

Directions

in Discovery

This month's "Directions in Discovery" column discusses current work in the use of capillary isoelectric focusing and mass spectrometry (CIEF-MS) as a multidimensional separation technique for proteomic studies.

Coupling Capillary Isoelectric Focusing with Mass Spectrometry

Identification of proteins involved in a disease state and elucidation of their structure and function is essential to the creation of appropriate therapeutic agents. For this reason, the emerging science of proteomics has become an important element in drug discovery. Expression proteomics is a subset of this field that seeks to identify specific proteins whose regulation is modified in a disease state or in response to drug treatment. To be successful, a protein expression study must be able to detect quantitative differences in expression of one or several proteins against the background of thousands of proteins found in a cell or tissue. To further complicate the problem, the protein of interest is likely to be present in only a few copies in a sample dominated by high-abundance proteins. Not surprisingly, separation techniques with high-resolution and high-detection sensitivity are key to expression proteomics.

Given the complexity of cell extracts, no single separation technique has the horsepower to sufficiently resolve all cellular proteins. Rather, two or more separation modes must be combined in a multidimensional separation system.

The classical approach is two-dimensional (2-D) gel electrophoresis (1), in which proteins are resolved on the basis of charge in the first dimension (isoelectric focusing [IEF]), and each resolved species is separated further on the basis of mass in the second dimension (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Although 2-D gel electrophoresis can resolve as many as several thousand proteins, it is time-consuming, labor-intensive, provides only semiquantitative information, and is not sensitive enough to display low-abundance species (2). For this reason, several proteomics labs have turned to multidimensional liquid chromatography (LC) as an alternative technique in which a first-dimension ion exchange separation is coupled to a second-dimension reversed-phase

separation (3). Multidimensional LC approaches using on-line column switching require complex hardware configurations and can be very time-consuming. On the other hand, multidimensional LC using direct coupled-column configurations with continuous elution simplifies the multidimensional LC experiment but eliminates the ability to monitor the first-dimension separation. Also, the first-dimension conditions that are compatible with direct coupling to the second dimension might not provide satisfactory resolution and recovery (4). In all cases, multidimensional LC with an ion-exchange first dimension does not provide isoelectric point information that can be valuable in protein database searches.

Capillary isoelectric focusing (CIEF) is fully analogous to the first-dimension IEF separation of 2-D gel electrophoresis. Proteins become focused at their isoelectric points within a stable pH gradient formed by a mixture of ampholytes. However, in contrast to IEF performed in gels or immobilized pH gradient strips, CIEF is performed in free solution without the need for a gel or support matrix. Once the focusing process is complete, the contents of the capillary can be mobilized through a concentration-sensitive detector to obtain qualitative and quantitative information on the resolved proteins. In most cases, a small segment of the capillary near the outlet end serves as the detection flow cell or "window." The mobilization process provides an opportunity to transport the capillary contents sequentially to a second detector. A mass spectrometer is the obvious choice for this second detector because the combined selectivity is similar to that of 2-D gel electrophoresis: charge-based separation in the first (CIEF) dimension and mass-based separation in the second (mass spectrometry [MS]) dimension. In contrast to capillary electrophoresis (CE) or CE-MS, the entire capillary is filled with the sample-ampholyte solution in CIEF, providing at

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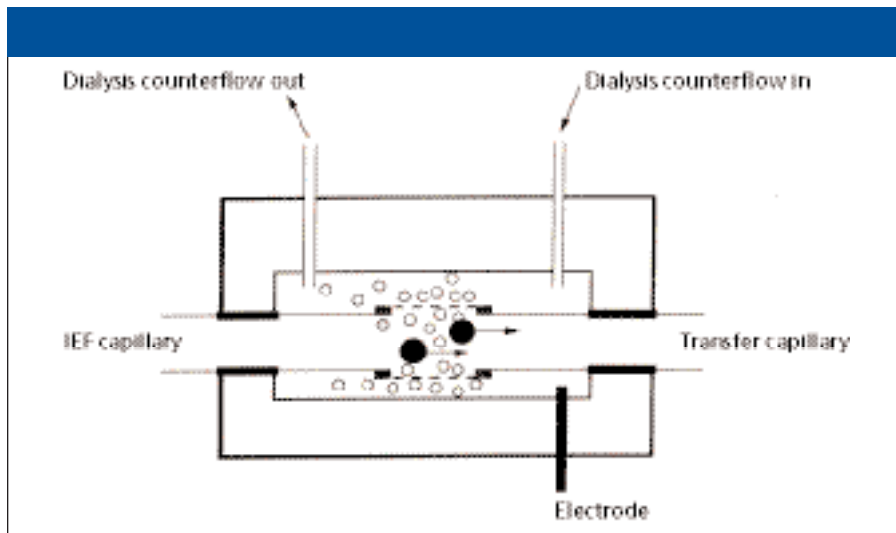


