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On-Column Sample Enrichment for Capillary Electrophoresis Sheathless Electrospray Ionization Mass Spectrometry: Evaluation for Peptide Analysis and Protein Identification

George M. Janini,*,† Ming Zhou,† Li-Rong Yu,† Josip Blonder,† Michelle Gignac,‡ Thomas P. Conrads,† Haleem J. Issaq,† and Timothy D. Veenstra†

Laboratory of Proteomics and Analytical Technologies and Image Analysis Laboratory, SAIC-Frederick, Inc., National Cancer Institute at Frederick, P.O. Box B, Frederick, Maryland 21702

Although several designs have been advanced for coupling sample enrichment devices to a sheathless electrospray ionization-mass spectrometry (MS) interface on a capillary electrophoresis (CE) column, most of these approaches suffer from difficulties in fabrication, and the CE separation efficiency is degraded as a result of the presence of coupling sleeves. We have developed a design that offers significant improvements in terms of ease of fabrication, durability, and maintenance of the integrity of the CE-separated analyte zones. Capillaries with different inside and outside diameters were evaluated to optimize the performance of the CE−MS system, resulting in a mass limit of detection of 500 amol for tandem MS analysis of a standard peptide using a 20-μm-i.d. capillary. The improved design incorporates an efficient method to preconcentrate a sample directly within the CE capillary followed by its electrophoretic separation and detection using a true zero dead-volume sheathless CE−MS interface. Testing of this novel CE−MS system showed its ability to characterize proteomic samples such as protein digests, in-gel-digested proteins, and hydrophobic peptides as well as to quantitate ICAT-labeled peptides.

Capillary electrophoresis (CE) is well known for its ability to provide highly selective separations. Its direct coupling with mass spectrometry (MS) has shown great potential in proteomic analysis as demonstrated by many laboratories[1−22] and detailed in several reviews.[23−26] CE−MS has many advantages compared to the more widely used technique of liquid chromatography (LC)−MS, including separation efficiency, speed, and simplicity. Since the CE capillary is constructed using open tubular fused silica, contamination due to carry-over effects from separation to separation is minimized. The time between consecutive CE experiments is much shorter than LC-based separations with gradient elution, since the CE capillary column does not require extensive reequilibration between analyses. On the negative side, however, CE has generally lagged behind LC in terms of ruggedness, sample capacity, and detection sensitivity.

While attomole mass limit of detection (MLOD) have been reported in several CE−MS applications,[1,2,4,8,16,20,21] the concentration limit of detection (CLOD) using conventional CE−MS capillaries is in the micromolar range compared to the nanomolar range for LC−MS. This disparity is mainly a result of the large sample volume capacity of a capillary LC column (i.e., microliters) compared to a CE capillary (i.e., nanoliters). This loading capacity limitation hampers the utility of CE−MS for the analysis of typical biological samples, where proteins and peptides are often present at submicromolar concentrations. In addition, most commercial CE−MS systems use coaxial sheath flow that lowers the overall

* To whom correspondence should be addressed. Phone: 301-846-7189. Fax: 301-846-6037. E-mail: Janini@mail.ncifcrf.gov.

† Laboratory of Proteomics and Analytical Technologies.

‡ Image Analysis Laboratory.

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sensitivity of the analysis due to the high sheath solvent flow rate and analyte dilution prior to ionization. A solution to this problem is to enrich the sample off-line prior to CE−MS analysis; however, this requires extra manipulations that invariably result in sample loss. On-column sample concentration is more desirable if it can be accomplished without a concomitant loss of CE separation efficiency.

There are two distinctly different methods for on-column sample enrichment, namely, electrophoretic stacking and chromatographic concentration.[27] In electrophoretic stacking, the capillary is partially filled with a solution of analytes, which are stacked in a narrow zone by exploiting differences in conductivity between the sample and CE buffer. In chromatographic concentration, analytes from a large-volume injection are concentrated on a miniature cartridge filled with chromatographic material placed near the injection end of the CE capillary. While electrophoretic stacking can be used to load larger amounts of sample in the CE capillary, it is difficult to fill more than half of the column volume without diminishing the effective separation length of the capillary and creating artifacts that disrupt the CE separation process. In contrast, it is possible to load multiple column volumes on a CE capillary equipped with a chromatographic cartridge and concentrate the analytes severalfold with minimal disruption to the CE separation process. On-column chromatographic concentration was first introduced by Guzman et al.[28] and further developed for CE−MS applications by others.[2,20−22,29−41] In most of these studies,[20−22,32−39] a solid-phase extraction (SPE) cartridge was constructed, where the solid packing material was held inside the capillary by two frit structures. An alternate packing material, namely, a polymeric membrane impregnated with a chromatographic stationary phase, such as C_{18}, was also used for CE−MS applications.[2,29−31,40,41] Membrane-based SPE devices do not require the use of retainers to hold the chromatographic material in position.

In a recent publication,[1] we described a novel sheathless CE electrospray ionization MS interface, where the separation capillary, an electrically conductive junction, and a spray tip are all integrated on a single piece of a fused-silica capillary with the same inside diameter along the entire length. The main advantage of this interface, besides ease of fabrication and durability, is that the CE-separated zones are not broadened in the interface prior to MS analysis. While this publication[1] reported on the use of a single-dimension capillary, we subsequently evaluated capillaries with different inside and outside diameters to optimize the overall performance of the CE−MS analysis and to demonstrate its utility for the study of peptides and proteins. In addition, the sensitivity of the original sheathless CE−MS design was improved by incorporating a membrane-based, solid -phase extractor (mSPE) to preconcentrate samples directly within the capillary prior to electrophoretic separation and MS detection.

EXPERIMENTAL SECTION

Materials. Sequencing grade-modified trypsin was from Promega (Madison, WI). Acetic acid and formic acid were obtained from Fluka Chemical Corp. (Milwaukee, WI), 48% hydrofluoric acid was obtained from Aldrich (St. Louis, MO), HPLC-grade acetonitrile (ACN) was purchased from EM Science, Merck (Darmstadt, Germany), 5-min epoxy was obtained from Devcon (Riviera Beach, CA). [Glu[1]]-fibrinopeptide B, equine apomyoglobin, horse heart cytochrome *c*, bovine serum albumin (BSA), lyophilized purple membranes from *Halobacterium halobium*, enriched for bacteriorhodopsin, and ammonium bicarbonate, were purchased from Sigma (St. Louis, MO). Immobilized monomeric avidin was obtained from Pierce Chemical Co. (Rockford, IL). The water used for all of the experiments was doubly distilled and deionized using a NANOpure Diamond water system (Barnstead Internations, Dubuque, IA).

