Development of New Capillary Electrophoresis-Based Techniques for Protein Analysis

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With the sequencing of human genome almost complete, the analysis of proteome is rather difficult since in human cells there are around 200 000 proteins which are expressed at any time at different levels of concentration with a wide dynamic range. In addition, a given proteome is not static but dynamic being defined by a combination of the cell’s genome environment and even its history. To meet the higher and higher requirements from proteome analysis in our recent work we have developed some capillary electrophoresis CE-based techniques for the analysis of complex protein samples.

Among the various CE modes, capillary isoelectric focusing CIEF is a powerful one that separates ampholytic substances based on their isoelectric points pIS. In CIEF commercial carrier ampholytes CAS are always consumed to establish a pH gradient which is considerably expensive and usually reduces the detection sensitivity due to CAS’ high absorbance at low wavelengths. To solve these problems recently in our group a monolithic column with immobilized pH gradient M-IPG was developed which can be repeatedly used with good stability linearity and reproducibility.

Capillary electrochromatography CEC which combines the high resolution of capillary high performance liquid chromatography chPLC and the high efficiency of CE has attracted more and more attention and showed promising in the analysis of biomolecules. Based on CEC pressurized CEC pCEC has also been proposed in which both pressure and electric field coexist offering more flexible adjustment on the retention of solutes. Recently we successfully coupled pCEC with electrospray ionization mass spectrometry ESI-MS by using the coaxial sheath liquid interface. However the detection sensitivity for protein analysis is generally poor. Therefore it is urgent to develop on-line concentration techniques of pCEC-ESI-MS.

It is well known that for proteome analysis no single chromatographic or electrophoretic method is able to separate such complex samples. Although two-dimensional electrophoresis 2D E has firmly held a central position in proteomics because of its unparalleled resolving power with up to 3 000 – 10 000 proteins visualized in a standard 2 D gel format it is manual time-consuming of poor sensitivity poor quantitation and limited dynamic range. Also it has difficulty in investigating hydrophobic proteins and proteins that occur in low copy number in the whole cell lysate. Another serious limitation is that it cannot be directly coupled with MS. All these drawbacks have undermined the prospects for 2D E as a dominant separation technique in proteomics and have stimulated the development of alternative technologies. Since 2D HPLC is generally mature and has been put on the market recently our research is focused on the development of 2D CE system.

1 Experimental

1.1 Instrumentation and reagents

The electrophoresis was performed on a set of TriSep-2000GV from Unimicro Technology Inc. Pleasanton CA USA equipped with a Data Module UV 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 375 μm o.d. were obtained from Ruiyang Chromatographic Co. Ltd. Yongnian China. Amphyole pH 3.0 – 10.0 was from BioChemika Switzerland. Cacodylic acid CACO 2-aminoo2-methyl-1β-propanediol AMPD tris hydroxymethylaminomethane Tris methyl cellulose MC and dextran M, 2 000 000 were all purchased from Sigma St. Louis MO USA. 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 treated ultrasonically for 15 min to remove bubbles prior to use. Caution should be taken in handling acrylamide and CA CO.

1.2 Preparation of soluble protein extract from antler

Fresh non-mature cartilage cut from hairy antler was frozen and dried at −40 °C and then freeze fracturing to 100 μm sized particles. The particles were dissolved in acetic acid buffer for 5 h at 4 °C and filtered with filter paper. Finally the filtrate was freeze drying to powder at −40 °C and stored in a refrigerator for analysis. Sample solution for CE was prepared by dissolving the powder in the buffer containing 50 mmol/L Tris HCl pH 6.8 1 mmol/L ethylene diamine tetraacetic acid EDTA and 5 mmol/L NaCl followed by sonication below 4 °C and filtration through 0.45 μm membrane. The solution was stored in the dialyzer nominal relative molecular mass cutoff 8 000 spun at 15 000 r/min 4 500 g desalted and concentrated at 4 °C until the total protein concentration determined by the Lawry method around 2.0 g/L. The sample solution of antler extract was prepared by dissolving the concentrated solution in relevant sample buffer.