Figure 1: Hollow fiber-based microdialysis device for on-line ampholyte removal in a CIEF-MS system. The dialysis counterflow is delivered by syringe pump. The transfer capillary provides the conduit from the microdialysis to the electrospray ionization source. (Reproduced from reference 13 with permission from Elsevier Science B.V., New York, New York.)

least a 10-fold increase in sensitivity. It is no surprise that several groups are exploring the feasibility of CIEF-MS as a 2-D separation technique for proteomic studies.

Early CIEF-MS Approaches

Coupling CIEF with MS presents several challenges. A common method of perform-

ing CIEF is to focus the proteins in one step, followed by mobilization as a second step. Mobilization can be achieved by changing the composition of the outlet electrolyte (usually the catholyte; this is variously termed chemical mobilization, electrophoretic mobilization, or ion-addition mobilization). Alternatively, mobilization

can be accomplished by displacing the contents of the capillary by the application of a force (pressure at the capillary inlet, vacuum at the outlet, or by gravity).

Successful coupling of CIEF with electrospray ionization (ESI) MS requires a means of performing focusing and mobilization with the capillary outlet interfaced with the electrospray ionization system. Initial attempts by Lee and colleagues (5) employed two-step CIEF with the catholyte reservoir placed in the ionization source during focusing. Upon completion of focusing, the reservoir was removed and mobilization was initiated by infusion of a coaxial sheath flow using water-methanol-acetic acid as the sheath liquid. The sheath flow also served as the electric connection for the ionization source. In a later modification of this system, gravity-assisted mobilization was used to compensate for moving-boundary effects caused by electromigration of sheath liquid ions into the capillary (6). This was accomplished by raising the inlet end of the capillary 8 cm relative to the height of the electrospray needle. This CIEF-MS interface was coupled to a triple-quadrupole MS system, and protein masses were determined by deconvolution of the multiply charged



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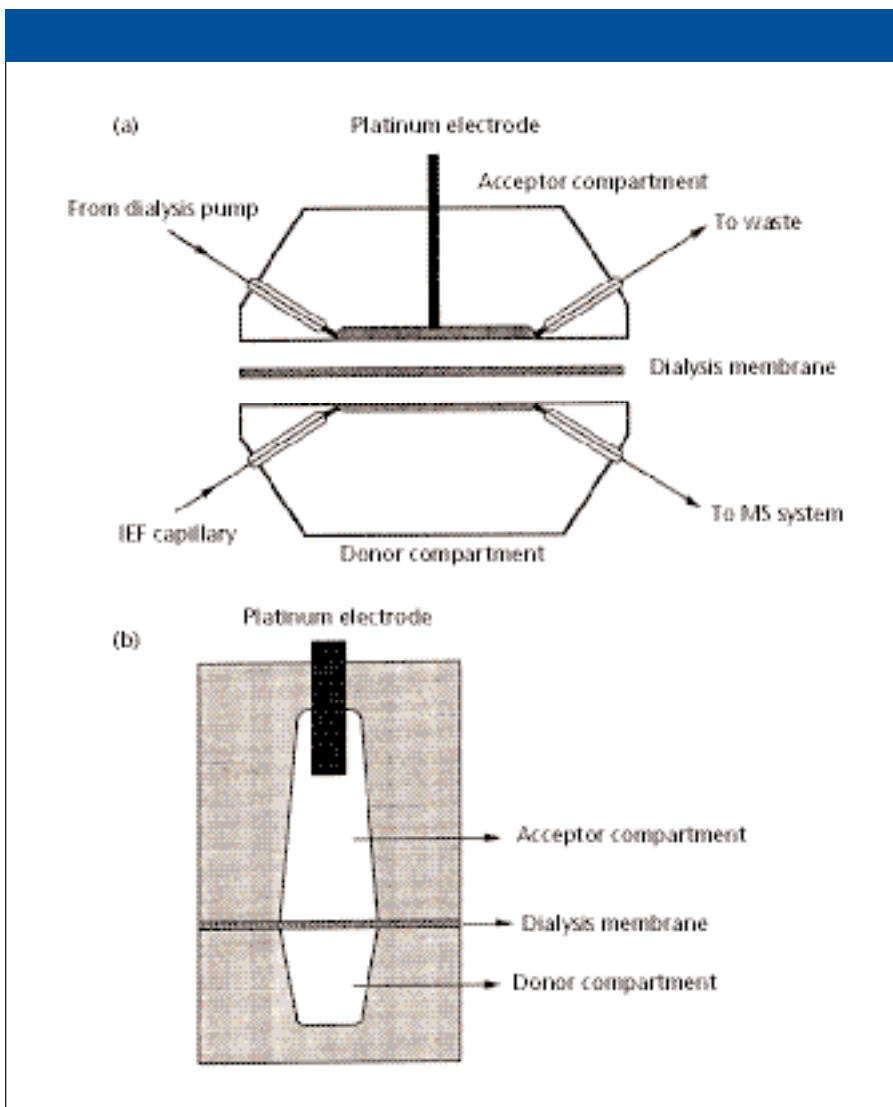