Interface Fabrication. A spray tip was made at the end of an 80-cm-long fused-silica capillary (Polymicro Technologies, Phoenix, AZ) by applying heat while pulling gently to form a long tapered tip, which was later trimmed to the desired inside diameter (i.d.) using a glass tube cutter. This step was only used with the more conventional 50- and 75-μm-i.d. capillaries to reduce the i.d. to ∼25−30 μm. For narrower i.d. columns, the capillary wall was thinned by etching with HF to reduce the outside diameter (o.d.) without affecting the i.d. Next, the polyimide coating was shaved off one side of the circumference of a 2−3-mm length of the capillary at a distance of 5−7 cm from the spray tip end, keeping the polyimide coating on the other side to protect it from HF etching. The capillary was then trimmed to the desired length and mounted on the porous junction assembly as shown in Figure 1. The porous junction assembly was constructed from a 4.5 cm × 1.5 cm Plexiglas slide, in which a groove slightly deeper than the capillary o.d. was milled along its length, and a 1.5 cm long × 1 cm i.d. Plexiglas reservoir that was attached to the top of the slide. The capillary was threaded into the groove until the exposed segment of the capillary was centered inside the reservoir. Epoxy glue was applied to the outside of the reservoir around the capillary to seal the two holes and hold the capillary firmly onto the slide. The HF etching reaction was started by filling the reservoir with 20% HF and incubating at ambient temperature.[2,42,43] (CAUTION: HF should only be handled with gloves in a ventilated hood). The inlet side of the capillary was threaded into the detector end of a windowless capillary cartridge of a P/ACE, System MDQ CE instrument (Beckman Coulter, Fullerton, CA) and the spray tip end extended to the outside of the instrument.

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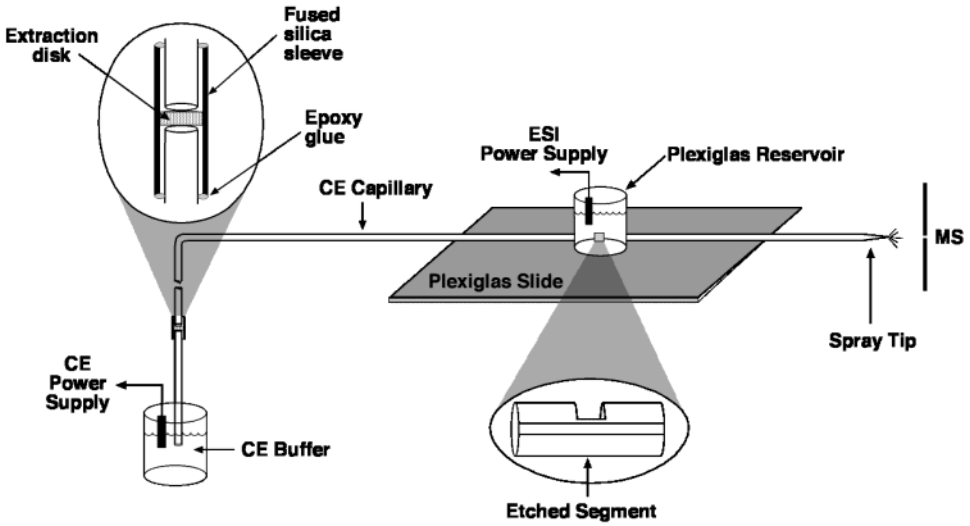

Figure 1. Schematic diagram of the CE capillary with on-column mSPE cartridge−sheathless ESI-MS interface.

After the capillary was washed with 0.1 M NaOH and water, the injection end was inserted into a vial filled with 1.0 M acetic acid. Low pressure was applied to fill the capillary with acetic acid and provide continuous flow throughout the duration of the etching procedure. A positive voltage (5−10 kV, depending on the capillary i.d.) was applied to the acetic acid vial, and a platinum electrode was inserted in the HF filled reservoir and connected to ground. The etching reaction was monitored by recording the MDQ current reading and by visual inspection of the spray tip. As long as there was no electrical contact through the capillary wall, the electrical circuit was not closed and the current reading was close to 0 μA. As soon as the capillary wall was thinned enough to be electrically conductive, the current reading instantaneously increased to several microamperes and droplets could be seen emitting from the spray tip. The etching reaction was immediately terminated by removal of the HF and rinsing the reservoir liberally with water. The total etching time using 20% HF can be as high as 360 min for capillaries with 360 μm o.d. × 50 μm i.d. and as low as 90 min for capillaries with 150 μm o.d. × 50 μm i.d. For capillaries of the same dimensions, the required etching time is reproducible to within ±10 min. Although etching proceeds much more rapidly with 48% HF,[43] its use was avoided because of its corrosiveness, which causes the Plexiglas to turn cloudy. After the porous junction is mounted on the nanospray assembly, the reservoir is filled with electrolyte and a platinum electrode is inserted in the reservoir and connected to a power supply to complete the CE electrical circuit and provide the spray voltage.

Surface scanning and cross-sectional scanning electron images of a 360 μm i.d. × 75 i.d capillary before and after HF etching were photographed with a Hitachi S-570 scanning electron microscope (Hitachi, Tokyo, Japan) operated at 10 kV after coating the capillary segments with gold−palladium in a vacuum evaporator.

Construction of an On-Column mSPE Cartridge. A miniaturized mSPE cartridge was attached to the CE capillary near the injection end as shown in Figure 1. The cartridge was constructed using a piece of a fused-silica capillary with an i.d. that matched the o.d. of the CE separation capillary. A C_{18}-impregnated Empore extraction disk, 47 mm × 0.5 mm thickness, (3M Filtration Products, St. Paul, MN) was placed on a clean, flat surface, and a 10-mm-long fused-silica capillary sleeve (185 um i.d. × 365 μm o.d.) was positioned over the disk, gently pushed down through it, pulled up several times to introduce multiple pieces of the disk inside the sleeve until ∼3 mm of the sleeve was packed. The polyimide coating was stripped off 3−4 mm from the injection end of the CE capillary and slid inside the sleeve behind the packing until all the polyimide-stripped length of the CE capillary was within the sleeve. Similarly, the polyimide coating was stripped off a short piece of capillary of the same dimensions as the CE capillary and slid in the other end of the sleeve such that it gently butted up against the packing material. The sleeve was glued to the capillaries by dipping it into a layer of epoxy that was smeared on a 10 mm × 5 mm piece of thin polystyrene sheet. The whole procedure can be completed within 15 min. If the cartridge gets clogged or contaminated with sample matrix, it can be easily cut and replaced on the same CE separation capillary.

Analytical Conditions for mSPE Sample Loading and CE Operations. The capillary used for the mSPE-CE−MS experiments was 60 cm long × 75 μm i.d. × 185 μm o.d. with a spray tip i.d. of 30 μm and a column volume of 2.6 μL. The mSPE cartridge was 1 cm long × 185 μm i.d. × 350 μm o.d. with an injection end that was 12 cm long × 75 μm i.d. × 185 μm o.d. The maximum loading capacity of the mSPE was estimated by multiplying the volume of the membrane plug (∼13.2 nL) by its density (∼0.74 g/mL). Assuming that it is possible to load ∼5% (w/w) of the plug packing weight, then the maximum loading capacity of the mSPE cartridge is ∼500 pg, which translates to ∼350 fmol for a peptide having a molecular mass of 1500 Da.

