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Capillary zone electrophoresis–mass spectrometry using a coaxial continuous-flow fast atom bombardment interface

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ABSTRACT

Mixtures of peptides have been analyzed by capillary zone electrophoresis in conjunction with mass spectrometry (MS) using an on-line coaxial continuous-flow fast atom bombardment interface. MS and MS–MS spectra have been acquired in electrophoretic real time from femtomole levels of the peptides, while maintaining separation efficiencies in excess of 100 000 theoretical plates.

INTRODUCTION

Capillary zone electrophoresis (CZE) has proven to be an exceptionally useful analytical tool for the analysis of mixtures of ionic analytes. The high separation efficiency of CZE permits the separation of complex mixtures, and its ability to analyze non-volatile and/or thermally labile species permits the study of biopolymers. The use of mass spectrometry (MS) as a detector for CZE is advantageous since qualitative information about the structure of the analytes can be obtained.

The first successful interface between CZE and MS was reported by Smith and co-workers[1–3] consisting of an electrospray ionization (ESI) interface at atmospheric pressure in conjunction with a quadrupole mass spectrometer. Lee *et al.*[4,5], have also reported the successful coupling of CZE with MS using another atmospheric pressure ionization source —an ion-spray interface (which is a variation of electrospray ionization) coupled with a triple quadrupole mass spectrometer.

In late 1988 work was begun in our laboratories on the coupling of CZE with MS using a modification of the coaxial system developed for coupling open tubular liquid chromatography with continuous-flow fast atom bombardment (coaxial CF-FAB)

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MS[6,7]. The online coaxial continuous-flow CZE–FAB-MS system has been shown[8,9] to be capable of separating peptide mixtures with separation efficiencies of hundreds of thousands of plates, with limits of detection of less than 10 fmol. Caprioli *et al.*[10] and Reinhoud *et al.*[11] have also coupled CZE with CF-FAB-MS using a liquid junction interface.

Since the initial reports of the coupling of CZE with MS using coaxial CF-FAB[8,9], several design modifications have been made to the interface probe. These modifications are reported here, along with applications of the system to the analysis of chemotactic peptides and neuropeptides, under both positive and negative ionization conditions. An example of the suitability of the CZE–coaxial CF-FAB-MS interface for the acquisition of MS–MS data in electrophoretic real time is also given.

EXPERIMENTAL

The fundamentals of our CZE–coaxial CF-FAB-MS interface have been previously reported[8,9]. In this coaxial interface the fused-silica CZE column (13 to 15 μm I.D., 150 μm O.D.) is inserted into the fused-silica sheath column (200 μm I.D., 350 μm O.D.) through which the FAB matrix solution is pumped via a syringe pump (μLC-500, Isco, Lincoln, NE, U.S.A.). All fused-silica capillary columns were obtained from Polymicro Technologies (Phoenix, AZ, U.S.A.). A 1/16-in. (1.59-mm) stainless-steel tee with vespel ferrules (400 μm I.D.) is used to couple the two capillaries. This tee is mounted in the plexiglass handle on the end of the FAB probe shaft. While in practice the CZE capillary can be threaded through the sheath column from either direction, it has been noted that threading from the 1/16-in. tee towards the FAB probe tip is preferred. Arcing between the inside and outside of the CZE capillary (leading to formation of a hole in the capillary wall) occurs more readily when the CZE column has been threaded from tip to tee.

The FAB matrix used with the CZE–FAB-MS system was glycerol–water (25:75), modified with either heptafluorobutyric acid (pH 3.5) or ammonium hydroxide (pH 9), at a flow-rate of approximately 0.5 μl/min. Proper flow-rate of the FAB matrix is readily determined by an observed lack of matrix ions in the mass spectrum (too little flow), or by CZE peak tailing (too much flow). The matrix solution transfer line from the pump to the tee incorporates a 0.5-μm in-line frit filter and a 3 m × 25 μm I.D. fused-silica pressure restriction column to provide sufficient backpressure to the syringe pump for stable operation. The FAB matrix modifiers served both to provide ions for electrical contact between the FAB tip and the CZE column effluent, and to modify the pH of the solution on the FAB probe tip, increasing the production of protonated or deprotonated molecular ions for the MS detection of analytes. Note that this allows the analytes to be separated as either negative or positive ions within the CZE column and detected as either negative or positive ions by the mass spectrometer.