Figure 2: Membrane-based microdialysis device for on-line ampholyte removal in a CIEF-MS system. (a) Sideview and (b) cross section of the microdialysis device. (Reprinted from reference 14 with permission from John Wiley & Sons, Hoboken, New Jersey.)

molecular ion envelopes. This system was applied to the analysis of a lysate of *E. coli*, expressing a recombinant fusion protein of glutathione-S-transferase and striped bass growth hormone (7). A 2-D display of mass versus isoelectric point presented an array of 104 protein “spots.” Other applications reported by this group included analysis of glycoforms and phosphorylated albumins (8). The same interface was coupled to a time-of-flight mass spectrometer for analysis of model proteins (9), and to a Fourier-transform ion cyclotron resonance mass spectrometer for analysis of a red blood cell lysate (10) and an *E. coli* lysate (11). In this last application, the bacterial culture was grown in media depleted of rare isotopes (for example, ^{13}C and ^{15}N). Isotope depletion increased the accuracy, sensitivity, dynamic range, and detection limit of mass measurements.

Clarke and Naylor (12) described a two-step CIEF system interfaced to a double-sector MS system in which only the composition of the sheath liquid was changed to effect mobilization. During focusing, the sheath liquid was methanolic ammonium hydroxide delivered at a reduced flow rate of $1\ \mu\text{L}/\text{min}$. In this step, the outlet of the separation capillary was withdrawn into the electrospray needle. This enabled formation of a static “hanging drop” of catholyte at the end of the separation capillary. At the completion of focusing, the capillary outlet was repositioned to extend $0.5\ \text{mm}$ outside the electrospray needle. The sheath liquid was changed to anolyte (methanolic acetic acid) delivered at a flow rate of $3\ \mu\text{L}/\text{min}$ to initiate electrophoretic mobilization into the electrospray ionization source. This CIEF-MS system was applied to the separation of human hemo-

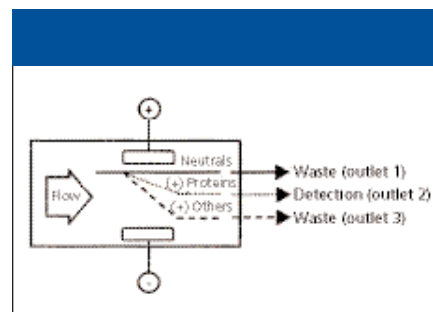


Figure 3: Trajectories of sample components in a chip-based free-flow electrophoresis cell for ampholyte removal. (Reproduced from reference 16 with permission from John Wiley & Sons.)

globin variants in a blood-cell lysate, proteins in cerebrospinal fluid, and glycosylated hemoglobins in blood samples obtained from normal and diabetic patients. To minimize interferences in the focusing process caused by the high salt content of body fluids, the samples were subjected to on-line desalting using a $0\text{--}10\ \text{kV}$ voltage gradient applied during the beginning of the focusing step.

Solving the Ampholyte Problem

The approaches just described have two limitations. First, they require operator manipulation in the transition from focusing and mobilization. Second, the presence of ampholytes compromises the performance of MS detection because they can contaminate the ionization source, destabilize the electrospray, suppress analyte signal intensity, and degrade mass resolution (5,7). Also, protein-analyte interaction shifts the multiply-charged molecular ion envelope to lower charge states (7).