Prior to loading the sample, the capillary was sequentially washed with 5 plug volumes of methanol followed by 10 plug volumes of wash solution (95% of 0.1% acetic acid in water/5% ACN). After the sample was applied, the capillary was rinsed with 10 plug volumes of wash solution to remove salts and matrix components. The capillary was then filled with separation buffer (95% 1.0 M acetic acid/5% ACN) followed by the introduction of 2 plug volumes of 20% of 0.1% acetic acid/80% ACN to elute the sample off the mSPE packing. These steps were accomplished by applying pressure to the capillary injection end for a specific period of time. The flow rates of the solvents delivered through

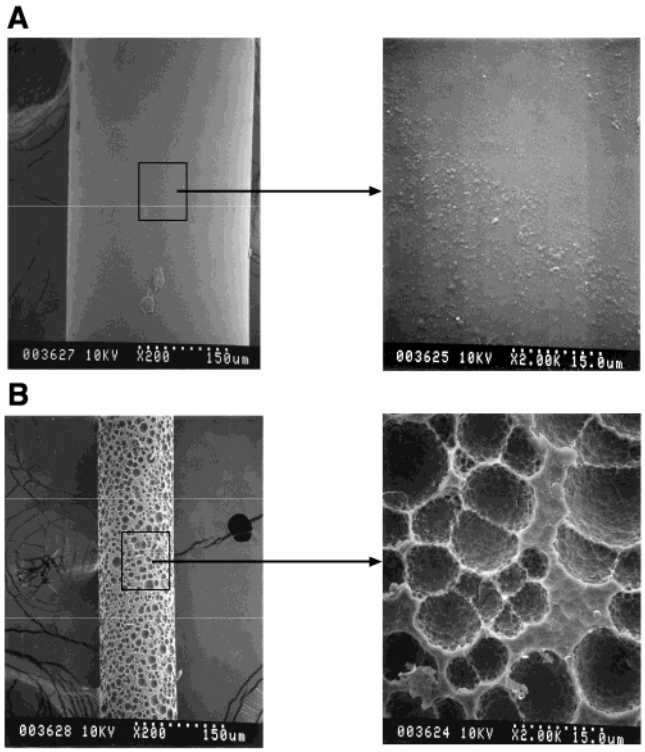

Figure 2. Surface view scanning electron images of a 360 μm o.d. $\times$ 75 μm i.d. fused-silica capillary (A) before and (B) after HF etching. Magnification and scales are given under the images.

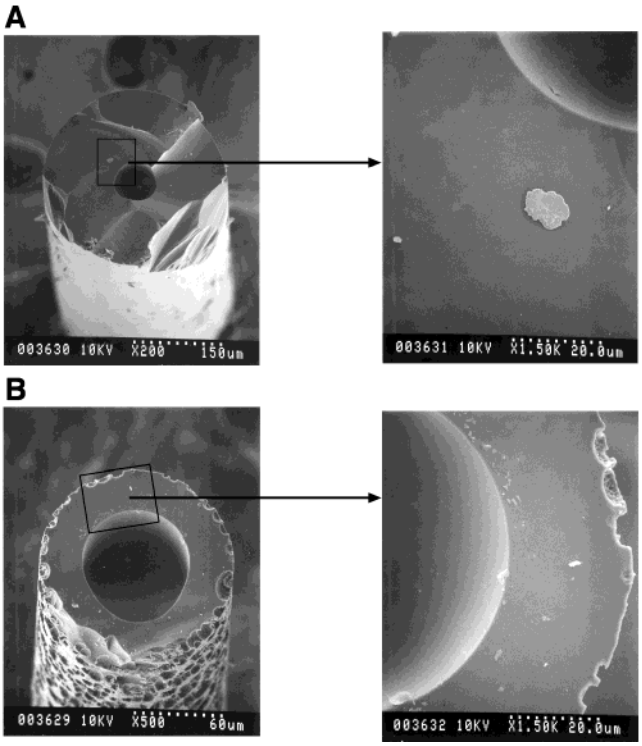

Figure 3. Cross-sectional view scanning electron images of a 360 μm o.d. $\times$ 75 μm i.d. fused-silica capillary (A) before and (B) after HF etching. Magnification and scales are given under the images.

the capillary were monitored by collecting and measuring the volume of the effluent from the spray tip in a graduated pipet. For the capillary described above, the flow rates were measured as 60 nL/min at 0.5 psi (1 psi = 6894.76 Pa), 120 nL/min. at 1 psi, 1.1 μL/min. at 10 psi, and 2.2 μL/min at 20 psi. The rinse steps were carried out using a pressure of 10 psi, the capillary was filled using 20 psi, and the sample was loaded onto the mSPE cartridge using 10 psi and bumped off the packing using 0.5 psi.

CE−MS. A P/ACE System MDQ CE instrument was used to conduct the CE separations (Beckman Coulter, Fullerton, CA). The MDQ instrument was configured to accept a windowless capillary cartridge, where the inlet side of the capillary was threaded into the detector end of the cartridge and the spray tip end extended to the outside of the instrument. An ion trap mass spectrometer (LCQ-DECA XP, ThermoFinnigan) equipped with a nanoelectrospray ionization source was used for all CE−MS and mSPE-CE−MS experiments. Both the MS and the CE instruments were controlled using ThermoFinnigan Xcalibur software (ThermoFinnigan, San Jose, CA). The porous junction assembly was mounted on the nanoelectrospray source and the capillary spray tip positioned using the XYZ stage. The reservoir was filled with the CE running electrolyte and the electrospray voltage was supplied by an external power supply (Series EL, Glassman High Voltage Inc., High Bridge, NJ 08829). For MS analysis, the electrospray voltage was adjusted between 1.5 and 2.0 kV for optimum electrospray stability using a capillary temperature of 180 °C for all experiments. The instrument was operated in a data-dependent tandem MS mode in which each full-scan mass spectrum was followed by a tandem MS scan of the most intense ion observed in the previous scan. Normalized collision energy was set to 38%. Fragment ion spectra were searched using SEQUEST (ThermoFinnigan).

Protein Digestion. Horse heart cytochrome *c* and equine apomyoglobin were dissolved in 100 mM NH_4HCO_3, pH 8.2, to a final concentration of 0.1 mg/mL and digested with trypsin for 18 h at 37 °C at a protein/trypsin ratio of 50:1 (w/w). The digest was lyophilized to dryness and resuspended in 0.1% formic acid prior to CE−MS analysis.

Isotope-Coded Affinity Tag Labeling. Fifty micrograms of BSA was labeled (1:1) with either the ^{12}C or the ^{13}C version of the isotope-coded affinity tag (ICAT) reagents as per manufacturer's instructions (Applied Biosystems, Framingham, MA). ICAT-labeled cysteinyl peptides isolated from the avidin affinity column were lyophilized to dryness and stored at −20 °C until CE−MS analysis.