The two coaxial capillary columns terminate at the stainless-steel FAB probe tip (Fig. 1). An important feature of this CZE interface is that the FAB probe tip is used as the electrical "ground" of the CZE system. Thus, active electrophoretic transport of the analytes occurs through the CZE column to the FAB probe tip where ion desorption takes place. This arrangement obviates the use of a transfer line from the end of the CZE capillary to the FAB probe tip. This precludes any zone broadening

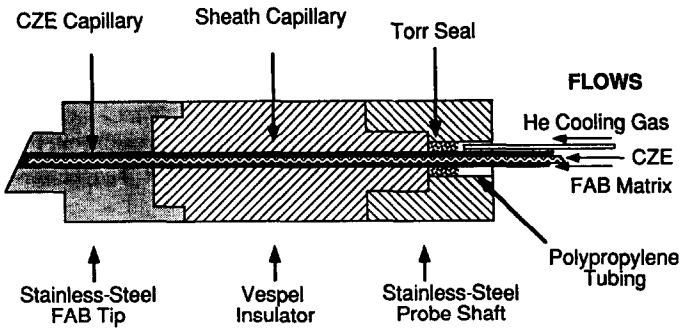

Fig. 1. On-line CZE–coaxial CF-FAB-MS probe tip.

that would occur within the transfer line used between the CZE column and the FAB probe tip, and in the connections between the CZE column and the transfer line. This FAB probe tip is electrically insulated from the probe shaft with a vespel insulator. The sheath column must fit very snugly within the FAB probe tip such that the FAB matrix solution cannot flow back inside the FAB tip. A loose fit between the sheath column and the probe tip will cause the ion source pressure to fluctuate, and consequently, ion intensities. Placement of a septum, teflon tape or Torr Seal in the probe tip helps prevent backflow.

The CZE high-voltage power supply (Glassman High Voltage, Princeton, NJ, U.S.A.) is maintained between -38 to $+38$ kV (depending upon the nature of the CZE–MS experiment), while the FAB probe tip is maintained at a potential of 8 kV for the detection of positive ions, and -8 kV for the detection of negative ions. The potential drop across the CZE column is $+/-30$ kV per meter. A safety interlock system incorporating a high-voltage relay (Model H-25, Kilovac, Santa Barbara, CA, U.S.A.) is used for operator safety, such that opening the lid of the sample/buffer box immediately shorts the CZE high-voltage electrode to ground potential. The high-voltage relay/safety interlock circuit incorporates a timer so that high-voltage switching can be performed for electromigration sample introductions.

Through the length of the FAB probe shaft, the two coaxial fused-silica capillary columns are contained within a polypropylene capillary (1.2 mm I.D., 1.9 mm O.D.), which serves to minimize the possibility of electrical shorts between the coaxial capillaries and the stainless-steel probe shaft. A fused-silica capillary column (200 μm I.D., 350 μm O.D.) is inserted into this polypropylene capillary from the plexiglass handle, terminating near the vespel insulator between the probe shaft and the FAB tip. This fused-silica capillary is used to purge the probe shaft with helium in order to cool the coaxial capillaries, and thus minimize the formation of temperature gradients within the capillaries.

Mass spectrometry

The mass spectrometer used in this work is a VG ZAB-4F (VG Analytical, Manchester, U.K.) four sector mass spectrometer of $B_1E_1E_2B_2$ geometry. An Ion Tech FAB gun was used with xenon as the FAB gas (8 kV at 1 mA). The desorbed ions were accelerated to 8 keV for analysis, and mass spectra were acquired by scanning MS-I (B_1E_1) and detecting the ions with a photomultiplier tube based detector located

in the third-field free region. MS–MS spectra were acquired by using MS-I to select the parent ions and focus them into the collision cell located in the third field-free region. Helium was used as the collision gas (50% parent ion beam suppression). Daughter ion spectra were acquired by a linked scan at constant B/E of MS-II (E_2B_2) with daughter ion detection in the fifth-field free region with a photomultiplier tube based detector. Nominal pressures were $2 \cdot 10^{-5}$ Torr in the ion source, and $5 \cdot 10^{-9}$ Torr in the analyzers.

Chemicals

The morphiceptin, proctolin, Phe–Leu–Glu–Glu–Ile (FLEEI), Met–Leu–Phe (MLF), Val–Gly–Val–Ala–Pro–Gly (VGVAPG), N-formyl Met–Leu–Phe (N-formyl MLF), and glycerol were obtained from Sigma (St. Louis, MO, U.S.A.) and were used as delivered. The water used in the preparation of all sample and buffer solutions had a resistance of >15 MΩ (Milli-Q System, Millipore, Bedford, MA, U.S.A.). Acetic acid, ammonium acetate, ammonium hydroxide and heptafluorobutyric acid were obtained from Aldrich (Milwaukee, WI, U.S.A.). Sample and buffer solutions are degassed and filtered (0.2 μm pore size) immediately prior to use.

