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Stable, reproducible, and automated capillary zone electrophoresis-tandem mass spectrometry system with an electrokinetically pumped sheath-flow nanospray interface

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Abstract

A PrinCE autosampler was coupled to a Q-Exactive mass spectrometer by an electrokinetically pumped sheath-flow nanospray interface to perform automated capillary zone electrophoresis-electrospray ionization-tandem mass spectrometry (CZE-ESI-MS/MS). 20 ng aliquots of an *E. coli* digest were injected to evaluate the system. Eight sequential injections over an eight-hour period identified $1,115 \pm 70$ (relative standard deviation, RSD = 6%) peptides and 270 ± 8 (RSD = 3%) proteins per run. The average RSDs of migration time, peak intensity, and peak area were 3%, 24% and 19% respectively, for 340 peptides with high intensity. This is the first report of an automated CZE-ESI-MS/MS system using the electrokinetically pumped sheath-flow nanospray interface. The results demonstrate that this system is capable of reproducibly identifying over 1,000 peptides from an *E. coli* tryptic digest in a one-hour analysis time.

1. Introduction

Capillary electrophoresis (CE) is an attractive separation technique for biological analysis [1–3]. The interface of CE with electrospray ionization and tandem mass spectrometry (CE-ESI-MS/MS) produces an interesting tool for protein and peptide analysis [4]. The development of improved CE-MS interfaces [5–7] has become an important step in the application of CE-ESI-MS/MS for protein and peptide analysis.

Two new electrospray interfaces have recently been developed for capillary electrophoresis. One is a sheathless interface based on a porous tip as the emitter [5, 8–10]. The separation buffer must support electrospray in sheathless electrospray interfaces, and a relatively small (30- μ m) inner diameter capillary and acidic buffer are usually employed to produce stable sprays. Lindner's group evaluated the interface for peptide analysis [8]. They compared this system with nano-liquid chromatography (nLC)-ESI-MS/MS for analysis of Arg-C-digested rat testis linker histones. With similar sample amounts, CE-ESI-MS/MS produced 60% more peptide IDs than nLC. They also analyzed several posttranslational modifications, including acetylation (lysine and N α -terminal), mono-, di-, and trimethylation, and phosphorylation derived from H1 and core histones by both CE-ESI-MS/MS and nano-LC-ESI-MS [9]. Yates' group used the same interface for an on-line solid phase microextraction, fractionation, and transient isotachopheresis CE-ESI-MS/MS analysis of a moderately

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complex protein mixture [10]. They compared their results with nLC, and found complementary identifications for 100 ng of a *Pyrococcus furiosus* tryptic digest.

Our group reported an electrokinetically pumped sheath-flow nanospray interface that employs an extremely low sheath flow-rate, which eliminates the large dilution typically produced by sheath-flow interfaces [6, 11–22]. In this interface, the separation capillary is inserted into a tapered glass emitter. Very stable sheath liquid flow is produced by electro-osmosis, which is generated at the inner wall of the emitter by the electrospray potential. The choice of capillaries and separation buffer are flexible. Capillaries with inner diameters from 10 μm to 50 μm and separation buffers with pH 2.0 to 8.0 have been used on this system. We have used the system for the analysis of the secreted protein fraction of *Mycobacterium marinum* [17] and for the analysis of more complex *E. coli* digests [18, 19], coupled with multiple reaction monitoring to generate detection limits of 335 zmoles of Leu-enkephalin [20, 21], used for fast top-down intact protein characterization [22], and demonstrated quantitative protein expression study of the differentiation of the very complex PC12 cell line following treatment with nerve growth factor [15].

To date, the capillary electrophoresis system has been manually operated. In this paper, we report the first automated system for capillary electrophoresis mass spectrometry using the electrokinetically-pumped sheath flow interface. This automation is important when analyzing complex proteomes that are pre-fractionated or when analyzing many samples in a quality-control environment.

2. Experimental

2.1 Chemicals and materials

Bovine pancreas TPCK-treated trypsin, bovine serum, urea, ammonium bicarbonate (NH_4HCO_3), dithiothreitol (DTT), and iodoacetamide (IAA) were purchased from Sigma–Aldrich (St. Louis, USA). Acetonitrile (ACN) and formic acid (FA) were purchased from Fisher Scientific (Pittsburgh, USA). Methanol was purchased from Honeywell Burdick & Jackson (Wicklow, Ireland). Water was deionized by a Nano Pure system from Thermo Scientific (Marietta, USA). Linear polyamide (LPA) coated-fused capillaries (50 μm i.d./150 μm o.d.) were purchased from Polymicro Technologies (Phoenix, USA). Complete mini protease inhibitor cocktail (provided in EASYpacks) was purchased from Roche (Indianapolis, USA).