To streamline the CIEF-MS system and resolve the problem of ampholyte interference, a variety of on-line ampholyte removal systems have been integrated into the CIEF-MS interface. Lamoree and colleagues (13) devised an on-line microdialysis system between the separation capillary and a transfer capillary connected to an ESI quadrupole MS system. The microdialysis system consisted of hollow fiber dialysis tubing sealed in a chamber infused with acetic acid, which served as the catholyte (Figure 1). The volume of the dialysis chamber was $25\ \mu\text{L}$. After focusing, the contents of the separation capillary were mobilized through the microdialysis device using $25\ \text{mbar}$ pressure, and ampholytes were removed by dialysis across the microdialysis tubing. The molecular mass cutoff of the dialysis tubing permitted

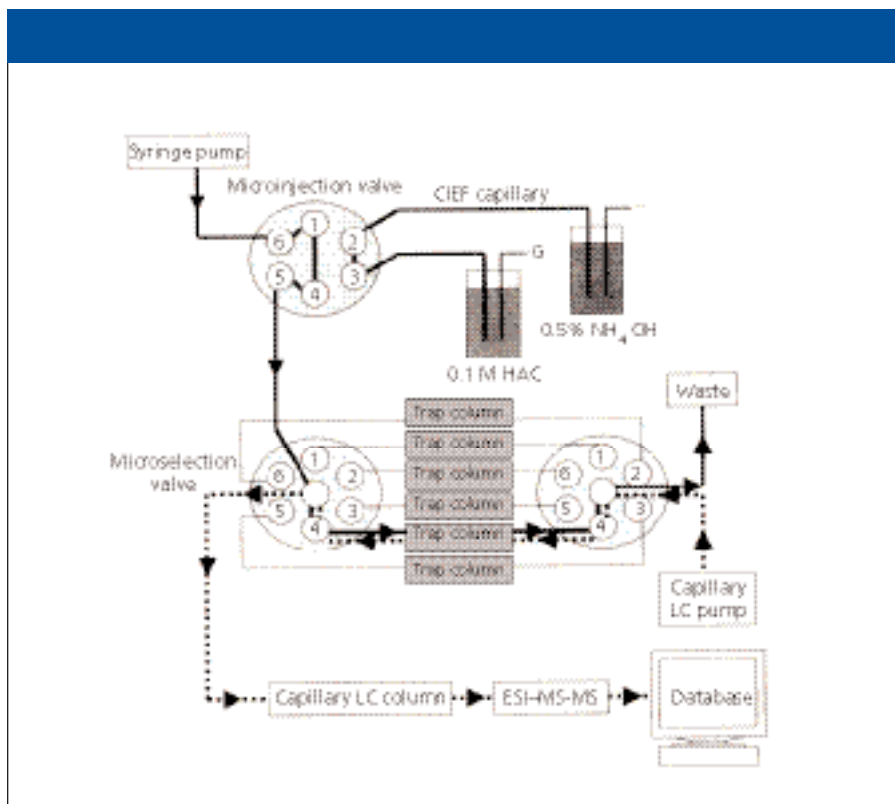


Figure 4: Schematic of on-line integration of CIEF with capillary reversed-phase HPLC as a concentrating and multidimensional separation platform. Solid and dashed lines represent the flow paths for the loading of CIEF fractions and the injection of fractions onto a capillary reversed-phase HPLC column, respectively. (Reproduced from reference 17 with permission from the American Chemical Society, Washington, DC.)

passage of ampholytes while proteinaceous analytes were retained within. A methanol-acetic acid sheath liquid provided electrical contact for the ESI source. Separations of three model proteins were demonstrated, and the efficiency of ampholyte removal was found to be dependent upon the flow rate of the dialysis liquid. A later modification of this system replaced the hollow fiber dialysis tubing with a flat dialysis membrane with a molecular mass cutoff of 5 kDa (14). In this miniaturized microdialysis device, the inlet separation capillary and outlet transfer capillary were connected to a donor compartment with a volume of 150 nL (Figure 2). The dialysis liquid (2% acetic acid) was pumped through the acceptor compartment at a flow rate of 5 $\mu\text{L}/\text{min}$. One limitation of this microdialysis device was band broadening introduced by the geometry of the donor chamber. In addition, analyte adsorption and electroosmotic flow associated with the plexiglass surface of the donor chamber contributed to band spreading.

