

On-line combination of capillary isoelectric focusing and capillary non-gel sieving electrophoresis using a hollow-fiber membrane interface: a novel two-dimensional separation system for proteins

Hechun Liu, Chun Yang, Qing Yang, Weibing Zhang, Yukui Zhang*

National Chromatographic R. & A. Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116011, PR China

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Abstract

A novel two-dimensional (2D) separation system for proteins was reported. In the system, a piece of dialysis hollow-fiber membrane was employed as the interface for on-line combination of capillary isoelectric focusing (CIEF) and capillary non-gel sieving electrophoresis (CNGSE). The system is similar equivalent to two-dimensional polyacrylamide gel electrophoresis (2D PAGE), by transferring the principal of 2D PAGE separation to the capillary format. Proteins were focused and separated in first dimension CIEF based on their differences in isoelectric points (pI). Focused protein zones was transferred to the dialysis hollow-fiber interface, where proteins hydrophobically complexed with sodium dodecyl sulfate (SDS). The negatively charged proteins were electromigrated and further resolved by their differences in size in the second dimension CNGSE, in which dextran solution, a replaceable sieving matrix instead of cross-linked polyacrylamide gel was employed for size-dependent separation of proteins. The combination of the two techniques was attributed to high efficiency of the dialysis membrane interface. The feasibility and the orthogonality of the combined CIEF–CNGSE separation technique, an important factor for maximizing peak capacity or resolution elements, were demonstrated by examining each technique independently for the separation of hemoglobin and protein mixtures excreting from lung cancer cells of rat. The 2D separation strategy was found to greatly increase the resolving power and overall peak capacity over those obtained for either dimension alone.

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1. Introduction

The resolution of complex samples into components requires sophisticated technologies. Most separation techniques are capable of resolving at most several dozen components. Giddings recognized that the combination of two separation techniques was important in the resolution of complex mixtures [1]. Provided that the separation mechanisms in the two dimensions are orthogonal, i.e., the two separation techniques are based on different physico-chemical characteristics of the analytes, the number of resolution elements of two-dimensional (2D) separation is given by the product of the resolution elements of both separation steps. For example, isoelectric focusing and

sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) can, individually, resolve ~50 components in a protein sample, then their combination, in 2D electrophoresis, can resolve several thousand components [2,3]. Mass spectrometry (MS) employing matrix-assisted laser desorption/ionization (MALDI) [4] and electrospray ionization (ESI) [5] has evolved to become an essential tool for protein identification and sequencing [6]. For the analysis of very complex mixtures of proteins such as cell lysates, 2D PAGE has excellent resolving power and is still the most powerful tool widely used in protein analysis [7] prior to MALDI–MS or ESI–MS.

However, classic 2D PAGE technique is time-consuming and labor-intensive [2]. Moreover, the planar 2D gel electrophoresis could not be easily coupled with MS to perform on-line identification. This technique in the post-genomic era as a routine method is not attractive.

Therefore, various coupled-column techniques combining different separation modes have been developed to achieve

* Corresponding author. Tel.: +86 411 3693427;
fax: +86 411 3693427.

E-mail addresses: hechunliu@163.com (H. Liu),
ykzhang1@online.ln.cn (Y. Zhang).

comprehensive multi-dimensional separations. These techniques include size exclusion chromatography (SEC) coupled with parallel reversed phase liquid chromatography (RPLC) [8], ion exchange chromatography coupled with RPLC [9–11] by using switching valves, strong cation exchange and reversed-phase packing materials packed in a capillary column called biphasic column [12], microcolumn LC coupled with CE by flow gating [13], optical gating [14], CIEF integrated with CRPLC system using a microinjector interface [15], and two different electrophoresis separations integrated on the zero-dead volume microfluidic device [16,17].

Although various non-gel-based 2D separation alternatives have been developed to overcome the disadvantages of slow speed and labor-intensive gel approach. Up to now, there are only a few reports concerning 2D CE. Michels et al. [18] first reported the development of a fully automated 2D CZE-CZE system for protein analysis using a modified version of Jorgenson's interface. In the system, proteins labelled with the fluorogenic reagent were separated in the first dimension, by submicellar CE at pH 7.5, and then the fractions were subsequently transferred to a second dimension capillary, where electrophoresis was performed at pH 11.1 to further separated proteins. Sheng and Pawliszyn [19] successfully coupled the micellar electrokinetic chromatography (MEKC), the first dimension 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 were added. Recently, Mohan and Lee [20] have employed microdialysis as the interface for on-line coupling of CIEF with transient capillary isotachopheresis (CITP)-CZE for the separation of tryptic digests of proteins. In our group, Yang et al. developed a similar approach for on-line hyphenation of CIEF with capillary gel electrophoresis (CGE), and on-line coupling of CIEF with CZE using a dialysis hollow-fiber interface to change the buffer solution in the interface before the second dimensional electrophoresis [21,22]. Our system differs from Michels and Sheng's in three respects: first, a simple interface device including a dialysis hollow fiber is employed to change buffer solution and achieve necessary electrical connection, which eliminates a relative complicated cross/valve interface. Second, in our 2D CE system, concentrating properties of CIEF is employed in the first dimension to enhance the content of sample injection and the detection sensitivity of the system. Third, the similar principal of 2D PAGE separation is transferred to the capillary format, e.g. the separation mechanisms of CIEF and CGE are completely orthogonal, and thus the overall peak capacity of the 2D separation system could be greatly increased over that of the individual 1D separation alone.