RESULTS AND DISCUSSION

The use of volatile buffers such as ammonium acetate has been found to be required for the long term stability of the CZE–MS analysis. The use of non-volatile buffers such as potassium phosphate leads to an unstable ion beam, as well as the formation of potassium adducts of the peptides, $(M + K)^+$, $(M + 2K - H)^+$ (ref. 9), which reduces the intensity of the protonated molecular ion, $(M + H)^+$. All data presented in this report were obtained using a 0.005 *M* ammonium acetate buffer, the pH of which was adjusted to 8.5 using ammonium hydroxide. At this pH all peptides had a net negative charge except for those peptides containing more than one arginine and/or lysine amino acid. Conversion of the negatively charged species to positive ions for MS detection was accomplished by using a FAB matrix acidified to pH 3.5 with heptafluorobutyric acid.

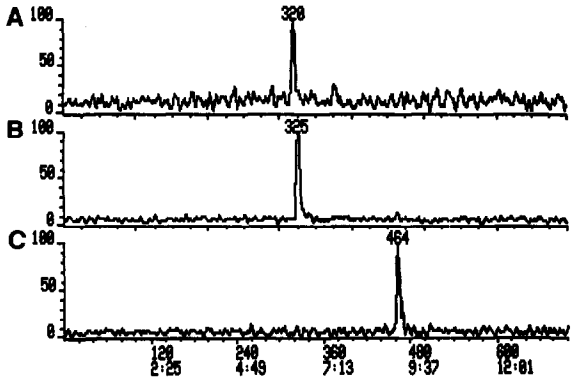

Fig. 2. Single ion electropherograms of the protonated molecular ions of (A) morphiceptin (30 fmol, number of plates $N = 91\ 000$), (B) proctolin (24 fmol, $N = 120\ 000$), and (C) FLEEI (24 fmol, $N = 190\ 000$). Time in min:s.

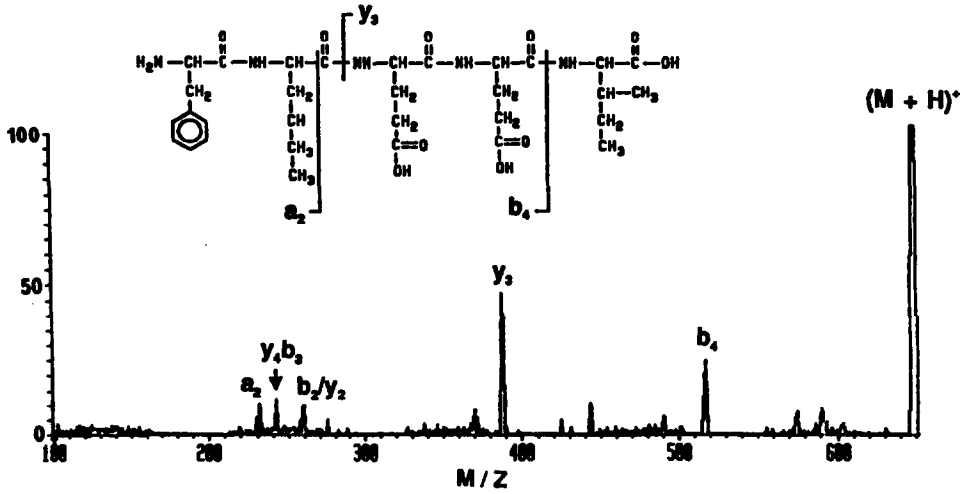

Fig. 3. On-line CZE–MS–MS spectrum of FLEEI.

The CZE–MS analysis of a mixture of three neuropeptides is given in Fig. 2. The sample ($2 \cdot 10^{-4}$ *M*) was introduced onto the CZE column using an electromigration sample introduction for 2 s at a potential of 6 kV. This corresponds to the introduction of between 24 to 30 fmol per component, with separation efficiencies ranging from 91 000 plates for morphiceptin to 190 000 plates for FLEEI.