1.3 Interface fabrication

As illustrated in Fig. 1-a two segments of capillaries were carefully inserted into a 10 mm length of self-prepared hollow fiber nominal relative molecular mass cutoff 10 000 and the gap between the two capillaries was 0.25 mm. Epoxy was then applied around the outside of the hollow fiber/capillary boundaries. After the epoxy was dried the connected capillaries were inserted through holes pierced on both sides of a plastic micro-centrifuge vial of volume about 2.0 mL with epoxy applied around the outsides of the holes/capillaries. The above junction housed in the micro-centrifuge vial was employed for the interface and the two capillaries were severed as the first and the second dimension separation columns respectively.

1.4 Configuration of 2D CIEF-capillary non-gel sieving electrophoresis CNGSE system

As shown in Fig. 1-b the first dimensional CIEF capillary 25 cm and the second dimensional CNGSE capillary 20 cm were connected by an interface. The inlet of the CIEF capillary was inserted into the inlet reservoir filled with 0.1 mol/L acetic acid solution. The outlet of the CNGSE capillary was passed through a UV detector and then inserted into the outlet reservoir filled with dextran solution buffer. The detector was placed 8 cm from the outlet reservoir. One platinum wire was inserted into the micro-centrifuge vial and served as the cathode common electrode and another two platinum wires were grounded and inserted into the inlet reservoir anode and outlet reservoir anode to provide the necessary electrical connections.

1.5 2D CIEF-CNGSE separation of proteins

Prior to perform 2D CE separation the hollow fiber junction and the micro-centrifuge vial

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![Fig. 1 Schematic diagrams of the dialysis hollow fiber membrane interface for 2D CE a and the configuration of the 2D CIEF-CNGSE system using a dialysis interface b](image-url)
housing the junction were initially filled with 1.0% v/v NH₄OH. The protein sample dissolved in the CIEF buffer was hydrodynamically introduced into CIEF capillary previously conditioned with 5 g/L methylcellulose overnight. CNGSE capillary coated with linear polyacrylamide LPAA was initially filled with 100 g/L dextran solution buffer by a syringe pump. The two capillaries were then connected. All the procedures should be operated cautiously to avoid bubbles formed in the separation channel. A high-voltage power supply with three electrodes was employed for providing the sequences of electric voltages during the 2D CE separation Table 1. Focusing was performed at a constant voltage of +10.5 kV ~ 400 V/cm over the entire CIEF capillary. Electroosmotic flow EOF mobilization was used to transfer the focused protein zones towards the interface. The current decreased continuously during the protein focusing. When the current reduced to ~10% of the original value and nearly remained constant the focusing was considered to be complete. The valve attached to the micro-centrifuge vial was opened and 50 mmol/L AMPD-CACO₃ pH 8.5 with 1.0% v/v sodium dodecyl sulfate SDS buffer was pumped into the micro-centrifuge by an HPLC pump Unimicro Technologies USA at about 1.5 mL/min. The high voltage was applied upon the first dimension for additional 2.0 min and then temporarily shut off. A high voltage ~11.5 kV ~550 V/cm was then applied over the second dimensional capillary for achieving CNGSE separation. The transferred proteins combined with SDS at the interface were further resolved in the second dimensional separation. The result was monitored at 280 nm. Focused zones were repeatedly transferred into the interface transfer time 1.5 min followed by CNGSE separation.