CE has many advantages over the slab electrophoresis with high column efficiency, high resolution, detection on-column, and conveniently coupling with MS. Therefore, the development technique of 2D CE has significance for the separation of proteins. Based on the benefits of CE

over its planar gel counterparts, we endeavored to transfer the main working principals of classic 2D PAGE to the capillary format.

The incorporation of 2D PAGE in the capillary format has some difficulties due to the anolyte and the catholyte of CIEF incompatible with the running buffer of CGE each other, which results in the difficulties involving the interface to switch the effluents from the first dimensional column into the second separation column. We have introduced an on-line 2D system consisting of CIEE and CGE. As all know, a gel-based capillary has some troublesome drawbacks. Typically, the gels produce exhibited tremendous variability [23,24], tedious bubbles are prone to form leading to gel shrinkage during the polymerisation, especially at the connection between CIEF and CGE resulting in experiment halted, and unavoidable cross-contamination in gels is easily caused by carryover between samples. In addition, chemically mobilization is less efficient and applicable for highly basic or acidic proteins.

In comparison with cross-linked polyacrylamide gel, non-gel sieving matrices employed for size-dependent separation of proteins avoid those drawbacks. In this paper, on-line combination of CIEF and capillary non-gel sieving electrophoresis (CNGSE) as a high-resolution 2D protein separation platform was constructed by using our self-prepared dialysis hollow-fiber membrane [25] as the interface and only one high-voltage power supply with three electrodes. Moreover, two UV detectors were used in the system, of which one mounted on the end of the first dimensional capillary allowed us to monitor the fractions transferred into the second dimensional capillary. Hemoglobin sample and mixtures of proteins excreting from lung cancer cells of rat were selected to demonstrate the 2D separation system's feasibility and resolving power.

2. Experimental

2.1. Materials and instrumentation

The electrophoresis was performed using a set of TriSep-2000GV from Unimicro Technology Inc. (Pleasanton, CA, USA) equipped with a Data Module UV-Vis detector and a high voltage dc power supply. A Workstation Echrom98 of Elite Instrument Inc. (Dalian, China) was used for data acquisition. Fused-silica capillaries (50 μm i.d. and 150 μm o.d.) were obtained from Ruiyang Chromatographic Co. Ltd. (Yongnian, China). γ -methacryloxypropyltrimethoxysilane (γ -MAPS), *N,N,N,N'*-tetramethylethylenediamine (TEMED, 99%), and ammonium persulfate (APS, +98%) were all obtained from Acros Organics (New Jersey, USA). Pharmalyte (pH 3.0–10.0) was obtained from BioChemika (Switzerland). Cacodylic acid (CACO), 2-amino-2-methyl-1,3-propanediol (AMPD), tris-(hydroxymethyl)aminomethane (Tris), methyl cellulose (MC), and dextran (M_r 2,000,000) were all

purchased from Sigma (St. Louis, MO, USA). Hemoglobins A, F, S and C were donated by Shangdong Institute of Pharmacy (Shangdong, China). The SDS-protein marker containing Rabbit Phosphorylase b (RPHB 97.4 kDa), Bovine Serum Albumin (BSA 66.2 kDa), Rabbit Actin (RA 43.0 kDa), Bovine Carbonic Anhydrase (BCA 31.0 kDa), Trypsin Inhibitor (TI 20.1 kDa) and Hen Egg White Lysozyme (HEWL 14.4 kDa) was purchased from Shanghai Xibasi Biotech Co. (Shanghai, China). Other reagents were of analytical grade unless otherwise stated. All solutions were filtered through a 0.45 μm pore size filter (Elite Co. Dalian, China) and were treated ultrasonically for 15 min to remove gas bubbles prior to use. Caution should be exercised in handling acrylamide and CACO, as they can be toxic.

2.2. Preparation of protein mixtures excreting from lung cancer cells of rat

Mixtures of proteins excreting from lung cancer cells of rat were supplied by Dalian Medical University. Sample solution containing proteins and culture medium were filled in the polystyrene film bag (nominal molecular weight cutoff, with 3,500) and dialyzed for desalting in the 50 mmol/L Tris–HCl buffer (pH 6.8) under 4 °C for overnight, then the polystyrene film bag containing the sample solution was wrapped with polyethylene glycol (PEG, 20,000) to dehydrate and concentrate the mixtures of proteins for CE use.

