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Received March 13, 2010
Revised May 13, 2010
Accepted May 14, 2010

Research Article

Two-dimensional capillary electrophoresis: Capillary isoelectric focusing and capillary zone electrophoresis with laser-induced fluorescence detection

CIEF and CZE are coupled with LIF detection to create an ultrasensitive 2-D separation method for proteins. In this method, two capillaries are joined through a buffer-filled interface. Separate power supplies control the potential at the injection end of the first capillary and at the interface; the detector is held at ground potential. Proteins are labeled with the fluorogenic reagent Chromeo P503, which preserves the isoelectric point of the labeled protein. The labeled proteins were mixed with ampholytes and injected into the first-dimension capillary. A focusing step was performed with the injection end of the capillary at high pH and the interface at low pH. To mobilize components, the interface was filled with a high pH buffer, which was compatible with the second-dimension separation. A fraction was transferred to the second-dimension capillary for separation. The process of fraction transfer and second dimension separation was repeated two dozen times. The separation produced a spot capacity of 125.

Keywords:
2-D CE / CIEF / CZE

DOI 10.1002/elps.201000151

1 Introduction

Multidimensional separations can provide exquisite resolution of complex mixtures. O'Farrell reported the archetypical example in 1975, in which a complex protein sample was first subjected to separation by isoelectric focusing followed by a second-dimension separation using PAGE [1]. This 2-D separation was performed on a rectangular electrophoresis plate, and resulted in the formation of a large number of spots, whose position was related to the isoelectric point and molecular weight of the proteins within the sample.

Giddings recognized that the spot capacity of a 2-D separation equals the product of the peak capacity of the individual separations for orthogonal separation mechanisms [2]. The combination of two high efficiency separation methods can result in very high spot capacity; IEF-PAGE can provide a spot capacity of over 10 000 [3].

Multidimensional chromatographic separations have also been developed for the analysis of complex mixtures of peptides produced by the proteolytic digestion of cellular homogenates [4, 5].

Detection remains an issue in the protein analysis. Classic gel electrophoresis typically employs staining technology to reveal proteins with a detection limit in the subpicomole range. MS can detect tryptic peptides in the high attomole range, although protein analysis tends to have lower sensitivity [6]. There are cases where higher sensitivity detection would be useful. As an extreme example, there is much interest in characterizing the protein content of a single cell, where the average protein abundance can be in the mid-zeptomole range [7–9].

CE coupled with LIF is an attractive alternative to the traditional proteomic techniques because it allows for analysis of small sample volumes, has high sensitivity, and can produce six or more orders of magnitude dynamic range [10, 11]. We have reported multidimensional separation systems using CE for sensitive and reproducible analysis of biological samples, including single-cell analyses [12–14]. In our first example, CZE at pH 7.5 was coupled with CZE at pH 11.1 for the 2-D separation of proteins [15]. We also reported the first coupling of capillary sieving electrophoresis, which is the capillary equivalent of PAGE, with CZE [16]. That system was employed for the high-resolution separation of a cellular homogenate prepared from *Deinococcus radiodurans*. Kraly characterized the reproducibility of that system for the analysis of proteins obtained from human biopsies [17]. The within-day migration time precision was better than 1% in both the dimensions and the LOD was in the high yoctomole (10^{-24} mol) range.

There have been a few efforts to couple CIEF with capillary sieving electrophoresis. Sheng and Pawliszyn reported a 2-D CE separation coupling MEKC in the first-dimension to CIEF using a 10-port valve interface with two loops [18]. Detection occurred on-column with whole-

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column absorbance imaging. There have been several reports of 2-D separations based on CIEF/CZE system that featured an etched porous interface to join two separations on a single capillary [19, 20]. Hydrodynamic mobilization was used to transfer plugs across the interface, and the analytes were detected on-column with a UV detector.

CIEF has had relatively limited application because of the poor sensitivity produced by absorbance detection across the narrow capillary diameter. Improved detection limits result from the use of labeling chemistry and LIF detection. Unfortunately, most fluorescent labels are incompatible with CIEF; those labels convert the cationic lysine residue into a neutral or anionic product, which generates very poor performance during IEF [21, 22]. Fortunately, a set of fluorogenic reagents has been developed by Wolfbeis that convert cationic lysine residues into cationic fluorescent products [23–24]. These reagents, the Chromeo dyes, preserve the IEF properties of proteins while providing outstanding sensitivity. We have reported three ultrasensitive CIEF separation systems coupled with LIF detection. In the first system, attomole detection limit of fluorescently labeled proteins was reported [25]. The sensitivity was limited by the background fluorescence generated by impurities found in the ampholytes. Several modifications were reported in the second system to reduce the background and improve sensitivity by an order of magnitude with concentration detection limits in the femtomolar range and mass detection limits in the zeptomole range [26]. These modifications included photobleaching the ampholytes to reduce impurities and replacing blue excitation with green, since the fluorescence generated by ampholyte impurities is more intense in the blue region of the spectrum. The most recent work employs an array of 32-capillaries for high-throughput analysis [27].