Table 1 Sequence of voltages applied during 2D separation

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Inlet reservoir</th>
<th>Interface reservoir</th>
<th>Outlet reservoir</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1 CIEF</td>
<td>point A</td>
<td>point B</td>
<td>point C</td>
</tr>
<tr>
<td>Step 2 Injection</td>
<td>ground</td>
<td>~10.5 kV</td>
<td>off</td>
</tr>
<tr>
<td>Step 3 CNGSE</td>
<td>off</td>
<td>~11.5 kV</td>
<td>ground</td>
</tr>
</tbody>
</table>

2 Results and discussion

2.1 Repeatedly usable monolithic capillary column with immobilized pH gradient

Glycidyl methacrylate GMA and ethylene dimethacrylate EDMA were used to synthesize a monolithic capillary column containing reactive epoxy groups. Glutaraldehyde was introduced and linked to the monolith after a process of amination. An aqueous solution of CAS ampholine was focused in such a polymer column. The primary amino groups of CAS reacted with glutaraldehyde along the capillary. CAS was immobilized at different positions in the column according to their pI resulting in an M-IPG. Due to the covalent attachment of the CAS to the monolith such an M-IPG column can be used repeatedly.

A practical sample was used to compare focusing in M-IPG. Velvet antler Cerus elaphus L. is an important substance in traditional Chinese medicines. It is considered as a tonic or healthy food in Asia. For protein analysis precipitation is always the result in the basic end of the capillary. To avoid this troublesome consequence a high concentration of urea 5 mmol/L was added to the buffer. All components were transferred by EOF. In M-IPG some basic components passed the detection window before focusing ~2 min while other components with lower pI eluted after replacing the catholyte ~13 min and ~24 min. The total separation procedure lasted less than 30 min with good stability linearity and reproducibility.

2.2 On-line concentration for pCEC coupled with ESI-MS by using the coaxial sheath liquid interface

Pressurized capillary electrochromatography a technique that applies both pressure and electric field to a capillary column offers more optimizable parameters to obtain successful separation. For practical samples the concentration of the protein is generally low and could hardly be analyzed directly by pCEC-MS. In order to improve the detection sensitivity on-line concentration by the chromatographic zone-sharpening effect and field-enhance sample-stacking effect have been developed. Parameters that affect the separation and detection such as pH and the concentration of the electrolyte in the mobile phase voltage and concentration time have been studied systematically.

With 70% v/v acetonitrile in 75 mmol/L formic acid-ammonium acetate at pH 3.4 as the mobile phase 3 min and 18 kV as the optimum concentration time and concentration voltage 0.5% v/v formic acid in methanol-water
30: 70 v/v as the sheath-liquid flow the analysis of a mixture of modified proteins at low concentration was achieved to prove the capability of our proposed techniques. Here three proteins were successfully separated by pCEC-ESI-MS with the detection limit improved by 20- to 100-fold. In addition a mixture of insulin and its modified product at the concentration of 20 mg/L was analyzed and the feasibility of such an online concentration method for pCEC-ESI-MS was illustrated.

2.3 On-line combination of 2D CIEF-CNGSE for proteins using a hollow fiber membrane interface

2.3.1 Characteristics and considerations of the dialysis hollow fiber membrane interface

Dialysis hollow fiber membrane which possesses semi-permeable properties controlled by the nominal relative molecular mass cutoff is widely used for desalting adjusting ionic strength and sample preparation. In our experiment dialysis hollow fiber was immersed in the micro-centrifuge vial containing buffer solution. By virtue of selective permeability external buffer ions from the reservoir could diffuse through the membrane and mixed with the running buffer while analytes of large molecule were prevented from “escaping” through the membrane channel. Furthermore the dialysis junction provided the necessary electrical connection across the dialysis membrane for defining the electric fields needed for CE separation. Therefore dialysis hollow fibers as interface are very suitable for 2D CE system.

The way to connect hollow fiber with capillaries is an essential factor to reduce dead volume across the interface. To deduce the dead volume at the interface junction self-prepared hollow fiber was used to connect the capillaries.

2.3.2 Driving force in the 2D system

In 2D CIEF-CNGSE separation system CIEF was executed by a one-step method combining the focusing and mobilization. Such a method has great advantages over traditional two-step CIEF method and proved to be more compatible with current commercial CE instruments for on-line 2D CE system. Since uncoated fused silica capillaries were used for CIEF polymers such as methyl-cellulose were necessary for preconditioning to reduce EOF and prevent proteins from being adsorbed onto the capillary wall. For preconditioning the capillary was flushed with 5 g/L methyl-cellulose solution overnight to obtain good stability and resolution.

