CESI-MS of Intact Proteins

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Introduction

There is a growing need for selective and sensitive analytical tools for the characterization of intact proteins. The effective coupling of capillary electrophoresis and electrospray ionization time-of-flight mass spectrometry (CE-ESI-TOF-MS) may offer an attractive option by providing both the separation and detection required to distinguish structurally related protein species.[1,2] In order to bring CE-ESI-MS to a high performance level, novel sheathless CE-MS interfacing was studied to achieve sensitive detection of intact proteins.

Based on a design of Moini,[3] a prototype CESI sprayer was recently developed in the laboratories of Beckman Coulter, Inc. Flow rates in CE are very low at nanoliter per minute, and the initial droplets formed during the sheathless electrospray process are small, which leads to more efficient ionization (i.e., nanospray). With sheathless[1] interfacing, the ESI [2] spray tip can be positioned close to the MS inlet, thereby improving ion sampling efficiencies. Overall, this can lead to enhanced sensitivity and lower limits-of-detection (LODs). The CESI sprayer provides electrical contact without the need for metal conductive coating, microelectrodes or liquid junctions. In the CESI design (Figure 1), the last 3–4 cm of the bare fused-silica capillary are etched with hydrofluoric acid until the section becomes conductive, producing a ~5 µm thick porous wall, which is conductive when in contact with an electrolyte. Capillaries with porous tips are simply inserted in a stainless-steel ESI needle filled with a static conductive liquid.

In this study, the performance of the CESI-MS of intact proteins was evaluated using some model proteins. Reproducibility, linearity and LODs were established. It is demonstrated that CESI-MS provides enhanced responses for intact proteins resulting in sub-nM detection limits. This is a 50- to 140-fold improvement compared to conventional sheath-liquid CE-MS interfacing using the same capillary dimensions.

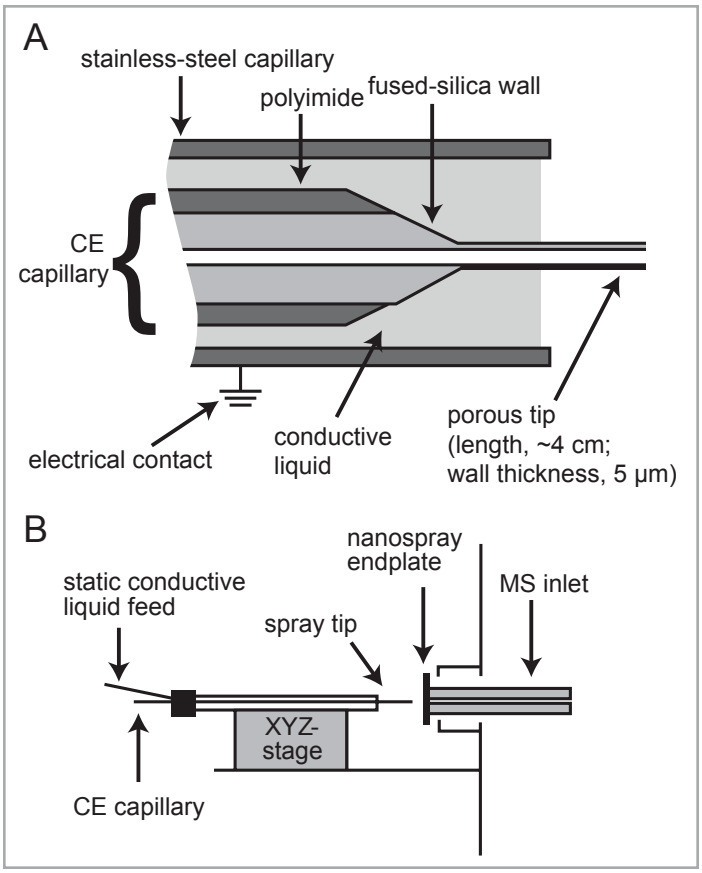

Figure 1. The PA 800 Plus System.

Materials and methods

Chemicals: Acetic acid (99.8%), ammonium hydroxide (25%) and isopropanol (IPA) were obtained from Merck. Insulin (bovine pancreas), carbonic anhydrase II (bovine erythrocytes), ribonuclease A (bovine pancreas) and lysozyme (chicken egg white) were from Sigma-Aldrich. Protein test mixtures were prepared in the appropriate concentration with deionized water. A background electrolyte (BGE) of 100 mM acetic acid (pH 3.1) containing 5% IPA was prepared by diluting 0.171 mL glacial acetic acid to 30 mL with deionized water/IPA (95/5, v/v) and adjusting the pH with ammonium hydroxide.

