/min; splitting ratio, 100:1; Mobile phase A consisted of 5% ACN/0.1% TFA, and mobile phase B consisted of 95% ACN/0.1% TFA. The solvent gradient used was as follows: 0–25% B in 25 min, and further increased to 100% B in 5 min.

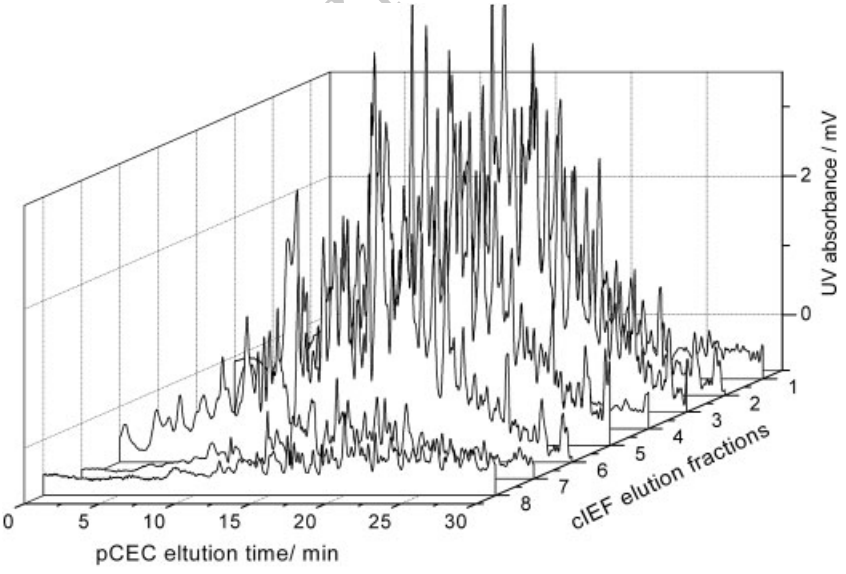

Figure 4. Analysis of BSA tryptic digest with on-line cIEF-pCEC 2-D system. Each number represents the sequence of cIEF fractions further analyzed by pCEC. Separation conditions of cIEF and pCEC are as described in Fig. 2 and Section 2.4, respectively. Sample concentration, 8 μg/μL.