Yang and colleagues (15) described a CIEF-ESI MS system using an in-line

microdialysis junction but without a sheath flow at the electrospray interface. The use of a coaxial sheath flow to establish electrical contact and mobilize analytes into the ionization source compromises

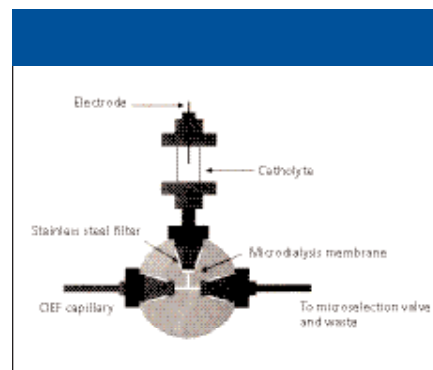


Figure 5: Diagram of a microdialysis membrane-based cathodic cell for CIEF-reversed-phase LC-MS. (Reproduced from reference 18 with permission from the American Chemical Society.)

sensitivity by diluting the analytes and introducing competing ions. The limitations of a conventional ESI interface with sheath flow were eliminated by the use of a microelectrospray interface with a tapered-tip emitter capillary. The emitter capillary was connected closely to the separation capillary within a section of 10-kDa-cutoff dialysis tubing, which was immersed in 10% acetic acid. The microdialysis liquid served as the analyte solution for CIEF and provided a proton source for positive electrospray ionization. A platinum electrode at the microdialysis junction served as the anode for the CIEF separation and the electrical connection for the electrospray. Mobilization was accomplished by applying 0.2 psi gas pressure at the capillary inlet. This design was compared with a conventional CIEF-ESI MS system

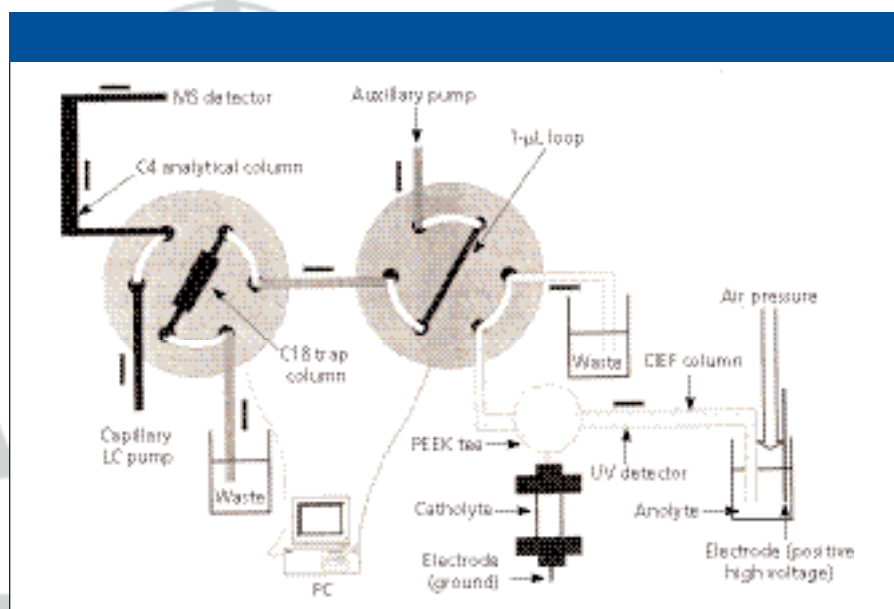


Figure 6: Diagram of a CIEF-reversed-phase LC-MS system incorporating the microdialysis cell. (Reproduced from reference 18 with permission from the American Chemical Society.)

using coaxial sheath flow, and a 10-fold increase in signal and reduced background noise were demonstrated for the microdialysis junction–microelectrospray system.