2.3. Preparation of polymer-coated capillaries

Fused-silica capillaries were coated internally with linear polyacrylamide (LPAA) covalently attached to the wall by a modification of the method of Hjärten [26]. Briefly, a 30 cm long capillary was first treated with 0.5 mol/L NaOH for 30 min and washed with 0.5 mol/L HCl, and then washed with deionized water and methanol, respectively, for 20 min. The capillary was then coated as following procedure. First, γ -MAPS with 50% (v/v) methanol was pushed through the capillary by a syringe, and then the capillary was kept at room temperature for 5 h with both ends sealed by a silicon septum. After reaction for 5 h, the residual γ -MAPS solution was removed from the capillary by pressure. Then the capillary was sequentially washed with methanol and deionized water, and dried in the GC oven at 60 °C. Last, the dried capillary was filled with a 4% acrylamide solution containing 0.1% (w/v) APS and 0.1% (v/v) TEMED. After polymerization at room temperature for 3 h, excessive polyacrylamide was pushed out of the capillary, and the capillary was rinsed with water.

2.4. Fabrication of the interface

As shown in Fig. 1A, two pieces of 2 cm long capillaries (50 μm i.d. \times 150 μm o.d.) were inserted into a piece of 10 mm long built in-house (self-prepared) polysulfone dialysis hollow fiber (160 μm i.d. \times 210 μm o.d., nominal molec-

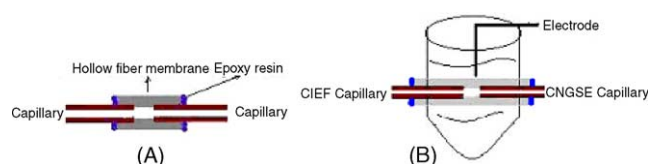


Fig. 1. Schematic of fabrication of the interface: A is a junction of hollow fiber with capillaries, and B is a interface for 2D CIEF–CNGSE.

ular weight cutoff 10,000), and the gap between the two capillaries was 1.5 mm. Epoxy was then applied around the outside of the fiber–capillary boundaries. The epoxy was allowed to dry and the above junction housed (through holes pierced on both sides of a microcentrifuge tube with a cover) in a microcentrifuge tube was employed for the interface (Fig. 1B).

2.5. Configuration of 2D CIEF–CNGSE separation system

As shown in Fig. 2, the above interface was employed for on-line combination of CIEF and CNGSE separation system. The first dimensional CIEF capillary (25 cm) and the second dimensional CNGSE capillary (length 19 cm) were closely connected to the capillaries of the interface by way of two pieces of 2 cm long Teflon (140 μm i.d. \times 1.2 mm o.d.), respectively. The inlet of the CIEF capillary was passed through a UV Detector 1 and then inserted into a reservoir filled with 15 mmol/L phosphoric acid (inlet reservoir). The Detector 1 was placed 3 cm from the interface. The outlet of the CNGSE capillary was passed through a second UV Detector 2 and then inserted into another reservoir (outlet reservoir) filled with CNGSE running buffer, and the Detector 2 was placed 6 cm from the outlet reservoir. The 2D electrophoresis was driven by a high voltage dc power supply. One platinum wire was inserted into the microcentrifuge tube and served as the cathode (common electrode), and another two platinum wires were grounded and inserted into

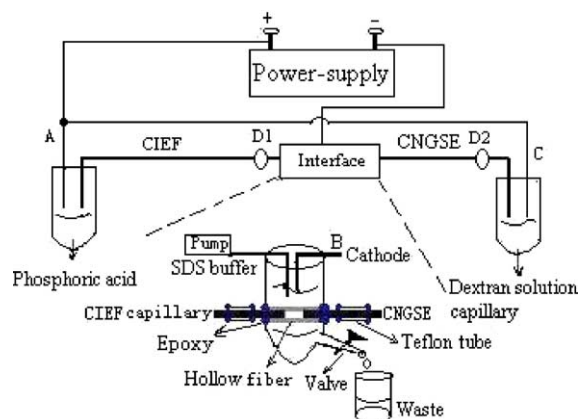


Fig. 2. Schematic of on-line combination of CIEF and CNGSE using a dialysis hollow-fiber membrane interface. Points A and C were connected to a ground, Point B was connected to a high-voltage power supply (Table 1).

the inlet reservoir (anode) and outlet reservoir (anode) to provide the necessary electrical connections.