***Halobacterium* Purple Membrane Preparation.** Five hundred micrograms of a lyophilized *Halobacterium* purple membrane sample was dissolved in 400 μL of 50 mM NH_4HCO_3, pH 7.9, and 600 μL of methanol. Tryptic digestion was carried out using a 1:20 (w/w) trypsin-to-protein ratio. Proteolysis was quenched after 6 h by rapidly freezing the digestate in liquid nitrogen, and the sample was stored at −80 °C until CE−MS analysis. Prior to CE−MS analysis, the sample was desalted using a C_{18} reversed-phase SPE column (Varian Inc., Harbor City, CA) as per the manufacturer's protocol. The peptides recovered from the SPE column were lyophilized to dryness and resuspended in 60% methanol/40% water containing 0.1% formic acid to a final concentration of ~0.4 μg/μL.

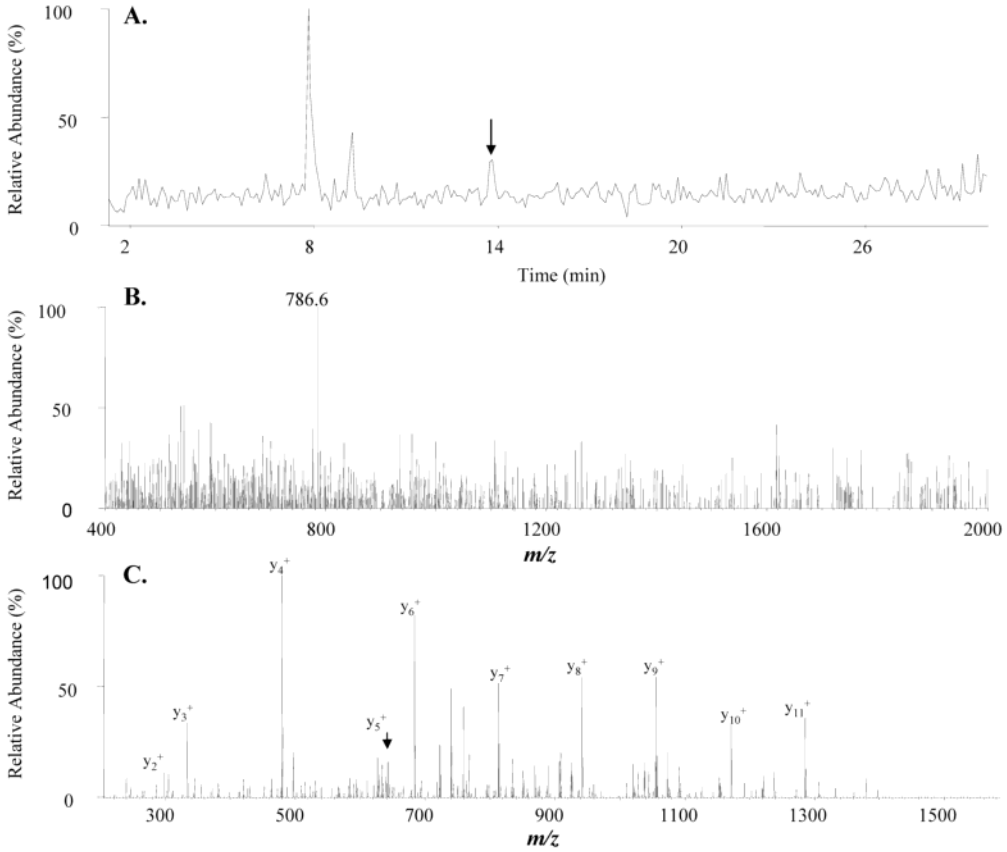

Figure 4. CE−ESI-tandem MS of 500 amol of [Glu[1]]-fibrinopeptide B. (A) Base peak electropherogram; (B) full-scan mass spectrum of the $[M + 2H]^{2+}$ [Glu[1]]-fibrinopeptide B molecular ion; and (C) tandem MS fragment ion spectrum of the *m*/*z* 786.6 ion. Conditions: column, bare fused-silica capillary, 60 cm × 185 μm o.d. × 20 μm i.d.; tip, 20 μm i.d. × 75 μm o.d.; separation voltage, 15 kV; observed CE current, 1.5 μA; running electrolyte, 1.0 M acetic acid, pH 2.4; temperature, 22 °C; sample concentration, 0.25 μM; injection time, 10 s at 2 psi (~2-nL total injection volume), Other experimental conditions, as in the Experimental Section.

Protein In-Gel Digestion. Coommassie blue-stained protein gel bands were excised and digested with trypsin, as described.[44] The digests were desalted with C_{18} ZipTips (Millipore, Bedford, MA) as per manufacturer's protocols, lyophilized to dryness, and stored at −20 °C until CE−MS analysis.

RESULTS AND DISCUSSION

Characterization of the Porous Junction. Surface scanning and cross-sectional scanning electron images of a 360 μm o.d × 75 μm i.d capillary before and after HF etching are shown in Figures 2 and 3, respectively. Over the course of the etching reaction, the capillary wall thins to about 25−30 μm. The wall thickness of the etched segment shown in Figure 3 (27 μm) is determined using the scale given in the enlarged segment of the figure. Examination of Figure 3B reveals micrometer-sized pores on the outer surface of the etched segment but no observable changes across the wall thickness, indicating that any pores across the wall would have diameters in the low-nanometer range, below the resolution limit of the electron microscope. During the course of this investigation, several interfaces were constructed with capillaries of different outer and inner diameters and were used over extended periods of time (>7 days). Although the thin wall is fragile, the porous junction is durable as long as it is handled gently.[1,2,42,43] It was also observed that porous junctions that were constructed from newly manufactured capillaries were more durable compared to those constructed from older capillaries that were stored on the shelves for extended periods of time.

Optimization of Capillary Parameters. To improve our original sheathless CE−MS design,[1] we investigated the effect of the capillary's dimensions (o.d. and i.d.) with respect to their durability, practicality, and sensitivity. In the original publication,[1] only 360 μm o.d × 75 μm i.d capillaries were used; however, in the present study, thinner capillaries were tested. The porous junctions made from capillaries with 150−200-μm o.d. are more durable and less prone to breaking during manipulation than the 360-μm-o.d. capillaries. This increased durability may be related to the increased flexibility of the narrower capillaries, allowing them to be manipulated without transmitting large mechanical forces to the porous junction. An added advantage is that thinner capillaries are more efficient in dissipating Joule's heating generated inside the capillary by the CE process. This heat dispersion is especially advantageous in CE−MS applications where part of the capillary is not covered by the cooling system of the CE instrument. The durability of the porous junction was also improved by modifying the procedure for the removal of the polyimide protective coating prior to HF etching, as discussed earlier in the Experimental Section. Retaining the coating on one side of the circumference of the capillary provided better support