2 Materials and methods

2.1 Chemicals and materials

Unless stated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solutions were made with distilled deionized water (Barnstead Nanopure, Boston, MA, USA) and vacuum filtered through a 0.22-μm filter (Millipore, Billerica, MA, USA). Biolyte ampholytes 5–8 and Biolyte ampholytes 7–9 where purchased from Bio-Rad (Hercules, CA, USA). P503 was purchased from Active Motif (CA, USA). Uncoated and LPA-coated fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). Drummond glass capillaries used in the interface were from Drummond Scientific (Broomall, PA, USA).

2.2 Sample and ampholyte preparation

Protein samples were originally dissolved in water at a concentration of 10 mg/mL, aliquoted, and stored at −20°C. Each day, a new sample was taken from the freezer and thawed at room temperature. Proteins were then labeled with Chromeo P503 [23, 24, 28–30]. To label proteins, 5 μL of protein solution was added to 15 μL of Na_2B4O_7 buffer (10 mM, pH 9.2) and 5 μL of a 1 mM solution of Chromeo P503 dye dissolved in methanol. The labeling reaction took 15 min at room temperature and was observed to be complete when the solution color changed from blue to pink; exposure to room light was minimized to avoid photobleaching. Once labeled, 175 μL of ddH_2O was added to quench the reaction. Just before an experiment, the sample was further diluted in 5% ampholyte solution containing 3% Pharmalytes 3–10, 1% each of Pharmalytes 5–8 and Pharmalytes 7–9 in 1% Triton X-100, 0.1% Tween 20.

2.3 CE instrumentation

Our LIF detectors have been described in detail [17, 31–32]. Briefly, analytes were detected using a postcolumn sheath-flow cuvette. Fluorescence was excited by a 473-nm solid-state diode-pumped laser (Lasermate Group, Ponoma, CA, USA), collected with an M-PLAN 60×, 0.7 NA microscope objective (Universe Kogaku, Oyster Bay, NY, USA), and filtered with a 580LP long-pass filter (Omega Optical, Brattleboro, VT, USA). Light was detected by an avalanche photodiode single-photon counting module (EG&G Canada, Vaudreuil, Canada). Voltage programming and fluorescence detection were controlled by home-built LabView software. The signal was corrected to account for the dead-time response of the photodetector.

We have previously described the instrumentation and interface for 2-D CE [15–16]. A few modifications were made to couple CIEF with CZE. The two-separation capillaries, with an id of 50 μm, were aligned at a buffer-filled interface. Both capillaries were 20 cm in length and coated with either polymerized *N*-acryloylaminopropanol using the Grignard reaction or linear polyacrylamide provided by Polymicro.

Capillaries were coated using a device similar to that reported by Gao and Liu [33], using a slightly modified procedure from that reported by Gelfi [34]. Thionyl chloride was pumped through 3–4 m capillaries for 4 h at 65°C, and the capillary was then purged with N_2 for 10 min (until bubbling could be seen at the outlet). The capillary was then filled with 1 M vinylmagnesium bromide in THF, which was allowed to react at 50°C for another 4 h. The capillary was then washed for 1 h with THF at 30 psi, followed by a rinse with H_2O for another hour. A solution of 0.1% TEMED, 0.1% ammonium persulfate, and 1.5% acryloylaminopropanol was prepared by mixing degassed solutions of each component (prepared immediately prior to this step), and pumped through the capillaries at 15 psi overnight. Capillaries were then washed with H_2O for 1 h, and stored filled with water.

2.4 One-dimensional separation

One-dimensional separations were performed on 30-cm long, 50-μm id fused-silica capillary coated with polyacrylamide as described earlier [25–26]. The buffer for the CZE was 20 mM NH_4OH with 3.5 mM SDS, pH 9. Sample was electrokinetically injected at −5 kV and separated at −15 kV. Anode detection CIEF was performed in the same capillary. The capillary was cleaned before each run with 3 M HCl for 2 min and ddH_2O for 5 min. The capillary was filled with ampholyte and sample mixture. The injection end of the capillary was placed in the catholyte, 40 mM NaOH, and the detection end of the capillary was placed in the cuvette where the sheath flow was the anolyte, 10 mM H_3PO_4. After focusing was complete, the sheath flow was switched from phosphoric acid to the mobilizing agent, 20 mM NH_4OH–3.5 mM SDS. Focusing and mobilization voltage remained constant at −15 kV.