2.6. CIEF and CNGSE separation of proteins

Prior to perform CIEF separation of proteins, the uncoated capillary was pre-conditioned with 0.5% (w/v) methyl cellulose overnight. During the CIEF experiments, the pre-conditioned capillary was initially filled with protein sample for CIEF from the inlet by a syringe pump. The two ends of the capillary were then placed into 15 mmol/L H_3PO_4 anolyte and 30 mmol/L NaOH catholyte, respectively. Focusing was started by applying electric field strength across the capillary. EOF mobilization was used to drive the formed pH gradient and focused protein zones to the detector windows. The result was monitored at 280 nm.

To perform the CNGSE separation of proteins, the CNGSE running buffer containing 60 mmol/L AMPD (CACO was used to adjust the pH to 8.5), 10% (w/v) dextran and 0.5% (w/v) SDS was initially filled into the capillary coated with LPAA. The SDS-protein sample was cathodically injected into the capillary by a certain high voltage and separated in the sieving matrix under negative separation voltage.

2.7. 2D CIEF–CNGSE separation of proteins

Prior to perform 2D separation, the hollow-fiber junction and the microcentrifuge tube housing the junction were initially filled with 30 mmol/L NaOH. The protein sample dissolved in the CIEF buffer was hydrodynamically introduced into the pre-conditioned CIEF capillary with a syringe and the CNGSE capillary coated with LPAA was initially filled with CNGSE running buffer by a syringe pump. The two capillaries were then connected to the interface described as above 2D configuration method (shown in Fig. 2). All the procedures should be operated cautiously to avoid bubbles in the separation channel. A high-voltage power supply with three electrodes was employed for providing the sequences of electric voltages needed during the 2D separation (Table 1). Focusing was performed at constant voltage of +10.5 kV (~ 400 V/cm) over the entire CIEF capillary. EOF mobilization was used to transfer the focused protein zones towards the interface. Once the first focused protein zone passed through the UV Detector 1 window, a high voltage -11.5 kV (~ 550 V/cm) was applied over the second di-

mensional capillary for achieving CNGSE separation, at the same time the valve attached to the microcentrifuge tube was opened and 60 mmol/L AMPD–CACO (pH 8.5) with 1.0% (v/v) SDS buffer was pumped into the microcentrifuge tube by a HPLC pump (Unimicro Technologies, USA) at about 1.5 mL/min. The flow rate out of the interface reservoir was adjusted to the same as that into the interface reservoir. Due to the first dimensional detector 3 cm from interface, the high voltage was applied upon the first dimension for additional around 2.0 min (the time was measured with thiourea) and then temporarily shut off. The transferred proteins complexed with SDS at the interface and were further resolved in the second dimension CNGSE. The result was monitored by the UV Detector 2 at 280 nm. Repeated focused zones transferred into the interface followed by CNGSE separations were performed until the entire CIEF capillary content was transferred and analyzed.

3. Results and discussion

Our purpose for developing such an on-line coupling CIEF with CNGSE system for complex protein analysis was to acquire as much relevant information regarding the sample as possible, and as quickly as possible. The 2D process could be simply divided into three steps: (i) loading of the mixed sample into the CIEF capillary, (ii) performing IEF and mobilizing the train of focused zones to the interface, and (iii) the transferred fraction performing CNGSE and detection. Each of these steps was initially optimized in detail in order to assure optimum performance.

3.1. Characteristics of 2D CIEF–CNGSE

In CIEF, the first separation dimension in this 2D system, proteins were focused and separated based on their differences in isoelectric points (pI), while in CNGSE, the second separation dimension, the focused proteins were further resolved by their differences in size. Thus, the similar principal of 2D PAGE separation is transferred to the capillary format via the hollow-fiber interface, i.e. the separation mechanisms of CIEF and CNGSE are completely orthogonal and the overall peak capacity of the 2D protein separation is the product of that of the individual 1D separation.

CIEF has a power to concentrate analytes with a typical concentration factor of 50–100 folds. Such a condensed and shortened analyte plug in a capillary is suitable for sample injection to other CE modes. Therefore, CIEF is a proper candidate as the first dimension in a multi-dimensional CE system.

A non-gel based, replaceable sieving matrix instead of the cross-linked polyacrylamide was employed in the second dimension. We have investigated the use of the cross-linked polyacrylamide gel [21]. While the gel could provide useful