An alternative approach to on-line ampholyte removal was developed by Chartogne and colleagues (16). These researchers employed a chip-based free-flow electrophoresis device to remove ampholytes prior to introduction of separated proteins into an ESI MS. Free-flow electrophoresis historically has been used as a preparative technique for protein purification. In free-flow electrophoresis, samples are injected into a thin film of buffer flowing between two closely spaced plates. An electric field is applied orthogonally to the direction of buffer flow, which causes deflection of charged sample components, with the angle of deflection dependent upon the electrophoretic mobility of the component. The free-flow electrophoresis device designed for ampholyte removal consisted of a 0.1 mm × 10 mm × 25 mm chamber with the free-flow electrophoresis carrier liquid (1% acetic acid) introduced at the inlet end and collected by three outlet capillaries at the opposite end (Figure 3). The sample stream from the outlet of the CIEF capillary was introduced in a perpendicular direction to carrier flow through the top of the chamber. A transverse electrical field was introduced across the chamber using electrodes at the lateral margins of the device. As focused proteins and ampholytes emerged from the separation capillary into the acid free-flow electrophoresis carrier liquid (which also served as catholyte for the CIEF system), both proteins and ampholytes acquired charge and migrated laterally as they were transported down the chamber. With optimized conditions, analyte proteins were transported to the central detection capillary and to the mass spectrometer. Neutral species and low-mass ampholytes exited the chamber through the two flanking waste capillaries.

A key element in optimizing performance of the CIEF–free-flow electrophoresis–MS system is the flow rate of the carrier liquid. The carrier flow introduces backpressure at the capillary outlet, and this pressure must be balanced carefully against the injection pressure at the capillary inlet to achieve simultaneous focusing and mobilization of proteins. The carrier flow rate also impacts the deflection amplitude of analytes and therefore their capture by the detection outlet of the free-flow electrophoresis. One consequence of an

electrophoretic mobility component to the transport of proteins through the free-flow electrophoresis chamber is that overall mobilization time no longer correlates with isoelectric point.

Adding a New Dimension

Insertion of a chromatographic separation after CIEF provides not only a means of removing ampholytes but of obtaining additional resolution before MS analysis. Reversed-phase HPLC is the obvious

choice, because the separation mechanism is orthogonal to that of CIEF, and the eluents are fully compatible with electrospray ionization. The hydrophilic and highly charged ampholytes are poorly retained on reversed-phase columns and can be pumped to waste before elution of polypeptides. The additional resolution provided by gradient reversed-phase HPLC also reduces the chance that comobilizing proteins from the CIEF separation will cause suppression of ionization in the elec-



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troscopy process.

Chen and colleagues (17) devised a CIEF-reversed-phase LC-MS system in which focused proteins were hydrodynamically mobilized onto a series of reversed-phase trapping columns. In this fashion, the contents of the focused CIEF capillary were fractionated into a series of 12 segments, each of which was "parked" on a trapping column while ampholytes were eluted to waste (Figure 4). The parked fractions were then separated by capillary or nano reversed-phase HPLC using gradient elution, and were detected by ESI MS-MS. This system was used to analyze tryptic peptides of the soluble fraction of a yeast lysate. Data from MS and MS-MS were used to identify 1894 unique peptides and from these, 1132 individual proteins (representing approximately 20% of the yeast proteome). The codon adaptation index values of the identified proteins were used to assess their expression levels. The codon adaptation index value of a protein is believed to be a measure of its abundance. Because 83% of yeast open reading frames have codon adaptation index values of 0.2 or less, most yeast proteins are low in abundance. The distribution of codon adaptation index values for the proteins identified in the CIEF-reversed-phase LC-MS experiment closely matched that of the entire yeast proteome, suggesting that this multidimensional approach can detect low-abundance proteins and can accurately represent the yeast proteome.

Real-time, on-line coupling of CIEF to reversed-phase LC-MS was achieved by Zhou and Johnston (18) by incorporating a microdialysis membrane-based cathodic cell between the CIEF separation capillary and the capillary reversed-phase LC system. The cell consisted of a PEEK tee with the CIEF separation capillary connected colinearly with a transfer capillary, which was in turn connected to a microselector valve (Figure 5). The catholyte cell and cathodic electrode, connected to the perpendicular port of the tee, were isolated from the separation capillary by a 3500 MW cutoff microdialysis membrane. The microselection valve was connected to a reversed-phase trapping column and the capillary LC-MS system (Figure 6). Following a 4-min focusing step, the CIEF capillary contents were sequentially pressure-mobilized to the LC-MS system in a 10-min "stop and go" regime. During the "go" step, pressure applied to the inlet of the separation capillary transported a segment of the pH gradient to the sample

loop of the microselection valve. Following the "go" cycle, mobilization pressure was turned off to initiate the "stop" cycle, while focusing voltage was maintained. During the "stop" cycle, the captured isoelectric point segment was transferred from the microselection valve to a reversed-phase trapping column by an auxiliary pump. After elution of ampholytes to waste for 2 min, the microselection valve was switched on-line with the capillary LC-MS system and captured proteins were separated by a 7-min gradient elution program. A sequence of seven "stop and go" segments was used to demonstrate CIEF-reversed-phase LC-MS fractionation of an eight-component mixture of model proteins within 2 h. An advantage of this 2-D system is that it was implemented using a commercial CE instrument.