Preparation of the *E. coli* sample—The *E. coli* digests were prepared as reported previously [18]. Briefly, frozen cultures of *E. coli* (Dh5-Alpha) were thawed and plated on solid lysogeny broth (LB) plates. After incubation at 37°C for 24 h, single colonies were selected and grown in tubes with 4 mL liquid LB medium and incubated in the shaker at 37°C overnight. When the content in the tubes turned opaque, the liquid medium with *E. coli* was transferred into new flasks, and shaken overnight at 37°C.

The *E. coli* samples were centrifuged, and the resulting pellets were washed with phosphate buffered saline three times. The pellets were then suspended in an 8 M urea and 100 mM Tris-HCl (pH 8.0) buffer supplemented with protease inhibitor, and sonicated for 15 min on ice. The resulting lysate was centrifuged at $18,000 \times g$ for 15 minutes. The supernatant was retained, and its protein concentration was measured by the bicinchoninic acid (BCA) method. An aliquot of proteins (240 μg) was precipitated by cold acetone overnight at -20°C . After centrifugation, the pellet was washed again with cold acetone. The resulting protein pellet was dried at room temperature.

The dried proteins (240 µg) were dissolved in 80 µL of 100 mM NH₄HCO₃ (pH 8.5) with 8 M urea, and denatured at 50 °C for 30 min followed by reduction with DTT (8 mM) at 60 °C for 50 min and alkylation with IAA (20 mM) at room temperature for 30 min in dark. Then, 320 µL of 100 mM NH₄HCO₃ (pH 8.5) was added to reduce the concentration of urea to less than 2 M. Finally, proteins were digested by incubating with trypsin at a trypsin/protein ratio of 1/30 (w/w) for 4 h at 37 °C. The digests were acidified with FA (final concentration of 0.5%) to terminate the reaction. The tryptic digests were desalted with tC18 SepPak columns (Waters, Milford, MA, USA), followed by lyophilization with vacuum concentrator (Thermo fisher Scientific, Marietta, OH, USA). The dried protein digests were dissolved in 25% (v/v) ACN and 0.05% FA before CZE-ESI-MS/MS analysis.

2.2 CZE-ESI-MS/MS

A PrinCE autosampler (Prince Technologies B.V., Netherlands) was used for automated sample injection and separation voltage control. The CZE-ESI-MS/MS interface was similar to that reported previously [6]. As the major difference from reference [6], 0.1% formic acid with 10% methanol was used as the sheath buffer instead of 10 mM aqueous acetic acid and methanol. The nanospray high voltage was supplied by a Spellman CZE 1000R power supply and voltage programming was controlled by LabView software. The LPA-coated separation capillary (60 cm, 50 µm i.d./150 µm o.d.) was coupled to a Q-Exactive (Thermo Fisher Scientific) with an electrokinetically driven sheath-flow nanospray interface [6, 11–16]. The emitter was pulled in a Sutter pipette puller to a ~8 µm i.d. tip. The nanospray voltage was 1.5 kV. The separation was performed at 24,000 V (400 V/cm). Sample injection was performed by pressure with 300 mbar for 0.05 min (injection volume 20 nL). 0.1% formic acid was used as the separation buffer.

Full MS scans were acquired in the Orbitrap mass analyzer over the m/z 380–1800 range with a mass resolution of 70,000 (at m/z 200). The target value was 1.00E+06. The twelve most intense peaks with charge state 2 were fragmented in the HCD collision cell with normalized collision energy of 28%. Tandem mass spectra were acquired in the Orbitrap mass analyzer with a mass resolution of 17,500 at m/z 200. The target value was 1.00E+06. The ion selection threshold was 2.50E+04 counts, and the maximum allowed ion accumulation times were 250 ms for full MS scans and 80 ms for tandem mass spectra. The dynamic exclusion time was set to 30 s.

2.3 Data analysis

Database searching of the raw files was performed in Proteome Discoverer 1.3 with MASCOT 2.2.4 against SwissProt database with taxonomy as *Escherichia coli* DH1 (4,160 sequences). Database searching for the reversed database was also performed to evaluate the false discovery rate [23, 24]. The database searching parameters included full tryptic digestion and allowed up to two missed cleavages, precursor mass tolerance 10 ppm, and fragment mass tolerance 0.02 Da. Carbamidomethylation (C) was set as fixed modifications. Oxidation (M) and deamidated (NQ) were set as variable modifications.