Table 1
Sequence of voltages applied during 2D separations

	Inlet reservoir (Point A)	Interface reservoir (Point B) (kV)	Outlet reservoir (Point C)
Step I: (CIEF)	Ground	-10.5	Off
Step II: (Injection)	Ground	-10.5	Ground
Step III: (CNGSE)	Off	-11.5	Ground

resolution for the separation of proteins, it had many drawbacks in capillary column, such as tremendous variability, tedious bubble formation, and cross-contamination in gels caused by carryover between samples, etc. In contrast, a SDS–CE using non-gel sieving matrices has many advantages over the gel-based CE and has gained popularity as an alternative to SDS–PAGE for the separation of proteins with M_r values of 14,000–200,000 [27]. There have been numerous investigations into replaceable sieving matrices for size-dependent separation in capillary electrophoresis. Such solutions, consisting of pullan [28], dextran [29], polyethylene glycol [29] and linear polyacrylamide [30] have been used in the separation of proteins. Dextran as a sieving matrix for SDS–proteins separation was shown in Fig. 4. The results indicated that dextran not only provided useful resolution for the separation of proteins, and also dextran solution possessed UV-transparent polymer networks and thus provides higher sensitive detection at 214 nm for a single CNGSE separation of proteins. Moreover, dextran solution had strong viscosity to allow itself stationary in the capillary and not to be pushed out of the capillary by the EOF.

3.2. Interface in 2D system

In this study, a 2D protein separation platform was developed with the help of a hollow-fiber membrane interface. Hollow-fiber membrane is frequently used as microdialyzers for desalting, adjusting ionic strength, and sample preparation [31–33]. Dialysis hollow fibers possess semipermeable properties. Low-molecular-mass components, such as H^+ , OH^- , SDS, etc. can pass through the pores of the fiber freely, while high-molecular-mass components, like proteins, can only transport into the second capillary via the channel of the fiber but not to be washed away through the wall of the fiber. Therefore, a hollow fiber as an interface is suitable for incorporation 2D CE system.

The feasibility of combination of CIEF and CNGSE is mainly attributed to the design of the membrane interface (the configuration of the interface and the 2D separation platform illustrated in Figs. 1 and 2). When the first focused protein zone was driven to pass through the UV Detector 1 by EOF, a high voltage should be started to apply upon the second dimensional capillary for achieving CNGSE separation, meanwhile the high voltage must be continued applying over the first dimension for additional several minutes and then be temporarily shut off. This additional time was needed to transfer the first zone to the interface since the Detector 1 was placed 3 cm from interface (the time measured with thiourea was shown around 2 min). Buffer solutions containing 1.0% (v/v) SDS was then continuously rinsed the outside of the hollow fiber. Small molecule, SDS passed through the wall of the fiber and hydrophobically bound to the proteins passing through the hollow fiber. The negatively charged proteins under the influence of electric field applied over the CNGSE capillary

were electromigrated towards the anode (outlet reservoir) and further separated according to their differences in size in CNGSE.

The dimension of the hollow fiber and the gap between the capillaries in the hollow fiber are essential factors to assure optimum performance of the 2D system. To minimize the dead volume of the interface, the hollow fiber with desired dimensions for matching capillaries should be employed to construct the 2D system. The self-prepared hollow fiber with dimension of 160 μm i.d. and 210 μm o.d. matched for capillaries was used in this 2D system rather than a commercial fiber used in our previous 2D CIEF–CGE [21]. The gap between the capillaries was 1.5 mm, e.g. the part of the fiber exposed to the buffer solution was 1.5 mm long. The electropherograms of 2D separation of hemoglobin variants (shown in Fig. 5) indicated that exposed part of the hollow fiber with 1.5 mm in length worked effectively and the buffer exchanging could be achieved quickly. Moreover, the 2D system showed a good separation resolution, and therefore it proved that no serious dilution and dispersion of the sample zone resulting in significant peak broadening at the interface.

3.3. Mobilizing the train of focused zones from the first dimension to the interface

In the 2D separation system, the CIEF was executed by a one-step method, which combined the focusing and mobilization steps [34]. EOF was used to drive the formed pH gradient and focused protein zones to the detector windows. It should be noted that the magnitude of EOF is not always constant throughout the entire capillary. The EOF will vary with the different position of the pH gradient due to the different extent ionization of the silanol groups. Despite of the small EOF at low pH, however, there still existed another force, i.e. cathodic mobilization, to drive the focused proteins, since catholyte, NaOH was displaced by SDS buffer through dialysis interface. Therefore, in this study, the transfer of all the analytes was achieved by both EOF and chemical mobilization.

In a one-step method, since uncoated fused silica capillaries are used for CIEF, polymers, such as methyl cellulose, are necessary for preconditioning and dynamic coating during CIEF, to reduce the EOF [34,35] and prevent proteins from adsorbing onto the capillary wall. One-step method combines the focusing and mobilization steps to simplify the operation of CIEF. Furthermore, the one-step method had great advantages over two-step method for the 2D system since EOF was employed to mobilized the focused zones to the interface, thus eliminating the mobilization by hydrodynamic force [36] or adding salts [36,37]. Salt mobilization is poor applicable to very acidic or basic proteins since mobilization speed is often found too slowly and inefficiently. In contrast, the one-step method is more compatible with current commercial CE instruments for on-line 2D system and thus facilitated automation.