Summary

Coupling of CIEF with MS is a promising tool for analysis of complex protein mixtures. The advantages of this approach include a high peak capacity in the IEF separation (resolution better than 0.02 isoelectric point units), high sensitivity due to the large amounts of sample injected into the capillary combined with the focusing effect, and the ability to couple directly to the mass spectrometer. The increased sample capacity in the first dimension provides a wider dynamic range for MS analysis. In contrast to multidimensional HPLC separations, the use of IEF as the first-dimension separation provides isoelectric point information for protein identification in database searches. The analysis of intact proteins in CIEF-MS makes the technique appealing for "top-down" proteomic studies.

There are two major limitations to CIEF-MS. The first is the need to remove ampholytes prior to introduction of the pH gradient into the MS to prevent ion suppression and fouling of the ionization source, and the second is ion suppression of low-abundance proteins caused by comigrating high-abundance proteins. The first limitation has been addressed by incorporating on-line dialysis systems to remove ampholytes; both membrane-based microdialysis systems and free-flow electrophoresis devices have been employed. The second limitation has been minimized by coupling the CIEF separation to capillary reversed-phase HPLC-MS. This additional separation dimension also provides the opportunity to remove ampholytes through the use of on-line trapping

columns. To date, none of these designs has been commercialized, but the recent advances in microfluidics and chip-based analyzers might lead to miniaturized instruments embodying these concepts.

References

- (1) P.H. O'Farrell, *J. Biol. Chem.* **250**, 4007-4021 (1975).
- (2) T. Wehr, *LCGC* **19**, 702-711 (2001).
- (3) T. Wehr, *LCGC* **20**, 954-962 (2002).
- (4) K.M. Gooding, B. Hodge, and R.I.K. Julian, Jr., *LCGC* **22**, 354-361 (2004).
- (5) Q. Tang, A.K. Harrata, and C.S. Lee, *Anal. Chem.* **67**, 3515-3519 (1995).
- (6) L. Yang, Q. Tang, A.K. Harrata, and C.S. Lee, *Anal. Biochem.* **243**, 140-149 (1996).
- (7) Q. Tang, A.K. Harrata, and C.S. Lee, *Anal. Chem.* **69**, 3177-3182 (1997).
- (8) J. Wei, L. Yang, A.K. Harrata, and C.S. Lee, *Electrophoresis* **19**, 2356-2360 (1998).
- (9) J.C. Severs, S.A. Hofstadler, Z. Zhao, R.T. Senh, and R.D. Smith, *Electrophoresis* **17**, 1808-1817 (1996).
- (10) P.K. Jensen, L. Paa-Toli, G.A. Anderson, J.A. Horner, M.S. Lipton, J.E. Bruce, and R.D. Smith, *Anal. Chem.* **71**, 2076-2084 (1999).
- (11) J. Wei, C.S. Lee, I.M. Lazar, and M.L. Lee, *J. Microcol. Sep.* **11**, 193-197 (1999).
- (12) N.J. Clarke and S. Naylor, *Biomed. Chromatogr.* **16**, 287-297 (2002).
- (13) M.H. Lamoree, U.R. Tjaden, and J. van der Greef, *J. Chromatogr., A* **777**, 31-39 (1997).
- (14) M.H. Lamoree, R.A. van der Hoeven, U.R. Tjaden, and J. van der Greef, *J. Mass Spectrom.* **33**, 453-460 (1998).
- (15) L. Yang, C.S. Lee, S.A. Hofstadler, and R.D. Smith, *Anal. Chem.* **70**, 4945-4950 (1998).
- (16) A. Chartogne, U.R. Tjaden, and J. van der Greef, *Rapid. Commun. Mass Spectrom.* **14**, 1269-1274 (2000).
- (17) J. Chen, B.M. Balgley, D.L. DeVoe, and C.S. Lee, *Anal. Chem.* **75**, 3145-3152 (2003).
- (18) F. Zhou and M.V. Johnston, *Anal. Chem.* **76**, 2734-2740 (2004).

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