The CZE–coaxial CF-FAB-MS interface has been used for the acquisition of MS–MS data in electrophoretic real time. In order to acquire CZE–MS–MS data with our system multiple analysis are performed. The first analysis is used to identify the mass–charge ratios of the analytes (parent ions). The analysis is then repeated, with the parent ions being subjected to collisionally activated dissociation, yielding an MS–MS daughter ion spectrum. As an example, Fig. 3 gives the on-line CZE–coaxial FAB-MS–MS spectrum of the pentapeptide FLEEI, obtained from a peptide mixture at a concentration of $1.5 \cdot 10^{-4}$ *M*. The MS–MS daughter ion spectrum contains sufficient structural information to confirm the identity of the parent ion.

The utility of CZE–MS for the analysis of low levels of peptides and their impurities is exemplified by the analysis of chemotactic peptides given in Fig. 4. This mixture of VGVAPG (14 fmol) and MLF (16 fmol) was found to contain an impurity peak, the mass–charge ratio of which is 16 units above that of MLF, suggesting the possibility of an oxidation product of MLF. MS–MS analysis of the impurity peak revealed that a fraction of the MLF in solution had been oxidized, with the site of oxidation being on the methionine side chain. The total amount of MLF injected onto the CZE column was approximately 16 fmol, and the signal-to-noise ratios of the two single ion electropherograms suggest that approximately 1/3 of the MLF had oxidized (assuming equivalent FAB–MS response factors for MLF and oxidized MLF).

In the majority of our applications, peptides are separated by CZE as negative ions but detected by MS as positive ions. The change in charge takes place on the tip of the FAB probe, where the basic CZE buffer mixes with the acidic FAB matrix (pH 3.5). Since the flow-rate of the FAB matrix solution is at least an order of magnitude higher than the CZE electroosmotic flow-rate, the buffer's capacity is exhausted, and the

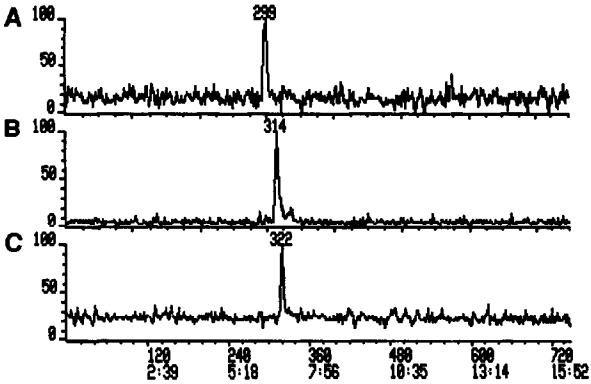

Fig. 4. Single ion electropherograms of the protonated molecular ions of (A) VGVAPG (14 fmol, N = 10 000), (B) MLF (<16 fmol, N = 31 000), and (C) oxidized MLF (<16 fmol, N = 32 000).

conversion to positive ion readily occurs. The CZE–coaxial CF-FAB-MS system can easily be used for the detection of negative ions by adding ammonium hydroxide to the FAB matrix (pH 9). This is illustrated by the CZE–MS analysis of MLF and N-formyl MLF as negative ions (Fig. 5). The detectivity of these negative ions (MLF, 150 fmol, N-formyl MLF, 46 fmol) with this system is within an order of magnitude of that observed when detecting them as positive ions.

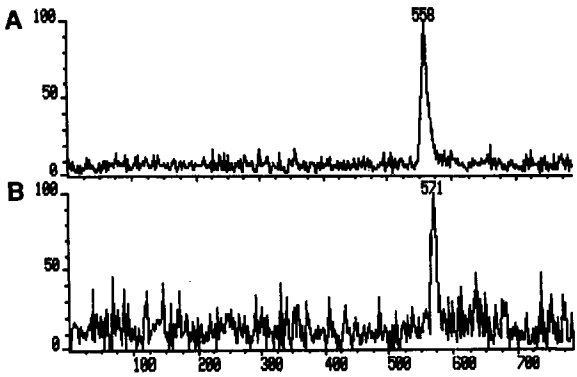

Fig. 5. Single ion electropherograms of the deprotonated molecular ions of (A) MLF (150 fmol) and (B) N-formyl MLF (46 fmol).

CONCLUSIONS

The on-line CZE–coaxial CF-FAB-MS system has been used for the acquisition of MS and MS–MS data in electrophoretic real time from femtomole levels of peptides, with separation efficiencies in excess of 100 000 plates. The ability of the coaxial interface to independently deliver the CZE effluent and the FAB matrix to the FAB probe tip permits the independent optimization of the composition and flow-rate of the two fluids, yielding experimental flexibility, as illustrated by the CZE separation of analytes as negative ions, with their MS detection as either positive or negative species.

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