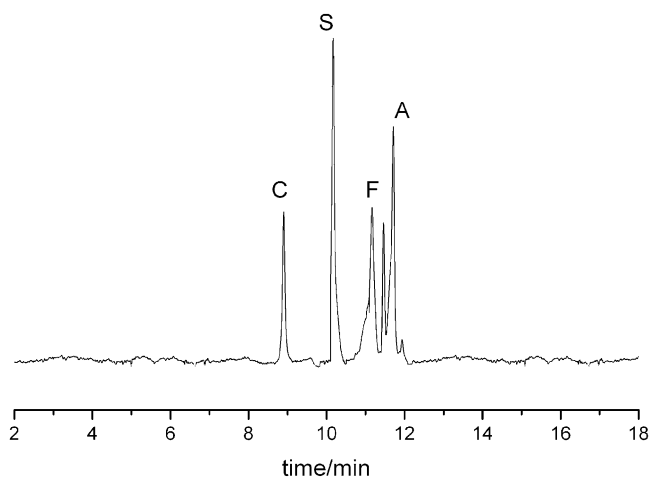


Fig. 3. CIEF separation of Hemoglobin. Experimental conditions: Capillary, pre-conditioned with 0.5% methyl cellulose, 50 μm i.d. \times 150 μm o.d., 32 cm total length, 25 cm from the anode to detection window; anolyte, 15 mmol/L H_3PO_4 , catholyte, 30 mmol/L NaOH; electric field strength, 400 V/cm; detection, UV, 280 nm; sample, 1.0 mg/mL hemoglobin dissolved in 50 mmol/L Tris-HCl (pH 6.8), 3% (v/v) pharmalyte (pH 3–10) and 1 mol/L ethylene glycol.

In our work, the dialysis hollow fiber interface was designed to achieve both combination of capillaries and buffer exchanging during the first dimensional effluents pumped into the hollow fiber by EOF. It was found, however, that the presence of methyl cellulose inside or outside the hollow fiber negatively affected the dialysis process. Possibly methyl cellulose blocked the pores of the hollow fiber resulting in less efficient buffer exchanging. In this case, methyl cellulose should not be present in the sample buffer. One way to solve this problem is to pre-condition the CIEF capillary prior to the CIEF separations. During the conditioning, the capillary was flushed with 0.5% methyl cellulose solution overnight. This approach was found to be sufficient to obtain good stability and resolution in CIEF separation, and the focusing of hemoglobin variants (shown in Fig. 3) proved it. In addition, during the CIEF process, all focused proteins at high concentration and in a state of near zero net charge for a long time increase their chance of precipitation. In order to improve resolution, an additive could be introduced into the CIEF sample to prevent the focused proteins from precipitating onto the capillary wall. In this study, ethylene glycol was added into the sample ampholyte mixture, and it was proved good efficient in improving resolution when its concentration of up to 1 mol/L.

3.4. Injection time and CNGSE speed

2D separation usually requires that the sample injection intervals to the second separation step be short and that the second step be a fast one. To achieve a fast speed in the second dimension, one way is achieved by applying higher electric field strength over the entire second dimensional capillary than that applied across the first dimensional capil-

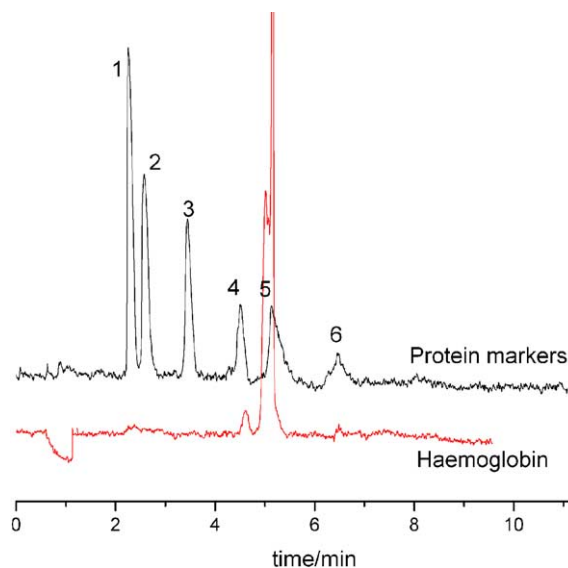


Fig. 4. CNGSE Separation of proteins. Experimental conditions: Capillary, LPAA coating, 50 μm i.d. \times 150 μm o.d., 21 cm total length, effective length 15 cm; electric field strength, 550 V/cm; injection, 5 s at 300 V/cm; sieving matrix, 10% (w/v) dextran and 0.5% (w/v) SDS dissolved in 60 mM AMPD-CACO (pH 8.5); detection, 214 nm. Protein markers, 0.5 mg/mL each protein dissolved in 50 mM Tris-HCl (pH 6.8) and 0.5% (w/v) SDS, peaks: (1) hen egg white lysozyme (14.4 kDa); (2) trypsin inhibitor (20.1 kDa); (3) bovine carbonic anhydrase (31.0 kDa); (4) rabbit actin (43.0 kDa); (5) bovine serum albumin (66.2 kDa); (6) Rabbit Phosphorylase b (97.4 kDa). (B) hemoglobin sample, 1.0 mg/mL dissolved in 50 mM Tris-HCl (pH 6.8) and 0.5% (w/v) SDS.

