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Evaluation of a commercial electro-kinetically pumped sheath-flow nanospray interface coupled to an automated capillary zone electrophoresis system

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Abstract

Capillary zone electrophoresis-electrospray ionization-mass spectrometry (CZE-ESI-MS) is attracting renewed attention for proteomic and metabolomic analysis. An important reason for this interest is the maturation and commercialization of interfaces for coupling CZE with ESI-MS. One of these interfaces is an electrokinetically pumped sheath flow nanospray interface developed by the Dovichi group, in which a very low sheath flow is generated based on electroosmosis within a glass emitter. CMP Scientific has commercialized this interface as the EMASS-II ion source. In this work, we compare the performance of the EMASS-II ion source with our in-house system. The performance of the systems is equivalent. We also couple the EMASS-II ion source with a PrinCE Next|480 capillary electrophoresis autosampler and an Orbitrap mass spectrometer, and analyzed these system's performance with in terms of sensitivity, reproducibility, and separation performance for separation of tryptic digests, intact proteins, and amino acids. The system produced reproducible analysis of BSA digest; the RSDs of peptide intensity and migration time across 24 runs were less than 20% and 6%, respectively. The system produced linear calibration curve of intensity across a 30-fold range of tryptic digest concentration. The combination of a commercial autosampler and electrospray interface efficiently separated amino acids, peptides, and intact proteins, and only required 5 μ L of sample for analysis.

Graphical abstract

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Compliance with ethical standards

Conflict of interests: Norman J. Dovichi, Liangliang Sun, and Guije Zhu are co-inventors on patents describing the electrospray interface and receive royalties on the sale of the CMP interface. Elizabeth H. Peuchen declares no conflict of interest.

Research involving Animals: All animal procedures were performed according to protocols approved by the University of Notre Dame Institutional Animal Care and Use Committee.

Keywords

Electro-kinetically pumped sheath flow interface; EMASS-II CE-MS interface; automated capillary zone electrophoresis-mass spectrometry; proteomics; intact protein analysis

1 Introduction

Capillary zone electrophoresis-electrospray ionization-mass spectrometry (CZE-ESI-MS) is renewed interest for biomolecules analysis [1–3]. One important reason for this renaissance is the maturation and commercialization of interfaces for coupling CZE with ESI-MS [4]. At least three interfaces have been commercialized: the co-axial sheath flow interface [5], the sheathless interface [6], and the electro-kinetically pumped sheath flow interface [7, 8].

Many interfaces for CZE-MS are laboratory-built. This group has published a number of papers describing the performance and application of an electro-kinetically pumped sheath flow nanospray CZE-MS system [7–12]. This system has been commercialized as the EMASS-II CE-MS interface by CMP Scientific. Very recently the commercial version has been used to characterize monoclonal antibodies [13] and complex glycosaminoglycan mixtures [14].

While the local-build instrument has been demonstrated in a large number of publications, it has not been compared directly with the commercial version. This comparison will be valuable for researchers contemplating application of capillary electrophoresis but who are uninterested in constructing a system from parts. In this work, we compared our locally built instrument with the commercial interface in terms of sensitivity and numbers of peptide identifications using a *Xenopus laevis* fertilized egg proteome digest as the sample. We also evaluated the EMASS-II based CZE-MS system for analysis of amino acids, peptides, and intact proteins. Finally, we coupled a PrinCE autosampler with the EMASS-II interface; this combination provides unattended operation for sequential separations.