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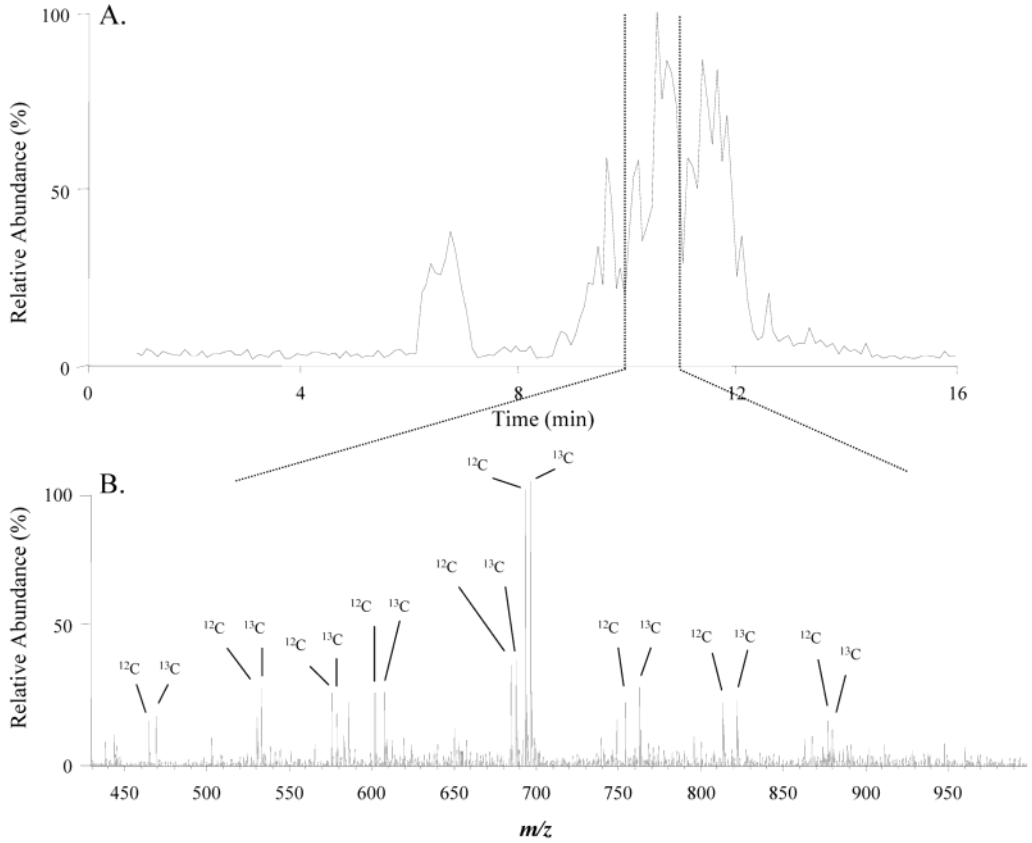

Figure 5. CE−ESI-tandem MS base peak of 4 fmol of a tryptic digest of ICAT-labeled BSA. (A) Base peak electropherogram; (B) section of the full-scan mass spectrum for the migration time window 10−11 min. Conditions: column, bare fused-silica capillary, 60 cm × 185 μm o.d. × 30 μm i.d.; tip, 30 μm i.d. × 75 μm o.d.; separation voltage, 15 kV; observed CE current, 2.5 μA; sample concentration, 1.5 μM. Other experimental conditions as in Figure 4.

for the etched capillary wall compared to the alternative procedure of completely stripping the polyimide coating.[1,2,42,43]

In general, ESI efficiency and CE−MS sensitivity increase with decreasing capillary i.d. since narrower i.d. capillaries emit smaller droplets from more focused analyte zones resulting in increased desorption and ionization efficiency.[4,45] Another advantage of smaller i.d. capillaries is that they generate lower CE currents, which are compatible with those generated during ESI. This compatibility is important since the CE low-voltage electrode and the ESI power supply electrode share a common point, making it necessary to balance the currents throughout the entire system.[46] To evaluate the effect of capillary i.d. on the sensitivity of the present CE−MS system, serial dilution experiments were conducted using the model peptide [Glu[1]]-fibrinopeptide B. Four capillaries of the same length (60 cm) with i.d.s of 75, 50, 30, and 20 μm were compared for MLOD. The results from an injection of 500 amol from a 250 nM solution of [Glu[1]]-fibrinopeptide B analyzed by CE−ESI-MS using a 20-μm-i.d. capillary are shown in Figure 4. The $[M + 2H]^{2+}$ molecular ion (m/z 786.6) shown in Figure 4B has a signal-to-noise ratio (S/N) = 3, which is typically considered a MLOD. Information from the fragment ion spectrum (Figure 4C) was used to search a nonredundant protein database (http://www.ncbi.nih.gov) using the program SEQUEST, which resulted in the positive identification of [Glu[1]]-fibrinopeptide B with a Xcorr score of 4.2 (data not shown). This result is in reasonably good agreement with the results of Figeys et al.,[8] who reported a MLOD of 600 amol for tandem MS mode using a 20-

Table 1. List of Peptides Identified from Tandem MS Analysis of ICAT-Labeled Bovine Serum Albumin

sequence	MH^+	charge state	Xcorr
R.LC#VLHEK.T	1077.61	2	2.11
K.SHC*IAEVEK.D	1242.62	2	2.63
K.SHC#IAEVEK.D	1251.64	2	2.17
K.C*C*TESLVNR.R	1478.72	2	2.14
K.SLHTLFGDELC*K.V	1589.80	2	3.48
K.SLHTLFGDELC#K.V	1598.82	2	3.34
K.YIC*DNQDTISSK.L	1613.75	2	2.33
K.YIC#DNQDTISSK.L	1622.77	2	2.90
K.DDPHAC*YSTVFDK.L	1724.76	3	2.28
K.DDPHAC#YSTVFDK.L	1733.78	3	2.23
K.LKPDPNTLC*DEFK.A	1746.88	3	3.03
K.LKPDPNTLC#DEFK.A	1755.90	3	3.10
R.MPC*TEDYLSLILNR.L	1894.94	2	3.72
R.MPC#TEDYLSLILNR.L	1903.96	2	3.99
R.RPC#FSALTPDETYVPK.A	2060.05	3	2.50
K.LFTFHADIC*TLPDTEK.Q	2078.03	3	2.80
K.LFTFHADIC#TLPDTEK.Q	2087.05	3	2.86
K.EC*C*HGDLLEC*ADDR.A	2259.99	3	2.70
K.C*C*AADDKEAC*FAVEGPK.L	2438.12	3	2.69
K.C#C#AADDKEAC#FAVEGPK.L	2465.18	3	2.55
K.DAIPENLPPLTADFAEDKDVC*K.N	2628.29	2	3.06
K.DAIPENLPPLTADFAEDKDVC#K.N	2637.31	2	3.65