lary. Due to the limitation of Joule heating, the electric field strength applied upon CNGSE was limited below 600 V/cm in this study. In addition, the separation speed of CNGSE can be further increased by using a shorter capillary if only obtaining good resolution.

Fig. 3 presents the single CIEF electropherogram of hemoglobin variants containing A, F, S and C with pI s 7.10, 7.15, 7.25 and 7.50, respectively. The average separation window of all the peaks was roughly 0.5 min, therefore 0.5 min was suitable for the subsequent injection period to transfer the focused zone into the interface. As shown in Fig. 4, the typical separation time was around 2–6.5 min in a single CNGSE (the column effective length 15 cm, electric field strength around 550 V/cm) for the protein markers with M_r range 14, 000–97, 000, and the CNGSE separation of hemoglobin was complete in 5 min. According to the single CIEF and CNGSE of hemoglobin, the transfer time was set 0.5 min, and the second dimension separation was for 5 min. The successive transfer and separation of fractions was repeated four times. Fig. 5 presents a 2D CIEF-CNGSE electropherogram of hemoglobin obtained with the sequential application of these two methods. The small peak observed between peaks F and A (Fig. 3) was not well resolved with peak A during CIEF, therefore it is not clearly shown up in the 2D electropherogram (Fig. 5). However, there are still some small peaks shown up between peaks F and A during CIEF-CNGSE.

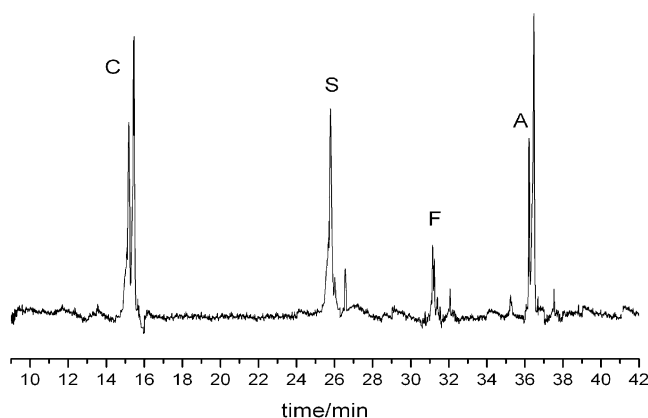


Fig. 5. Separation of hemoglobin in 2D CIEF–CNGSE. Experimental conditions: CIEF, pre-conditioned capillary, 25 cm; CNGSE, capillary coated with LPAA, effective length 15 cm; detection 280 nm; the transfer time was set 0.5 min, the second dimension separation was for 5 min, and the successive transfer and separation of fractions was repeated four times. Other conditions are same as those in Figs. 3 and 4 (for details, see CIEF–CNGSE separation of proteins in experimental section).

3.5. Application

Hemoglobin is a relative complex system containing abundant variants [38]. Hemoglobin samples from different sources and/or purification will amplify the content complexities and separation difficulties. Therefore, hemoglobin is a good candidate to evaluate the feasibility and the separation power of the 2D CE system. Figs. 3 and 4 present the single CIEF and CNGSE separation of hemoglobin variants containing A, F, S and C. Hemoglobin with different variants was preliminary separated by CIEF, and then further resolved based on their differences in size by CNGSE. Fig. 5 presents separation of hemoglobin in 2D CIEF–CNGSE system. At least four groups of peaks were obtained from the 2D separation system, and each group peaks have good resolution.