2 Materials and Methods

2.1 Materials

Bovine pancreas TPCK-treated trypsin, bovine serum albumin (BSA), ubiquitin, cytochrome c, myoglobin, carbonic anhydrase, superoxide dismutase, alpha casein, ammonium bicarbonate (NH_4HCO_3), dithiothreitol (DTT), iodoacetamide (IAA), and amino acids (Gly,

Pro, Cys, Ile, Leu, Asp, Lys, Glu, His, Phe, and Arg) were purchased from Sigma-Aldrich (St. Louis, MO USA). Acetonitrile (ACN), acetic acid, formic acid (FA), and hydrofluoric acid (HF) were purchased from Fisher Scientific (Pittsburgh, PA USA). Methanol was purchased from Honeywell Burdick and Jackson (Wicklow, Ireland). Water was deionized by a NanoPure system from Fisher Scientific. Complete mini protease inhibitor cocktail (provided in EASYpacks) was purchased from Roche (Indianapolis, IN USA). Fused silica capillaries (50 μ m ID/360 μ m OD and 30 μ m ID/360 μ m OD) were purchased from Polymicro Technologies (Phoenix, AZ).

2.2 Sample preparation

Xenopus laevis fertilized egg proteome digest was prepared with similar protocols as references [8,15]. All animal procedures were performed according to protocols approved by the University of Notre Dame Institutional Animal Care and Use Committee. Fifty fertilized *Xenopus laevis* eggs at development stage 1 were collected in minimal cell culture buffer in an Eppendorf tube and 800 μ L of mammalian Cell-PE LB buffer plus complete protease inhibitor was added before vortexing for 1 min to preliminarily lyse the embryos. Embryos were homogenized by a Powergen Model 125 homogenizer (Fisher Scientific, Pittsburgh, PA USA) for 1 min on ice and further sonicated for 10 min on ice with a Branson Sonifier 250 (VWR Scientific, Batavia IL USA) to lyse the eggs completely. Lysate was then centrifuged at 10,000g for 10 min and three layers were obtained: lipids (top layer), protein (middle layer), and pellet (bottom layer). The protein layer was collected in an Eppendorf tube and a small fraction of the protein was subjected to a bicinchoninic acid (BCA) analysis. The remaining protein was subjected to acetone precipitation to purify the proteins; 1 mL of cold acetone was added to 200 μ L of protein solution (~1 mg of protein), and the mixture was kept at -20°C overnight. After centrifugation, the supernatant was removed and the protein pellet was washed with cold acetone again to remove the detergent in the lysis buffer. After centrifuging again, the supernatant was removed and protein pellet was placed in the fume hood at room temperature to dry the sample. A 1 mg aliquot of the protein was dissolved in 250 μ L of 8 M urea dissolved in 100 mM NH_4HCO_3 (pH 8.0) via vortexing and sonication. The protein was denatured at 37°C for 40 minutes, followed by reduction with 8 mM DTT at 37°C for 40 minutes and alkylation with 25 mM IAA at room temperature in the dark for 20 minutes. Two μ L of 1 M DTT was added to the protein solution to block excess IAA at room temperature for 20 minutes. 100 mM NH_4HCO_3 (pH 8.0) was used to dilute the urea concentration to ~2 M. Protein digestion was performed at 37 °C with trypsin at a trypsin:protein ratio of 1:40 (w/w) overnight before being acidified with FA. Protein was desalted with C18-SepPak columns (Waters, Milford MA USA) and then lyophilized with a vacuum concentrator (Thermo Fisher Scientific, Marietta OH USA). Dried protein digest was stored at -80 °C before use. 160 μ g of protein digest was dissolved in 5 mM NH_4HCO_3 to a final concentration of ~4 μ g/ μ L and kept at 4°C for analysis.

The standard protein digests were prepared with the same protocol as reference [8]. For BSA digest demonstrating the repeatability of the autosampler and interface, 0.5 mg/mL in 0.1% formic acid was used. The protein- digest mixture in 5 mM NH_4HCO_3 containing 1 mg/mL of BSA digest, 0.5 mg/mL of cytochrome c digest and 0.1 mg/mL of alpha casein digest was

prepared, and diluted 3 times, 10 times, 30 times and 100 times with 5 mM NH_4HCO_3 followed by CZE-MS analysis.