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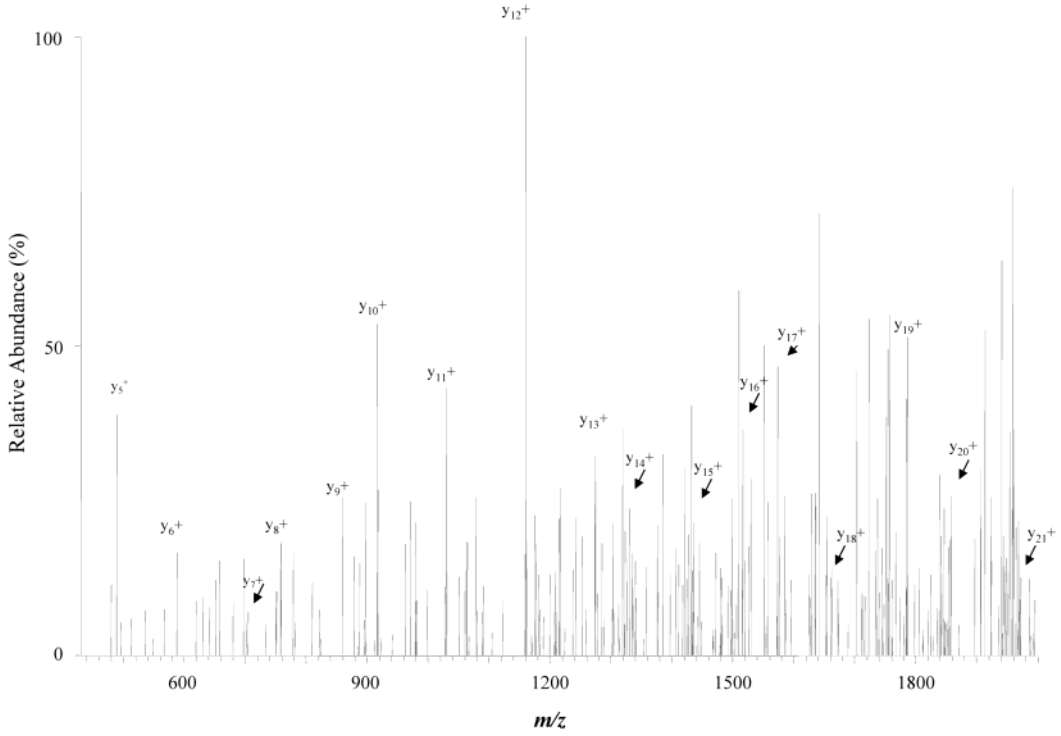

Figure 6. Tandem MS fragment ion spectrum of a hydrophobic tryptic peptide from bacteriorhodopsin. Boldface letters refer to lipid bilayer-embedded amino acid residues.

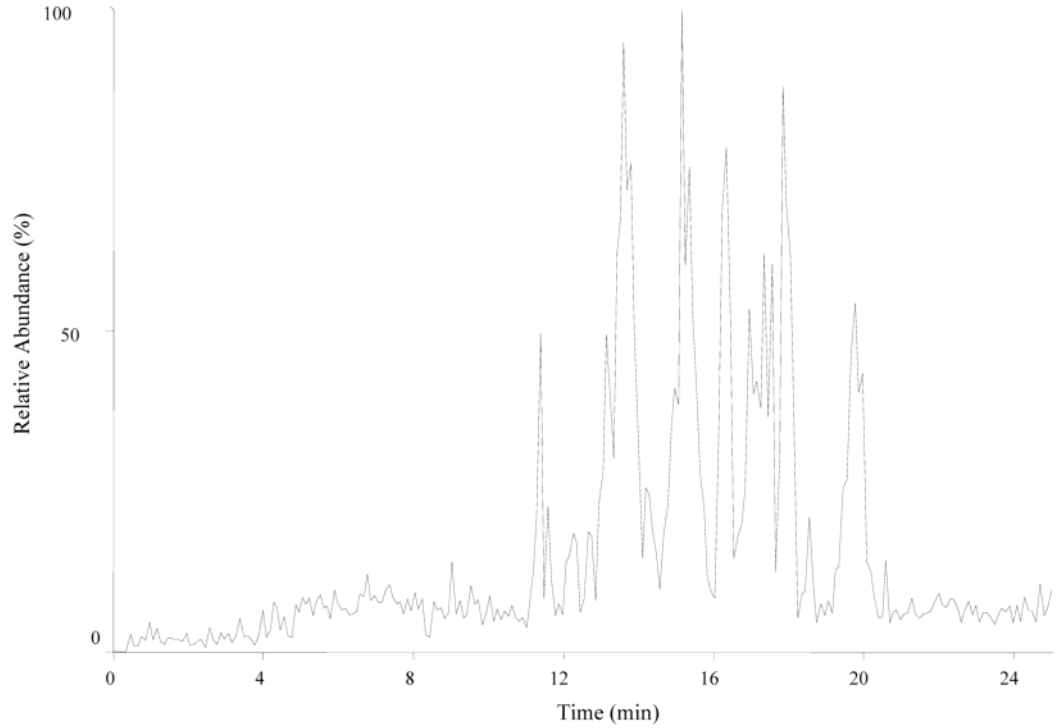

Figure 7. Base peak electropherogram from mSPE-CE−MS analysis of the tryptic digest of a 10 nM solution of cytochrome *c* and apomyoglobin. Conditions: sample, 55 fmol of each protein digest; sample loading, 5 min at 10 psi (~5.5 μL); running electrolyte, 95% 1 M acetic acid, pH 2.4/5% ACN; separation voltage, 15 kV; observed CE current, 11 μA; temperature, 22 °C; supplementary pressure, 0.5 psi; supplementary flow, 60 nL/min. Other experimental conditions as in the Experimental Section.

μm capillary. The MLOD was determined to be 625 amol for the 30-μm capillary, 900 amol for the 50-μm capillary, and 5 fmol for the 75-μm capillary using different concentrations of the same peptide standard solution. An obvious benefit of the use of narrower i.d. capillaries is the ability to obtain meaningful CE−MS data on lower quantities of material; however, there are other factors to be considered for the selection of the optimum capillary i.d. for CE−MS applications, including practicality and durability.