To further demonstrate the applicability of the proposed 2D system, more complex mixtures of proteins excreting from lung cancer cells of rat were used to analyzed. The complex mixtures were preliminarily dialyzed and concentrated to eliminate salts, culture medium and other unwanted components (details see Section 2.2). The protein sample mainly contained proteins with relative molecular weight range 8,000–15,000 (the data was obtained by SDS–PAGE and supplied by Dept. Biochem, Dalian Medical Univ.), therefore the single CNGSE of the protein mixtures should be complete in 2.5 min under the same experimental condition as Fig. 4. The single CIEF separation of the protein mixtures provided a poor resolution for the proteins mixtures (shown in Fig. 6), which indicates that the sample is a complex system. To overcome the limited resolving power of 1D, the proposed 2D CE system was employed to separate the relative complex system. In order to reduce the total analysis time, the transfer time of injection ef-

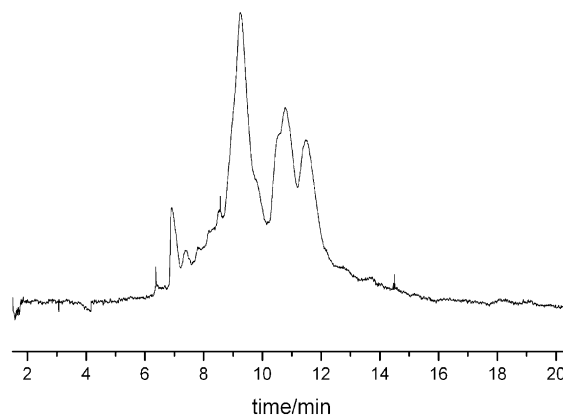


Fig. 6. CIEF separation mixtures of proteins excreting from lung cancer cells of rat. Experimental conditions are same as those in Fig. 3.

fluent into the interface was set about 1 min and the injection fractions into the second capillary at 2.5 min intervals. The successive injection and separation of fractions was repeated five times. The transfer times were chosen to ensure that the slowest component of the first fraction did not re-mix with the fastest component of the second fraction. Fig. 7 presents a 2D result of the protein sample acquired with the sequential application of the above two methods.

In comparison with the electropherograms of the single CIEF, CNGSE and 2D CIEF–CNGSE, respectively, the above results demonstrate that the coupled 2D CIEF–CNGSE system does possess higher resolving power and overall peak capacity than those of the single separation modes.

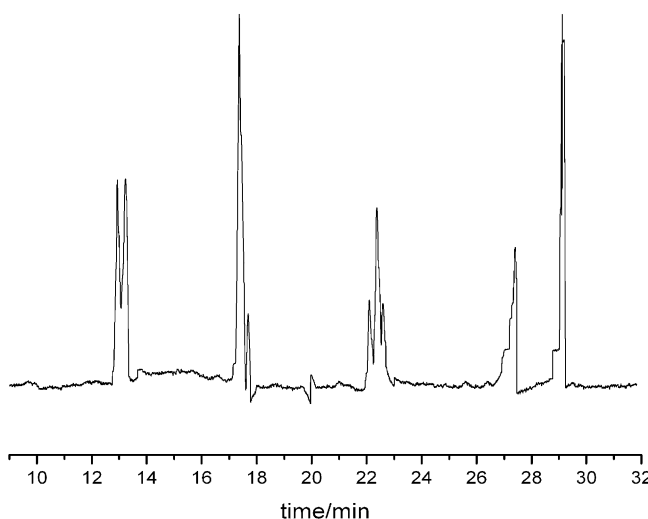


Fig. 7. 2D CIEF–CNGSE separation of mixtures of proteins excreting from lung cancer cells of rat. Experimental conditions: Except that the transfer time of injection effluent into the interface was set 1 min, the injection fractions into the second capillary at 2.5 min intervals, and the successive transfer and separation of fractions was repeated five times. Others are same as those in Fig. 5.

4. Conclusions

The feasibility of a complete on-line combination of CIEF and CNGSE system for protein analysis has been demonstrated. In principle, this 2D prototype system is similar equivalent to the classic 2D PAGE. Separation according to pI s is accomplished in the first dimension by CIEF and then separation by CNGSE according to differences in size follows in the second orthogonal dimension. The coupling of the two techniques was made possible via a novel interface device, dialysis hollow-fiber membranes. The promising marriage of this two separation techniques is attributed to the ease of fabrication, speed of mass delivering and convenience of column switching of the interface device, as well as orthogonal principle of CIEF–CNGSE. This 2D separation platform is of interesting and valuable for the instrumental proteomics. A multidimensional system involving more than 2D CE could be achieved with this dialysis hollow-fiber interface.

It is worthy of to be recognized one primary limitation of this 2D system. The orthogonal coupled CIEF–CNGSE, while possessing higher resolving power and overall peak capacity than 1D, provides very little information concerning the identity of the separated component. While Clarke et al. [39] have developed an simple, robust and efficient micro-electroelution technique for the coupling of SDS-containing gels with MALDI–TOF–MS analysis of proteins, at present in our group, no way could make mass spectrometry directly coupled to the 2D system to identify the separated effluents due to the using SDS in the system. The objective of the study mainly proposed and demonstrated an interesting, higher resolving power and overall peak capacity 2D CE platform. There is still a lot of further research work, such as, optimization of experimental conditions, reproducibility, and on-line identification of the effluents etc., to realize the potential applicability of the 2D CIEF–CNGSE system in our future deeper study.

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