CE system: Experiments were carried out on a Beckman Coulter capillary electrophoresis instrument. The separation voltage was -30 kV and the capillary temperature was 20 °C. Fused-silica capillaries (total length, 100 cm; inner diameter, 30 µm; outer diameter, 150 µm) equipped with the porous tip [3] (length, 3–4 cm) were supplied by Beckman Coulter. The capillaries were precoated with polyethylenimine (PEI). At the beginning of each day, the coated capillary was conditioned by flushing the capillary at 50 psi with air (10 min), methanol (20 min), deionized water (5 min) and BGE (10 min). Before each run, the capillary was flushed for 3 min (50 psi) with fresh BGE. The sample was injected for 10 s at 5 psi.

CE-MS: MS detection was performed using a Bruker Daltonics micrOTOF orthogonal-accelerated time-of-flight (TOF) mass spectrometer.

Sheathless CE-MS: The capillary with the porous tip was placed in a grounded stainless-steel needle that could be positioned by an XYZ-stage (Beckman Coulter) fitting the Bruker micrOTOF instrument (Figure 1B). A nanospray endplate and gas diverter were installed to allow nano-electrospray ionization (nano-ESI). The porous tip protruded the grounded needle approximately 0.5 cm and the needle was filled with BGE to establish the electrical contact. The optimized spray conditions were as follows: dry gas temperature, 180 °C; dry gas nitrogen flow, 3.0 L/min; nebulizer pressure, 0.0 bar. Electrospray in positive ionization mode was achieved using an ESI voltage of -2.1 kV.

Important:

- A separation current above 5 µA might cause permanent damage to the separation capillary.
- Generally, please do not apply >2000V to generate electrospray as it may result in capillary damage.

Sheath-liquid CE-MS: For sheath-liquid interfacing, the capillary with porous tip was placed in a grounded coaxial CE-MS sprayer (Agilent Technologies) installed on a conventional ESI source comprising the standard ESI endplate and capillary cap. A flow of 2 µL/min of IPA-water-acetic acid (75:25:0.1, v/v/v) was applied as sheath liquid. The optimized conditions for sheath-liquid interfacing were: dry gas temperature, 180 °C; dry gas nitrogen flow, 4 L/min; nebulizer pressure, 0.4 bar; ESI voltage, -4.0 kV.

Data analysis: CESI-MS data were analyzed using Bruker Daltonics data analysis software. Base-peak electropherograms (BPE) were constructed in the range m/z 1000–3000. For determination of detection linearity and LOD, extracted-ion electropherograms (EIE) for the 4 model proteins were constructed from their most abundant m/z signals. These were m/z 1147.7 and 1434.1 for insulin; m/z 1210.4, 1262.9, 1320.3, 1383.1 and 1452.2 for carbonic anhydrase II; m/z 1521.2, 1711.1 and 1955.5 for ribonuclease A and m/z 1431.6, 1590.34 and 1789.0 for lysozyme.

Results and discussion

The porous tip capillary was placed in a grounded needle that was positioned on an XYZ-stage (Figure 1B). The electrical contact was established by filling the stainless-steel needle with BGE. The liquid hardly evaporated from the needle and the same liquid could be used for an entire day of measurements. The porous tip capillaries were coated with positively charged PEI, which exhibits a net positive charge when an acidic BGE is used. Under these conditions, adsorption of peptides and proteins to the capillary wall will generally be avoided due to electrostatic repulsion. Most stable electrospray formation and highest analyte signals were obtained with the capillary tip at a distance of 3 mm to the MS inlet.