In the second dimensional separation CNGSE small molecules such as SDS were hydrophobically bound to the proteins after passing through the hollow fiber. In the electric field negatively charged proteins migrated towards the anode outlet reservoir and were further separated according to their sizes.

2.3.3 Injection time and CNGSE speed

In 2D separation it usually requires high speed in the second dimensional separation. Although high separation voltage is helpful to speed up the separation in CNGSE due to the limitation of Joule heating it had to be limited below 600 V/cm. Another way is to shorten the capillary length at the cost of losing resolution. In this study the typical CNGSE separation of protein markers with M_r ranging from 14000 to 97000 was completed in 5 min with an effective column length of 12 cm.

2.3.4 Applications

To demonstrate the applicability of the proposed 2D system complex mixtures of proteins extracted from antler were separated by CIEF and 2D CIEF-CNGSE. The transfer time of injection effluent to the interface was set at 1.5 min and the results are shown in Fig. 2 and Fig. 3 individually which could demonstrate the higher resolving power and overall peak capacity of 2D-CE than those of the single separation mode.

![Fig. 2 CIEF separation of soluble proteins extracted from antler](image)

Experimental conditions capillary 50 μm i. d. × 375 μm o. d. 32 cm total length 25 cm from the anode to detection window pre-conditioned with 5 g/L methyl cellulose anolyte 0.1 mol/L HAc catholyte 1.0% v/v NH₃·H₂O electric field strength 400 V/cm detection UV 280 nm sample protein extract of antler dissolved in 50 mmol/L Tris-HCl pH 6.8 2.0% v/v amphotolyte pH 3–10 and 1 mol/L ethylene glycol.
Fig. 3 Separation of soluble proteins extracted from antler in 2D CIEF-CNGSE

Experimental conditions: CIEF preconditioned capillary, 25 cm CNGSE capillary coated with linear polyacrylamide effective length 12 cm, detection UV 280 nm. Other conditions are the same as those in Fig. 2 for details see CIEF-CNGSE separation of proteins in the Experimental section.

References

6. Liang Zhen Zhang Lihua Yan Chao Zhang Weibing Zhang Yukui. Submitted to Electrophoresis

Biographical Sketch

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Dr. ZHANG Lihua obtained her bachelor degree of science in 1995 from Jilin University China. Then she pursued her Ph. D. in Dalian Institute of Chemical Physics DICP Chinese Academy of Sciences CAS. During this period she visited GSF National Environment and Health Research Center Germany for cooperative research. In 2000 she obtained her Ph. D. degree from DICP CAS. After that she went on her postdoctoral research in Department of Medicinal Chemistry Faculty of Pharmaceutical Sciences the University of Tokushima Japan. In 2003 she went back to China and was promoted to an associate professor of DICP CAS.

Her research has been focused on the following aspects:
1. Theoretical study and development of new technologies of capillary electrophromatography CEC. By the self-designed capillary liquid chromatography-CEC-pressureurized CEC instrument systematic study on the retention mechanism of neutral solutes in the above mentioned three modes has been carried out by linear solvation energy relationship method. In addition new technologies such as gradient elution mixed-packing column fast analysis on-line concentration and monolithic column of CEC have been developed.
2. Establishment of CE-MS method for the analysis of proteins related to environmental pollution which could offer high accuracy high sensitivity and high speed for environmental samples and food analysis.
3. Microchip and its application in DNA analysis. Stepwise polymer concentration gradient separation has been developed and the advantages in the analysis of complex DNA samples were shown. In addition fast DNA analytical system has been proposed including DNA extraction amplification on-chip enzymatic digestion and electrophoresis-based separation.

Up till now Dr. ZHANG has published over 70 papers joined the writing of 7 books and applied for 5 patents. In 2004 she was selected to join 100 Persons Project of CAS.