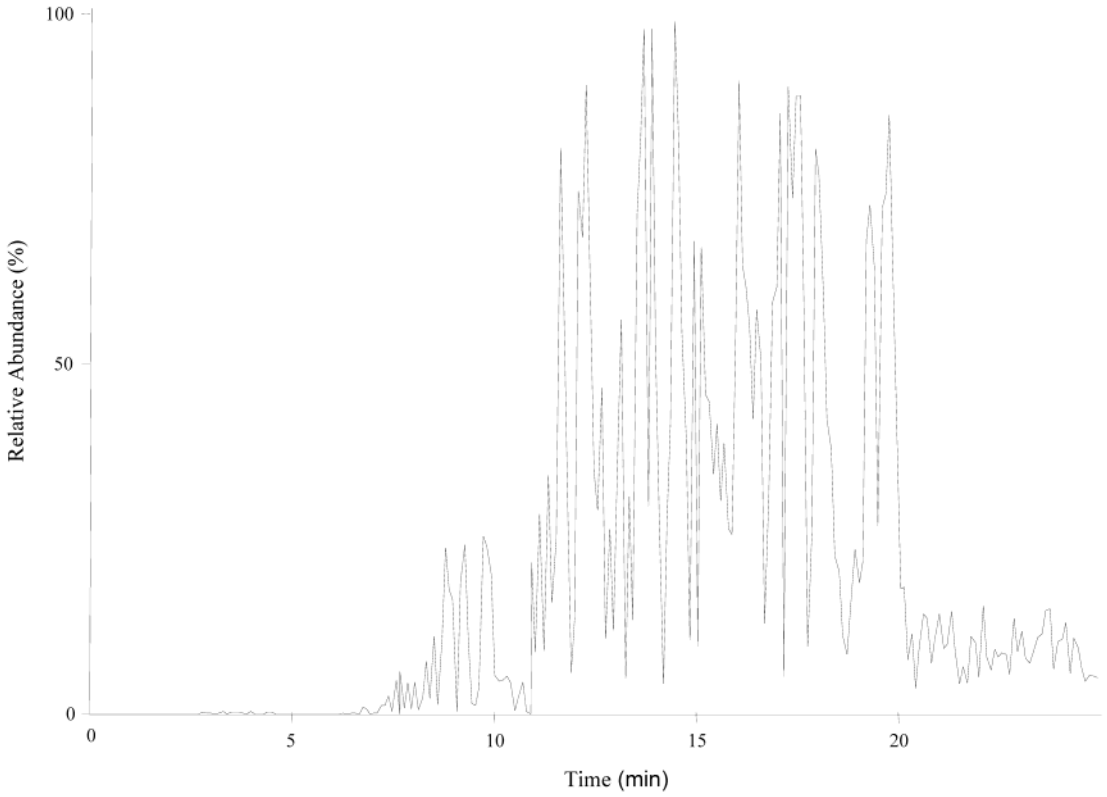

Figure 8. Base peak electropherogram from mSPE-CE−MS analysis of 5 μL (75 ng) from in-gel trypsin digestion of a Coomassie blue-stained protein spot.

The disadvantage of employing narrow-diameter capillaries ($\leq$20 μm) in our experience, as well as others,[4] is that they are less durable because they are prone to blockage. Moreover, it is difficult to maintain a stable spray with these capillaries because of the low electroosmotic flow rate. Although slightly less sensitive, 30-μm capillaries were selected for the applications presented in the following examples because they were found to be more durable and robust than the 20-μm capillaries.

Application to ICAT-Labeled Proteins. The use of ICAT for comparative quantitation of global protein expression is a standard practice in proteomics research.[47,48] To the best of our knowledge, CE−MS has never been tested for the analysis of ICAT-labeled proteins. Two identical aliquots of BSA were labeled with the heavy (^{13}C) and light (^{12}C) versions of the ICAT reagents and analyzed by CE−tandem MS. The base peak electropherogram generated from an injection of 4 fmol of the ICAT-labeled protein digest is shown in Figure 5A. A section of the full-scan mass spectrum for the retention time window 10−11 min is given in Figure 5B, showing the presence of at least nine pairs of ICAT-labeled peptides. Inspection of the reconstituted ion electropherograms of the observed doublets (data not shown) shows that each member of the individual isotopic pairs comigrate during the CE separation. Thirteen ICAT-labeled peptides were identified in the CE−tandem MS analysis (Table 1), covering 51% (18 out of 35) of the cysteine residues within BSA.

Application to Membrane Proteins. The intrinsic hydrophobicity of integral membrane proteins presents a challenge for LC−MS analysis with regard to their isolation, solubilization, separation, and identification.[49] The ability of the improved sheathless CE−MS system to analyze membrane proteins and identify extremely hydrophobic peptides was evaluated using a sample consisting of a tryptically digested preparation of *Halobacterium* purple membranes. The tryptic peptides were prepared using a novel detergent-free approach for the isolation and solubilization of membrane proteins.[50,51] Twenty-five femtomoles of a 5 μM solution of the tryptically digested membrane preparation was injected onto the CE capillary. The hydrophilic peptides were separated using a 1.0 M acetic acid running electrolyte; however, a 50% (v/v) isopropyl alcohol was added to the electrolyte to keep the hydrophobic peptides in solution. The tandem MS spectrum of a large, hydrophobic, triply charged peptide is shown in Figure 6. The high positive GRAVY value of 1.119 for this peptide is a measure of its extreme hydrophobicity.[50,51] Searching the fragment ion spectrum against the *Halobacterium* proteomic database identified this peptide as amino acid residues 96−142 of bacteriorhodopsin.

Applications with mSPE-CE−MS. As described previously, the CE−MS system had a CLOD of 250 nM when [Glu1]-fibrinopeptide B was analyzed using a 20-μm-i.d. capillary. When using conventional capillaries of with i.d.s of 50 and 75 μm, the CLOD was in the micromolar range. This CLOD limitation hampers the utility of CE−MS for the analysis of very dilute biological samples. To decrease the CLOD of this sheathless CE−

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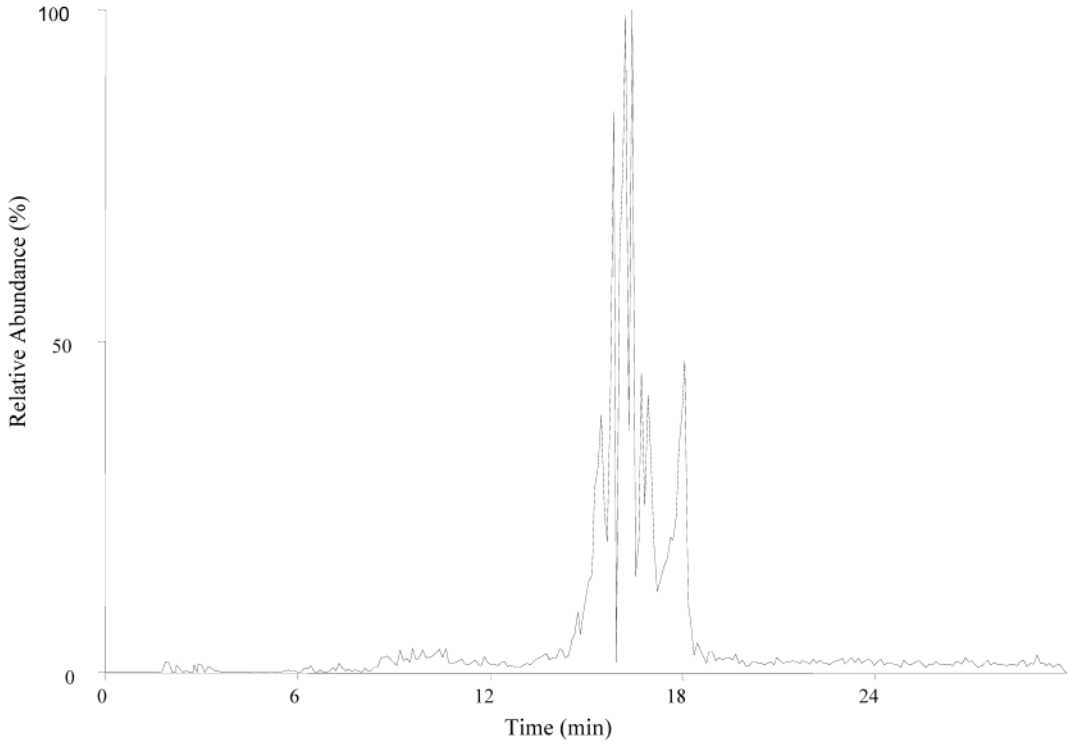

Figure 9. Base peak electropherogram from mSPE-CE−MS analysis of 5 μL (15 ng) from in-gel trypsin digestion of a Coomassie blue-stained protein spot.