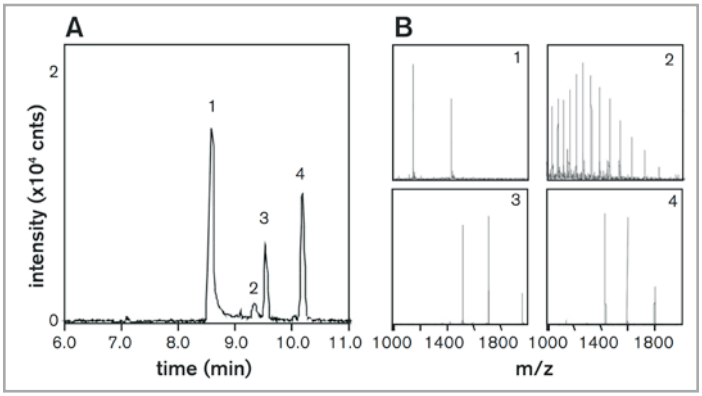

Figure 2. (A) BPE obtained with sheathless CESI-MS of a mixture of (1) insulin; (2) carbonic anhydrase II; (3) ribonuclease A and (4) lysozyme (each 5 µg/mL). (B) Mass spectra obtained at the apices of peaks 1–4. For further conditions, see the materials and methods section.

A mixture of insulin, carbonic anhydrase II, ribonuclease A and lysozyme was analyzed by sheathless CESI-MS. Using a BGE of 100 mM acetic acid (pH 3.1) with 5% IPA, the 4 proteins were baseline separated within 11 min (Figure 2A). IPA was added to the BGE as it provided a modest gain in protein signal intensities. Good quality mass spectra were obtained under these conditions (Figure 2B). Deconvolution of the mass spectra yielded masses of 5733.6 Da (insulin), 29024.6 Da (carbonic anhydrase II),

13681.8 Da (ribonuclease A) and 14304.5 Da (lysozyme), respectively, which agreed well with their expected molecular masses. The overall performance of the sheathless CESI-MS system was further evaluated by assessing the repeatability, detection linearity and LODs for the test proteins. Migration time RSDs for all 4 proteins were less than 0.8% (n=5) and peak area RSDs were within 8% (n=5). Plate numbers ranged from 0.5 x 105 (insulin) up to 1.5 x 105 (ribonuclease A). Clearly, the CESI-MS system allows repeatable and efficient analyses of intact proteins. To check for detection linearity, protein mixtures with concentrations between 0.05 and 25 µg/mL of each protein were prepared and each solution was analyzed in triplicate. For all proteins, good linear relationships (Table 1) between injected concentration and obtained peak areas were obtained. Table 1 also lists the LODs (S/N=3) achieved with sheathless CESI-MS. Sub-nM levels could still be detected for carbonic anhydrase II, ribonuclease A and lysozyme, indicating very favorable LODs for CESI-MS of intact proteins.

In order to assess the gain in signal provided by CESI-MS, a comparison with sheath-liquid CE-MS was made. The same capillary (i.e., equipped with the porous tip) was placed in a coaxial sprayer installed on a conventional ESI source. A sheath liquid of IPA-water-acetic acid (75/25/0.1, v/v/v) was applied at a flow rate of 2 µL/min. These conditions had shown to be optimal for intact protein analysis in a previous CE-MS study.[4] A baseline separation within 10 min (Figure 3) and linear protein signals (Table 1) were obtained for the test proteins, indicating the proper functioning of the sheath-liquid interfacing.

Protein signals obtained with sheath liquid interfacing were lower by a factor of approximately 10 to 50 with respect to CESI-MS (cf. Figure 2A and Figure 3; note that injected protein concentrations are 5 and 50 µg/mL, respectively). In particular, insulin showed reduced response with sheath-liquid CE-MS. The lower protein signals are the overall result of the dilution of the CE effluent by the sheath liquid and the lower ionization and ion sampling efficiencies obtained using conventional ESI instead of nanospray. It should be noted that the IPA in the sheath liquid strongly enhances protein ionization, as without IPA, protein signals are even much lower. Furthermore, the baseline noise observed is more favorable in CESI-MS (Figure 4).

The application of sheath liquid causes significant chemical noise, which is avoided with CESI-MS. Overall, the LODs achieved with CESI-MS are considerably lower than for sheath-liquid CE-MS (Table 1).

Conclusion

The performance of CESI-MS for the analysis of intact proteins was evaluated. Sub-nanomolar LODs were obtained with the CESI-MS system using a nanoESI source. These are highly favorable sensitivities that, to our knowledge, have not been achieved before with CE-MS for intact proteins. The gain in performance of the CESI-MS system with respect to sheath-liquid CE-MS can be attributed to the reduced noise levels and increased analyte responses. The very favorable LODs and overall performance indicates that CESI-MS can be highly useful for intact protein analysis.