2.5 2-D separation: CIEF/CZE

In 2-D separations, the cathode end of the capillary was placed in sodium hydroxide (40 mM, pH 12) and the anode end was in the interface filled with phosphoric acid (10 mM, pH 2). Capillary 2 was filled with the zone separation buffer, NH_4OH and SDS (20 mM and 3.5 mM, pH 9). Before each experiment, the capillaries were rinsed with citric acid (100 mM) or 1% Triton X-100 for 4 min by using a syringe. The capillaries were then rinsed with ddH_2O for 5 min. Capillary 1 was filled with a mixture of 5% ampholyte solution and sample by purging the solution through the capillary for 2 min at 5 psi. While filling capillary 1 with the ampholytes, a syringe was used to manually pump the anode into the interface, which flushed the matrix from capillary 1 through the interface and to waste. Focusing voltage was held constant at 660 V/cm for 7 min, or until the current stabilized. Chemical mobilization at the anode (interface) was used for migration of the proteins by changing the interface buffer from phosphoric acid to the second-dimension buffer, NH_4OH with SDS.

After focusing, fractions were electrokinetically transferred to capillary 2 by the application of 15 kV for 10 s. Sample was separated in capillary 2 by applying field strength of 600 V/cm for 180 s. During this time, the net potential across capillary 1 was held at 0 V, preventing migration from capillary 1. These transfer and CZE separation cycles were repeated until all components from capillary 1 had been analyzed. The voltage program is illustrated in Fig. 1.

2.6 Data processing

Data were first corrected for the non-linear response of the avalanche photodiode photon counting modules [11, 35]. The corrected data were then treated with a five-point median filter to remove noise spikes from particles and convoluted with a Gaussian filter that had a 0.1-s SD. The reconstructed 2-D electropherogram was convoluted with a 2-D Gaussian filter with a 0.4 transfer SD in the IEF dimension and a 40-ms SD in the zone electrophoresis dimension.

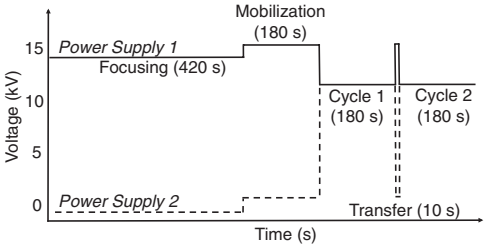

Figure 1. Voltage program for 2-D CE. Analytes were focused in the first capillary during the 420-s focusing step. After focusing, a series of mobilization and second-dimension separation cycles were performed. In these cycles, sample as transferred for 10 s and then subjected to a 180-s duration second-dimension separation.

A non-linear least-squares routine was used to fit a Gaussian surface to the spots in the 2-D electropherogram. The function used for the fit is

$$\text{Signal} = \text{Amplitude} * e^{[-0.5*(\hat{t}_{cIEF} - t_{cIEf})^2/\sigma^2_{cIEF}]} * e^{[-0.5*(\hat{t}_{CZE} - t_{CZE})^2/\sigma^2_{CZE}]}$$

where amplitude is the peak maximum, t_{cIEF} the migration time in the cIEF dimension in units of fractions transferred, $\hat{t}_{cIEF}$ the peak center in the IEF dimension, σ_{cIEF} the SD in the cIEF dimension, t_{CZE} the migration time in the CZE dimension in units of seconds, $\hat{t}_{CZE}$ the peak center in the CZE dimension, and σ_{CZE} the SD in the CZE dimension. The SDs of the Gaussian surface in the two dimensions were used to estimate spot capacity.

3 Results and discussion

3.1 One-dimensional separations of P503-labeled proteins

Two standard proteins (β-lactoglobulin (pI = 5.1) and ovalbumin (pI = 4.7)) were labeled with P503 and separated by CZE and CIEF (Fig. 2). The electropherograms for both separations are shown in Fig. 2. Zone electrophoresis shows two relatively sharp peaks while the IEF separation presents a much more complex electropherogram.

3.2 2-D CIEF/CZE separation of standard proteins spiked with a pI standard

Chemical mobilization of the components from capillary 1 across the interface to the capillary 2 requires compatibility with all the buffers involved, including the catholyte,

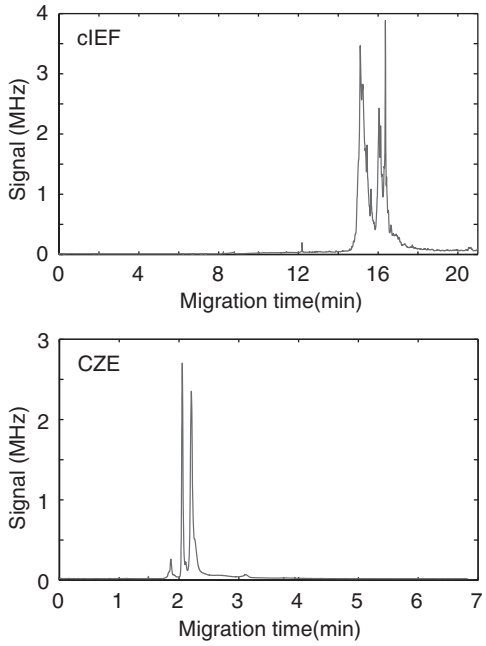

Figure 2. The top trace shows the CIEF separation of ovalbumin (7 nM) and β-lactoglobulin (1 nM). Bottom trace shows CZE separation of ovalbumin (83 nM) and β-lactoglobulin (34 nM), injected at −5 kV for 2 s.