On the peptide level, peptide confidence value as high as possible was used to filter the peptide identification, and the corresponding false discovery rate on peptide level was less than 1%. On the protein level, protein grouping was enabled.

Other data analysis was performed using locally constructed scripts with Matlab.

3. Results and Discussion

3.1 Automated CZE-MS/MS system

In this work, a PrinCE autosampler was coupled to a Q-Exactive mass spectrometer by an electrokinetically pumped sheath-flow nanospray interface to perform automated CZE-MS/MS. The automated sample injection, separation method, and sequential runs were programmed and controlled by Wprince 7.1 software. We attempted to use the external events of PrinCE autosampler to trigger the Mass Spectrometer software with a close contact signal. This trigger has been successfully applied on an Orbitrap-Velos mass spectrometer. However, due to incompatibility with the Windows system, this triggering was not accessible on the Q-Exactive mass spectrometer. Instead, eight runs were acquired as one .raw file. The electropherograms were then separated manually.

As an important different from our manual system, the automated system requires a capillary that is at least 60 cm in length, which is twice the length of the manual system. As a second difference, we used a relatively low acetonitrile concentration in the protein sample to minimize the effects of evaporation over the eight-hour analysis time.

3.2 Reproducibility of automated system

Figure 1 shows eight sequential base peak electropherograms of the *E. coli* digest. The relative standard deviation of the base peak intensity is 14%. After database searching, $1,115 \pm 70$ peptides and 270 ± 8 protein groups were identified ($n=8$). The number of peptide and protein identifications are slightly lower than our earlier report [19]. The difference in identification number is likely due to the relatively low acetonitrile concentration in the sample buffer, which reduced stacking.

A total of 2,027 peptides and 401 proteins groups were identified over the eight runs. Figure 2 plots the cumulative number of peptide and protein IDs vs. the number of replicates. The identification of the first run (1,219 peptides, 290 protein groups) covers 60% of the total peptide and 70% of the total protein identifications. Over 80% of the peptide identifications and up to 90% of the protein identifications were obtained from the first three runs.

The similar number of peptide IDs for the eight runs may be a result of the limited tandem spectra acquisition rate of the mass spectrometer. Figure 3 presents a smoothed plot of the ms/ms duty cycle for the first electropherogram. Very few tandem spectra were taken before 20 minutes. The instrument then approached 100% duty cycle, where nine tandem spectra were generated per second, and the acquisition rate across the majority of the separation exceeded 50%. A higher speed mass spectrometer will be needed to delve deeper into the proteome, or the sample must be prefractionated into simpler mixtures that are better suited to the separation speed and data acquisition rate of the capillary electrophoresis/mass spectrometer combination.

340 of the most abundant peptides identified in all runs were extracted from the electropherograms. A nonlinear least-squares regression analysis was used to fit a Gaussian function to the peaks:

$$I = H \times e^{-0.5 * t(-t_0)^2 / \sigma^2}$$

where I is intensity and H is the peak height, t_0 is the peak center and σ is the standard deviation of the Gaussian function. An unsupervised fit was used where the initial guesses for height and t_0 were the height and migration time of the maximum value in each electropherogram and the starting value of σ was set as 1 second.

As shown in Figure 4, the average relative standard deviations of migration time, peak intensity, and peak area are 3%, 24% and 19%, respectively. The main source of variability is likely associated with temperature changes in the room in which the system is located and solvent evaporation over the eight hour period required for the analyses. Similar precision is reported for capillary RPLC-MS/MS for peptide analysis [25].

The emitter in our CE-MS interface is typically used continuously for 2–3 days. The cost of emitter is low (about 20 cents/each) when the emitter puller is available. Roughly 10 min are required to prepare and change the emitter.

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Highlights

- First automation of electrokinetically-pumped sheath-flow interface
- Eight analyses of *E. coli* digest performed in eight hours
- An average of 1,115 peptide IDs per run

Fig. 1.
Base peak electropherograms of 20 ng *E. coli* digests analyzed by automated CZE-ESI-MS/MS.

Fig. 2.
The cumulative number of peptide (A) and protein (B) IDs vs. the number of replicates. The smooth curves are the least squares fit of a saturation curve ($y = \frac{a x}{b + x}$) to the data.

Fig. 3. Tandem mass spectra generation duty cycle. The data were smoothed with a lowess filter with a span of 45 points.

Fig. 4.
The relative standard deviation distribution of migration time (A), peak amplitude (B) and peak area (C) of 340 *E. coli* peptides with high intensity identified in eight runs.