Protein	Sheathless interface[c]		Sheath liquid interface[d]	
	R^2	LOD	R^2	LOD
insulin	0.999	1.28	0.992	106
carbonic anhydrase II	0.989	0.58	0.981	79
ribonuclease A	0.992	0.62	0.989	33
lysozyme	0.997	0.50	0.990	41

Table 1. Linearity (R2)a and LODs (nM)b for the 4 model proteins obtained with CESI-MS and CE-MS with sheath-liquid interfacing.

a. Concentration range, 0.05–25 µg/mL (CESI-MS) and 1–100 µg/mL (sheath liquid).

b. Concentration to yield S/N ratio of 3 as calculated by extrapolation from 1-µg/mL injection. For carbonic anhydrase analyzed with sheath-liquid CE-MS, a 5-µg/mL injection was used.

c. BGE, 100 mM ammonium acetate (pH 3.1) containing 5% (v/v) isopropanol.

d. BGE, 100 mM ammonium acetate (pH 3.1).

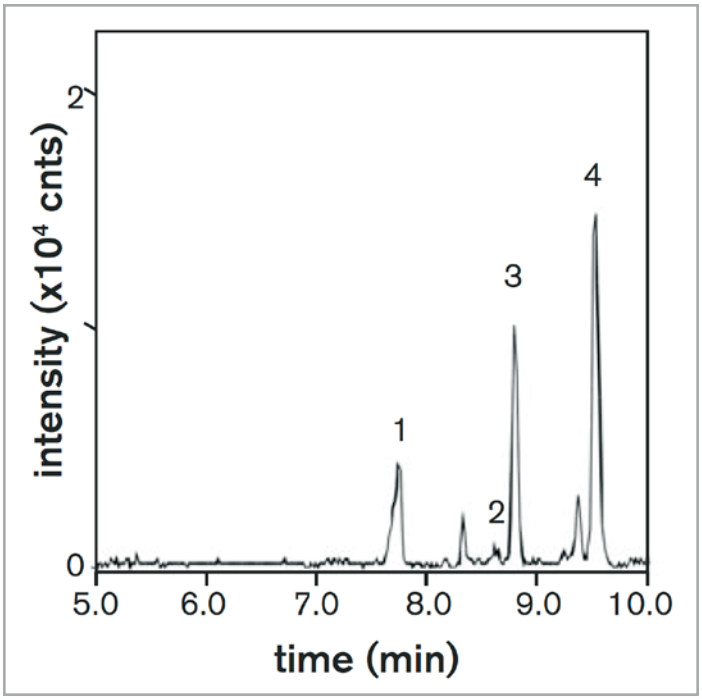

Figure 3. BPE obtained with sheath-liquid CE-MS of a mixture of (1) insulin; (2) carbonic anhydrase II; (3) ribonuclease A and (4) lysozyme (each 50 µg/mL) using a conventional ESI source and a sheath liquid of isopropanol-water-acetic acid (75/25/0.1, v/v/v). For further conditions, see the materials and methods section.

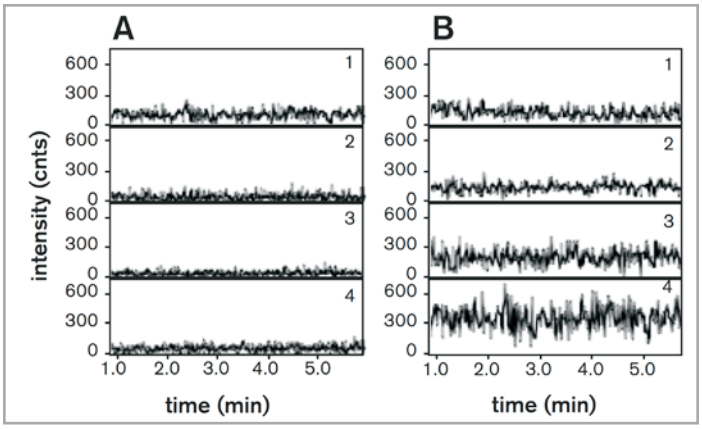

Figure 4. Baseline signal and noise in the 0.9–5.9 min interval during (A) CESI-MS and (B) sheath-liquid CE-MS of the protein test mixture. Traces represent extracted-ion traces for (1) insulin (m/z 1434.1); (2) carbonic anhydrase II (m/z 1262.9); (3) ribonuclease A (m/z 1711.1) and (4) lysozyme (m/z/ 1590.3). For further conditions, see the materials and methods section.

References

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