An amino-acid mixture in 0.1% FA containing Gly, Pro, Cys, Ile, Leu, Asp, Lys, Glu, His, Phe, and Arg (45 μM concentration for each amino acid) was used for CZE-MS analysis.

A standard-intact-protein mixture in 0.1% FA containing 0.2 mg/mL ubiquitin, 0.2 mg/mL cytochrome c, 0.4 mg/mL myoglobin, 1.4 mg/mL carbonic anhydrase, and 0.2 mg/mL superoxide dismutase was used for CZE-MS analysis.

2.3 CZE-ESI-MS/MS

A Prince Technologies' PrinCE Next480 capillary electrophoresis autosampler (Prince Technologies, B.V., The Netherlands) was used for automated sample injection and separation voltage control in all experiments. The locally constructed electro-kinetically pumped sheath flow interface and its commercialized version (EMASS-II CE-MS interface, CMP Scientific, Corp., Brooklyn, NY, USA) were employed for coupling CZE to an Orbitrap Velos mass spectrometer. The high voltage power supply in the autosampler was connected to the proximal end of the capillary for CZE separation, and another power supply from CMP Scientific, Corp. (Brooklyn, NY USA) was used at the distal end of the capillary for electrospray. The sheath electrolyte was 10% methanol in 0.1% FA, and the background electrolyte for the CZE separation was 5% acetic acid. For all the experiments, spray emitters with a 20- μm -diameter orifice were used; emitters were pulled with a Sutter pipette puller. The distal end of the separation capillary was etched with HF to reduce its outer diameter from 360 μm to ~70 μm for 50 μm ID capillary and to ~50 μm for 30 μm ID capillary [16]. CAUTION: Appropriate safety procedures should be followed when working with HF.

CZE conditions varied for different samples. For analysis of BSA digests, a 60 cm long (50 μm ID/360 μm OD) capillary with linear polyacrylamide (LPA) coating on the inner wall was used [17]. The BSA digest was injected at 200-mbar pressure for 5 seconds before a separation voltage of 20 kV was applied for 1800 seconds. For the intact protein mixture, a 60 cm long (50 μm ID/360 μm OD) LPA-coated capillary was used. The intact protein mixture was injected at 200-mbar pressure for 5 seconds before a separation voltage of 20 kV was applied for 3600 seconds. For the standard-protein-digest mixture, a 60 cm long (50 μm ID/360 μm OD) LPA-coated capillary was used. The standard-protein-digest mixture was injected with 250-mbar pressure for 10 seconds before a separation voltage of 20 kV for 4800 seconds was applied. For the *Xenopus laevis* fertilized egg proteome digest, an 85 cm long (50 μm ID/360 μm OD) LPA-coated capillary was used. The digest was injected with 400-mbar pressure for 6 seconds before a separation voltage of 26 kV was applied for 7200 seconds. For the amino acid mixture, a 60 cm long (30 μm ID/360 μm OD) LPA-coated capillary was used. The amino acid mixture was injected with 400-mbar pressure for 5 seconds before a separation voltage of 20 kV was applied for 1860 seconds. The Electronic Supplementary Material (ESM, Supplemental 1) provides information for each of the samples tested. The nanospray voltage was set to 1.8 kV for all experiments.

For the standard-protein-digest mixture, the intact-protein mixture, and the amino-acid mixture experiments, only parent ion mass spectra were acquired. The Orbitrap mass analyzer was used with a m/z range of 250–2000 for analysis of the BSA digest, a m/z range of 70–400 for the amino-acid mixture, a m/z range of 500–2000 for the intact-protein mixture, and a m/z range of 350–1500 m/z for the standard-protein-digest mixture. Mass resolution was set to 60,000 (at m/z 400). The Automatic Gain Control target value for mass spectrum acquisition was $1.00\text{E}+06$. The C-trap pressure was decreased to $\sim 1/3$ of the original pressure for intact protein analysis.