MS design, a mSPE cartridge was incorporated in-line with the CE capillary. The incorporation of the mSPE cartridge increases the loading capacity from dilute solutions. The back pressure in the system also increases, depending on the column dimensions, the length of the packing plug, and the degree of its compactness. For example, the pressure needed to generate a flow of 2.2 μL/min through a 60 cm long × 75 μm i.d capillary is 0.5 psi without a cartridge and 20 psi when a 3-mm packing plug is included. Narrower capillaries (≤30-μm i.d.) are not suitable for this application because they generate excessively high back pressure, making them impractical. Thus, this type of application is limited to capillaries of a minimum i.d. of 50 μm.

The proper operation of the mSPE-CE−MS system was tested by conducting a validation experiment using a 75-μm capillary. This step is necessary to confirm that analytes from a volume exceeding that of the capillary are bound to the mSPE bed in the sample loading step, eluted from the packing plug by the organic solvent, and eluted from the capillary as a single peak under the CE separation step. Five hundred nanoliters of a 100 nM solution of [Glu^1]-fibrinopeptide B (50 fmol) was loaded on the mSPE and analyzed as described in the Experimental Section. The experiment was repeated twice by loading the same amount of sample (50 fmol) from 5 μL of a 10 nM solution and 10 μL of a 5 nM solution. In all three experiments (data not shown), the sample peak migration times, areas, and widths were similar, providing credible evidence of the validity of this approach for the analysis of peptides present in very dilute solutions. It is clear from these results that even lower CLOD can be achieved by increasing the injected sample volume.

The mSPE-CE−MS system was tested for the separation and identification of peptides generated from tryptic digestion of a protein mixture. A solution of ∼0.1 mg/mL each of horse heart cytochrome *c* and equine apomyoglobin (∼10 μM each) was digested with trypsin. After diluting the sample 1000-fold, 5.5 μL (corresponding to ∼55 fmol of each protein digest) was loaded onto the mSPE cartridge and analyzed by CE−tandem MS. The resulting base peak electropherogram is shown in Figure 7. A number of peptides were identified from each of the protein samples as shown in Table 2. The total protein coverage obtained for apomyglobin and cytochrome *c* was 61 and 67%, respectively. In contrast, no peaks were observed and no peptides were identified when the same diluted solution was analyzed with an identical CE capillary that was not equipped with an mSPE

Table 2. List of Peptides Identified from Tandem MS Analysis of the Tryptic Digest of Cytochrome *c* and Apomyoglobin

sequence	MH^+	charge state	Xcorr
Protein: Cytochrome *c*			
R.EDLIAYLK.K	964.54	2	3.09
K.TGPNLHGLFGR.K	1168.62	2	3.36
K.KGEREDLIAYLK.K	1434.80	3	3.74
K.TGQAPGFSYTDANK.N	1456.67	2	2.80
R.KTGQAPGFSYTDANK.N	1584.77	3	3.57
K.IFVQKCAQCHTVEK.G	1633.82	2	3.01
K.GITWGEETLMEYLENPK.K	2009.95	3	2.90
K.GITWGEETLMEYLENPKK.Y	2138.05	2	4.49
Protein: Apomyoglobin			
K.HLKTEAEMK	1087.30	2	2.97
K.YKELGFQG	941.94	2	3.12
K.HGTVVLTALGGILK.K	1378.84	2	4.58
K.HPGDFGADAQGAMTK.A	1502.67	2	3.09
K.VEADIAGHGQEVLIR.L	1606.86	3	3.70
-.GLSDGEWQQVLNVWGK.V	1815.90	2	4.40
K.YLEFISDAIIHVLHSK.H	1885.02	2	4.13

cartridge. The same mSPE-equipped capillary was also used for the analysis of peptides generated from an in-gel tryptic digestion of two distinct proteins. Two Coomassie blue-stained protein spots of different intensities (~5 and 1 μg, respectively) were excised from a gel obtained from an ongoing research project in our laboratory. The proteins were digested in situ with trypsin, desalted using C_{18} ZipTips, lyophilized to dryness, and resuspended in 15 μL of 0.1% formic acid in water. One microliter of each sample was diluted 1:10, and 5 μL of each (~75 ng from the high-intensity spot and 15 ng from the low-intensity spot) was loaded on the mSPE cartridge and analyzed by CE−tandem MS. The base peak electropherograms resulting from the analysis of the higher intensity spot and the lower intensity spot are shown in Figures 8 and 9, respectively. Information from the tandem MS spectra was searched against a nonredundant protein database using SEQUEST. Ten peptides were identified from the high-intensity spot correctly identifying this protein as Ig κ chain V region (Mab 13−1). Similarly, seven peptides were identified from the low-intensity spot, correctly identifying this protein as ras-related nuclear protein. No peaks were observed and no peptides could be identified when the samples were analyzed with an identical CE capillary that was not equipped with an mSPE cartridge.

In both examples described above, supplementary pressure was used to augment the low electroosmotic flow in the CE capillary. The use of supplementary pressure was found to be necessary to eliminate air bubbles and to stabilize the electrospray.[44] The CE separation efficiency attained under these conditions was not as high as expected from typical CE; however, the loss of efficiency is partially compensated by the higher peak capacity obtained through the two-dimensional aspect of CE−MS.

CONCLUSION

A novel capillary electrophoresis column−sheathless electrospray ionization interface equipped with a membrane-based on-column solid-phase extractor is developed. Mid-attomolar mass limit of detection and low-nanomolar concentration limit of detection were achieved. The system was applied for the analysis of proteomic samples including ICAT-labeled, membrane, solution, and in-gel-digested proteins. Despite the emphasis in this paper on proteomic applications, the system can also be adapted for the concentration, separation, and analysis of other types of analytes including small molecules. The system presented here offers significant improvement in terms of fabrication, durability, and overall CE performance. The on-line solid-phase extractor, CE column, and interface can be easily assembled from readily available inexpensive materials.

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