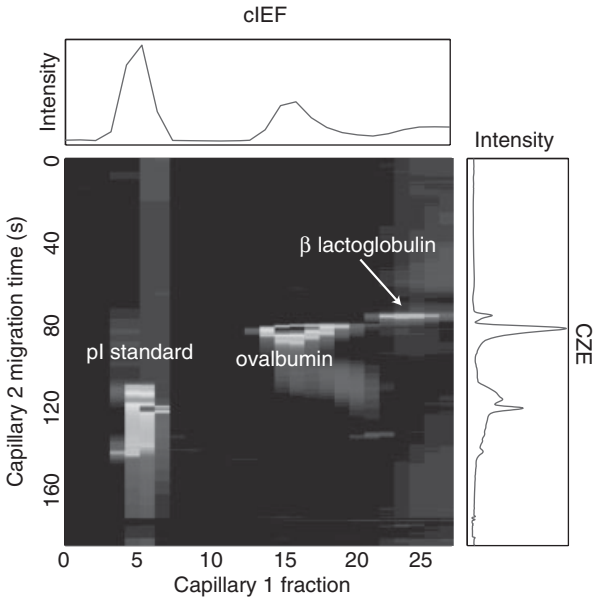

Figure 3. 2-DCIEF/CZE separation of a 4.0 p*I* standard, ovalbumin (p*I* = 4.7), and β-lactoglobulin (p*I* = 5.2). The p*I* trace at the top is the summed electropherogram along that axis. The trace on the right is the summed CZE signal. The p*I* standard was present at 1 ng/mL, the labeled ovalbumin at 5 nM and β-lactoglobulin at 2 nM.

anolyte, mobilization buffer, and the second-dimension buffer. Chemical mobilization in the traditional CIEF involves displacing the catholyte with a salt solution or acidic solution that matches the anolyte [36, 37]. The ions disrupt the pH gradient and allow migration of the focused zones. Coupling CIEF with another separation mode requires either the physical separation of the two dimensions through a dialysis loop or that the mobilization buffer matches the second-dimension buffer. To simplify the instrument, we employed a basic mobilization buffer, which also served as the separation buffer in CZE mode. SDS was added to the CZE buffer to improve the separation by complexing with the proteins.

The two standard proteins were labeled with Chromeo 503 and separated by 2-D CIEF/CZE. The sample was spiked with a fluorescent p*I* standard (4.0) synthesized and donated by Šlais [38]. Figure 3 presents the 2-D electropherogram. The separation in both the dimensions was reasonably good. We used a non-linear least-squares regression routine to fit a Gaussian surface to the three spots. The results of this fit are summarized in Table 1.

The SD of the spots was 1.2 ± 0.2 transfers in the cIEF dimension and 1.4 ± 0.4 s in the CZE dimension. Note that these are the SD of the spots; the full width at half height is a factor of 2.3 larger and the width at baseline is a factor of 4 larger. The measured peak capacity in the cIEF dimension is 4 and in the CZE dimension is 33, for an overall spot capacity of approximately 125.

The IEF dimension produces poorer resolution in the 2-D separation compared with the 1-D separation. It is clear that the resolution is degraded by the relatively large volume of the fraction transferred to the second capillary. Decreasing this transfer volume will improve resolution, albeit at the expense of longer analysis time.

Table 1. Least-squares fit of a Gaussian surface to the spots of Fig. 3

Spot	σ_{cIEF} (transfer)	σ_{CZE} (s)
β-Lactoglobulin	1.4	1.0
Ovalbumin	1.2	1.7
Standard	0.9	1.6

The LIF detector worked well in this experiment. After filtering, the noise in the baseline was extremely low, generating an SD of ∼600 Hz. The maximum signal was 3 MHz, corresponding to a *S/N* ratio of over 5 000 and a concentration detection limit (3σ) of ∼5 pM for ovalbumin. The detection limit was poorer than our previous report [26]; that report employed photobleached ampholytes and green laser excitation, which dramatically improved detection limits.

This work was supported by grants from the National Institutes of Health (P50HG002360, R33CA122900). The authors gratefully acknowledge the donation of fluorescent p*I standards by Karel Šlais from the Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, Brno.*

The authors have declared no conflict of interest.

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