For analysis of the *Xenopus laevis* fertilized egg proteome digest, a data-dependent-acquisition was performed. The S-lens RF level was 50.0. A top 20 method was used over a mass range of 350–1500 m/z with a resolution of 60,000. The number of microscans was set to 1. The target intensity value was $1.00\text{E}+06$. The 20 most intense peaks with a charge state 2 were selected for sequencing and fragmented in the ion trap with a normalized collision energy of 35% and target value of $1.00\text{E}+04$. The ion selection threshold was 500 counts and maximum time allowed for ions to accumulate was 500 ms for full scans and 100 ms for collision-induced dissociation. Dynamic exclusion was enabled and peaks selected from fragmentation more than once within 20 seconds were excluded from selection for 60 seconds.

2.4 Data Analysis

Raw data files from the *Xenopus laevis* fertilized egg proteome digest sample were analyzed by MaxQuant [18] version 1.5 with the Andromeda search engine [19], against the *Xenopus laevis* protein database derived from mRNA data [20] (158,428 entries including forward and reverse sequences). The protocol described in reference 8 was used for the search.

3 Results and Discussion

3.1 Comparisons between commercialized and locally constructed interfaces

The locally constructed interface uses a cross (Fig 1A) whereas the EMASS-II CE-MS interface (Fig 1B) employs a tee to introduce the separation capillary into the emitter. One arm of the cross connects to a syringe containing sheath liquid for system flushing in the locally-constructed interface. System flushing for the EMASS-II CE-MS interface is carried out by applying pressure into the sheath liquid vial via an external syringe.

We first compared the systems for analysis of a *Xenopus laevis* fertilized egg proteome digest in terms of the sensitivity, identification capacity, and separation performance, Fig. 2. The locally constructed and the EMASS-II CE-MS interfaces generated comparable base peak intensities, Fig 2A and B. The peptide intensities were in good agreement for the two interfaces ($r^2 = 0.95$), ESM Fig. S1.

Although the EMASS-II CE-MS interface electropherogram (Fig 2A) appears to be more complex than the electropherogram generated with the locally constructed interface (Fig 2B), the two systems produced nearly identical numbers of protein and peptide identifications: 399 protein identifications/1372 peptide identifications (EMASS-II CE-MS interface) vs. 388 protein identifications/1258 peptide identifications (locally constructed

interface). The two systems identified the same pool of proteins from this complex proteome digest, Fig 2C.

Based on these results, we conclude that the EMASS-II CE-MS interface based CZE-ESI-MS system provides essentially indistinguishable sensitivity and peptide identifications as the locally constructed interface for bottom-up analysis of complex proteomes.

3.2 Reproducibility of the automated CZE-MS system based on a PrinCE autosampler coupled with the EMASS-II CE-MS interface

We next evaluated the reproducibility of the PrinCE autoinjector - EMASS-II CE-MS interface based automated CZE-MS system using a BSA tryptic digest as the sample. The system yielded reproducible separation profiles and base peak intensity across twenty-four runs of CZE-MS analysis, Fig. 3A. We extracted the intensities of three arbitrarily chosen peptides (m/z 547.32, 997.59, and 480.61), and observed that the relative standard deviations (RSDs) of peptide migration time and intensity across 24 runs are less than 6% and 20%, respectively. Figure 3B presents the repeatability for the peptide at m/z 547.32, reproducibility data for other peptides can be found in the ESM (Supplemental 1). Migration time showed a modest drift to shorter times, presumably due to temperature drift in our mass spectrometry facility.

3.3 Calibration curve for the standard-protein-digest mixture

We used the commercial CZE-MS system for analysis of a tryptic digest of a standard protein mixture. The stock solution contained 1 mg/mL of a BSA digest, 0.5 mg/mL of a cytochrome C digest, and 0.1 mg/mL of an alpha casein digest in 5 mM NH_4HCO_3 . A calibration curve was generated by diluting the stock solution 3 times, 10 times, 30 times, and 100 times with 5 mM NH_4HCO_3 followed by CZE-MS analysis in quadruplicate.