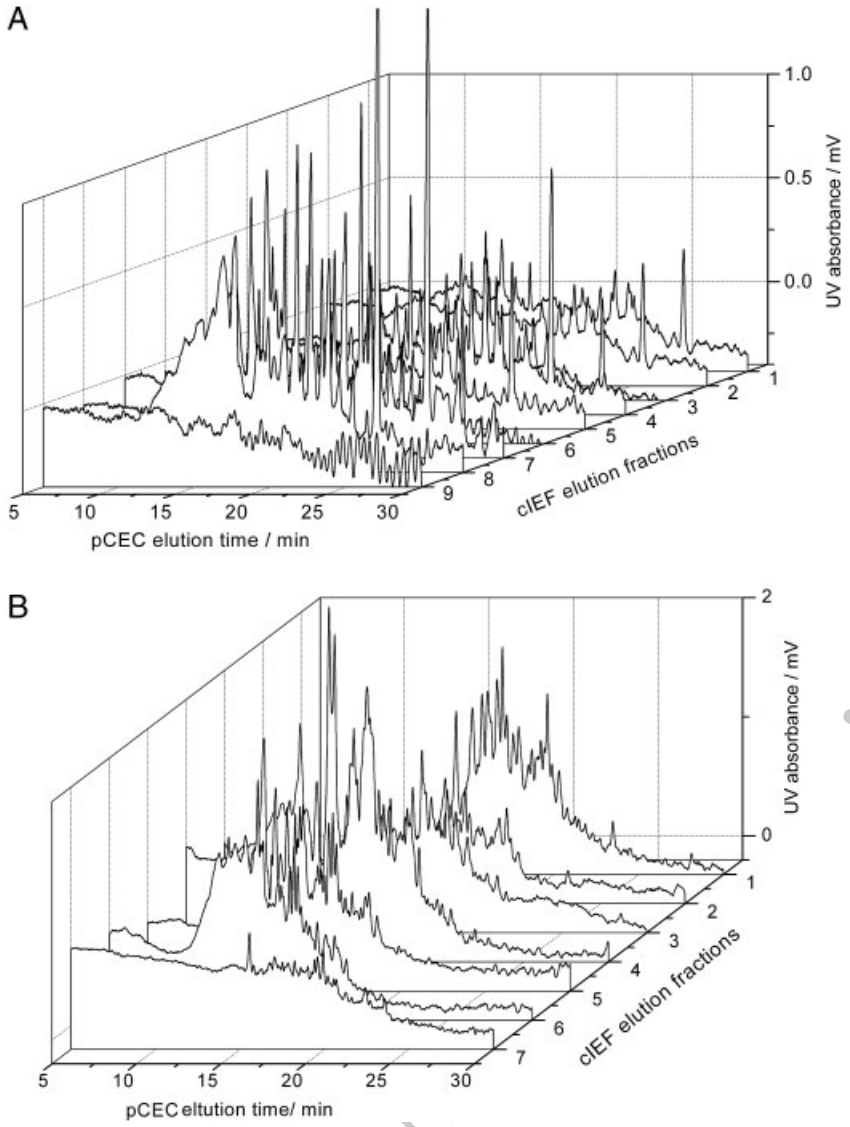

Figure 5. Analysis of HRBC tryptic digests (A) and proteins (B) with on-line cIEF-pCEC 2-D system. Each number represents the sequence of cIEF fractions further analyzed by pCEC. Separation conditions of cIEF and pCEC are as described in Fig. 2 and Section 2.4, respectively. Sample concentration, 6 μg/μL.

separation power can be realized by further increasing the cIEF fractionation steps coupled with more sensitive detection technique and using highly permeable monolithic column with strong EOF at low pH instead of C_{18} particle-packed column in the pCEC dimension to meet the rate of cIEF fractionation, thus eliminating the need of voltage interruption.

The reproducibility of the combined cIEF-pCEC system was also assessed by performing multiple runs of identical BSA tryptic digest samples. Five consecutive runs of this 2-D system showed that for recognizable peaks of BSA peptides, the average RSD values for elution time and peak height in pCEC were about 6.1 and 15%, respectively. The fluctuation in electric currents for both two dimensions and the intermittent operation-mode of cIEF fractionation might make contributions to some migration time deviations. Relatively high deviations for peak height may be partly attributed to splitting a first-dimensional peak into two adjacent fractions. The repeatable cutting position in the cIEF dimension for fraction transfer was difficult to be controlled precisely. Employing a higher pCEC sampling rate would be useful to eliminate this problem.

4 Concluding remarks

In this study, on-line coupling of cIEF with pCEC has been developed by employing potential-free valve interface and simple system design. The feasibility and performance of the proposed 2-D system have been demonstrated by the analyses of proteins and peptides. Although only average eight cIEF fractions were processed by pCEC dimension due to the limited sensitivity of UV detection, which resulting in sacrificed separation capability, the orthogonally coupled cIEF-pCEC holds considerable promise for application to proteomic research with high-efficiency separation. In the future study, the resolving power of this system will be further enhanced by increasing the number of cIEF

fractions under more sensitive detection and employing monolithic column with strong EOF in the pCEC dimension. In addition, this 2-D separation system will be further coupled with ESI-MS or MALDI-MS for protein/peptide identification. In the coupling scheme, a positive voltage was applied to the inlet end of pCEC column, whereas the outlet end of the column was grounded and connected to an electrospray source or interfaced with MALDI-MS. The valve interface was set to ground potential to protect the pump from any damage from the high voltage. This combination clearly results in a potentially powerful analytical platform. Further studies on these interesting topics will be the subject of future investigation in our laboratory.

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The authors have declared no conflict of interest.

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