Fig. 4A presents the base peak electropherograms of the standard protein digest mixtures. Peptide intensity increased linearly across a 30-fold range of peptide concentration, Fig. 4B. The r^2 for the three standard proteins was 0.97 or higher.

3.4 Amino acid and intact protein analysis

We also applied the automated EMASS-II CE-MS system for analysis of both amino acids and intact proteins. A mixture of 11 amino acids was analyzed by the system, Fig. 5A. The mixture generated eight major peaks, and the migration time is roughly related to their isoelectric points, Fig. 5B. The automated EMASS-II CZE-MS system efficiently and reproducibly generated baseline separation of the intact standard proteins, Fig. 6.

3.5 Sample volume

Although the injection volume used in CZE-MS is typically a few nanoliters, much larger volumes are required for sample injection. Typically, sample injection requires that the capillary be dipped into a microcentrifuge tubes that contains $\sim 10 \mu\text{L}$ or larger volumes, and pressure or voltage is applied to inject the nanoliter volume sample into the capillary for analysis. We observe that only 5 μL sample volume is sufficient for reproducible injection using the PrinCE autoinjector. Smaller sample volumes were not consistently injected. This

two-fold reduction in sample size compared with manual injection will be valuable whenever minute samples must be analyzed.

4 Conclusions

The EMASS-II electrospray interface provides virtually identical performance as the locally constructed electrokinetically-pumped nanospray interface for tryptic digests of complex proteomes. The commercial interface also provides excellent performance for analysis of amino acid mixtures and for separation of intact proteins. The PrinCE Next|480 autosampler provided unattended and stable operation when combined with the EMASS-II interface.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.
Schematic drawing of locally constructed electrokinetically pumped sheath flow interface (A) using a cross connection with one arm of the cross connected to sheath liquid [7] and the EMAS-II CE-MS interface (B) containing a T-connection.

Fig 2.

Total ion electropherograms generated with the commercial (Fig 2A) and locally-constructed interfaces (Fig 2B). For figures 2A and 2B, ~175 ng of *Xenopus* protein digest was loaded into the capillary. Fig 2C shows a comparison of the protein identifications for the interfaces. A total of 388/1372 and 399/1256 proteins/peptides were identified in the locally constructed and commercial interfaces, respectively.

Fig 3. Reproducibility of the commercial system. Fig 3A contains example base peak electropherograms of a BSA digest. Fig 3B presents the repeatability of migration time and peak intensity.

Fig 4. Calibration curve using the automated commercial system. Fig 4A presents the base peak electropherograms of the standard protein digest mixtures. A seven point Gaussian filter was applied to each electropherogram. Fig 4B presents calibration curves for peptide signals from the BSA digest (547.32 m/z), the cytochrome C digest (824.9 m/z), and alpha casein digest (985.0 m/z).

Fig 5.

Analysis of 11 amino acids using the automated EMASS-II CZE-MS system. Fig 5A presents the base-peak electropherogram. Amino acids are labeled according to their one-letter abbreviation (K: lysine, H: histidine, R: arginine, G: glycine, L: leucine, I: isoleucine, F: phenylalanine, E: glutamic acid, P: proline, C: cysteine, D: aspartic acid). A five point Gaussian filter was applied to the electropherogram. Fig 5B plots migration time vs. isoelectric point.

Fig 6.

Triplicate base-peak electropherogram of a five-component mixture of intact proteins.

Proteins (concentrations) are: A - ubiquitin (0.2 $\mu\text{g/mL}$), B - cytochrome C (0.2 $\mu\text{g/mL}$), C - myoglobin (0.4 $\mu\text{g/mL}$), D - carbonic anhydrase (1.4 $\mu\text{g/mL}$), and E- superoxide dismutase (0.2 $\mu\text{g/mL}$).