Chapter 8

High-Throughput Capillary Electrophoresis–Mass Spectrometry: From Analysis of Amino Acids to Analysis of Protein Complexes

Mehdi Moini

Abstract

Recent advances in capillary electrophoresis–mass spectrometry (CE-MS) interfacing using porous tip is leading to commercialization of CE-MS with a sheathless interface for the first time. The new sheathless interface in conjunction with CE capillary coatings using self-coating background electrolytes (BGE) has significantly simplified CE-MS analysis of complex mixtures. CE-MS, with its high separation efficiency, compound identification capability, and ability to rapidly separate compounds with a wide range of mass and charge while consuming only nanoliters of samples, has become a valuable analytical technique for the analysis of complex biological mixtures. These advances have allowed a single capillary to analyze a range of compounds including amino acids, their D/L enantiomers, protein digests, intact proteins, and protein complexes. With these capabilities, CE-MS is poised to become the multipurpose tool of separation scientists. More recently, an eight-capillary CE in conjunction with an 8-inlet mass spectrometry has allowed 8 CE-MS analyses to be performed concurrently, significantly increasing throughput.

Key words: Capillary electrophoresis, Electrospray ionization, Mass spectrometry, Amino acids, Enantiomers, Biological clocks, Dating, Self-coating background electrolyte, Peptides

1. Introduction

A quarter century after its first introduction, capillary electrophoresis–mass spectrometry (CE-MS), with its high separation efficiency, compound identification capability, and ability to rapidly separate compounds with a wide range of mass and charge while consuming only nanoliters of samples, has become a valuable analytical technique for the analysis of complex biological mixtures. Capillary electrophoresis–mass spectrometry is the only analytical

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technique with which one can analyze amino acids, their racemic mixtures, peptides, proteins, intact protein complexes, and intact cells simply by changing the composition of the background electrolyte (BGE). This allows a single capillary to analyze a range of omics, such as metabolomics, proteomics (both top-down and bottom-up), functional proteomics, and cellomics. In this regard, CE-MS is considered the multipurpose tool of the separation scientists. CE-MS is especially useful for the analysis of charged compounds which are not easily retainable under reverse-phase high-performance liquid chromatography (HPLC) and in this regard is considered a complementary technique to HPLC. Comparison between CE-MS and HPLC-MS in proteomics area has been discussed (1, 2). In addition to its multipurpose characteristics, CE-MS also offers many other important advantages compared to HPLC-MS such as the following:

- High separation efficiency because of its flat flow profile
- High-speed separation allowing sample to be analyzed in minutes
- Low-pressure operation allowing injection of nL or less of the samples or injection of the intact cells
- Ease of changing BGE (1–2 min)
- Higher sensitivity under ESI due to lower flow rates
- Ease of operation (no fittings, no leaks, etc.)

While these advantages were well known to the practitioners in the field, the widespread use of CE-MS and its commercialization remained elusive due to the difficulties of interfacing CE to MS. This changed in 2007 when a simple, reproducible CE to MS interface design using a porous tip was introduced. The new design provided a path for the commercialization of sheathless interface, which in turn will provide a turn-key sheathless CE-MS instrument for wide usage within the scientific community and later in clinical laboratories.

1.1.1. Sheathless CE-I Interface Using a Poro Tip

1.1. CE-MS Interface Design

An important feature of any CE/MS interface is the method by which electrical current is provided to the CE outlet/ESI electrode. Over the past 25 years, a variety of CE/ESI-MS interfaces have been introduced, various reviews of which have been published and will not be discussed here (3–10). These interfaces are divided into three general categories: sheath-flow, sheathless, and split-flow interfaces. Sheathless interfaces are especially popular due to their high sensitivity of detection (resulting from the absence of sheath liquid to dilute the CE effluent), and most interfaces introduced in the past 25 years deal with developing this type of interfaces; however, novel sheath-flow designs that minimize the dilution of the analyte by sheath liquid are still being pursued and published (11, 12). In split-flow interfaces the electrical connection

to the CE capillary outlet is achieved by diverting part of the CE background electrolyte (BGE) out of the capillary through an opening near the capillary outlet. The CE buffer exiting the opening contacts a sheath metal tube which acts as the CE outlet/ESI shared electrode. In cases in which the ESI source uses a metal needle, the voltage contact to the CE buffer is achieved by simply inserting the outlet of the CE capillary, which contains an opening, into the existing ESI needle. As a result of the concentration-sensitive nature of ESI, splitting a small percentage of the CE flow has minimal effect on the sensitivity of detection. In addition, because the liquid is flowing through the opening and out of the capillary, there is no dead volume associated with this interface. Moreover, bubble formation due to redox reactions of water at the electrode does not affect CE/ESI-MS performance, because the actual metal/liquid contact occurs outside of the CE capillary. Despite these advantages, split-flow interface is a multistep interface design since it requires a separate step for sharpening the capillary outlet outer diameter (o.d.) tip for efficient ESI. In addition, reaching a consistent split ratio is currently difficult to achieve (13).

1.1.1. Sheathless CE-MS Interface Using a Porous Tip

To address the deficiencies mentioned above, in 2007 we introduced porous tip interface as a robust and reproducible, single-step sheathless design. In this design, the electrical connection to the capillary outlet is simply achieved by etching ~4 cm of the capillary outlet using a solution of 49% HF until it becomes porous. The etching process also simultaneously reduces the outlet tip for efficient ionization, eliminating the separate tip-sharpening process of the older designs. Small ion transport through the porous section of the capillary in contact with the conductive solution in the ESI needle provides voltage to the solution inside the capillary for ESI. In CE, in addition to providing voltage for ESI, ion transport through the porous tip closes the CE electrical circuit (14). Figure 1 shows the rate of HF etching for 150-μm-o.d., 20-μm-i.d. fused-silica capillary, with its polyimide coating (~10 μm) removed prior to etching. The procedure for HF etching is provided in Appendix 1. As shown, to etch porous a nominal 150-μm-o.d., 20-μm-i.d. capillary requires about 30 min. Once etched, the capillary is inserted inside an existing ESI needle filled with a conductive solution (0.1–1 M formic or acetic acid). Figure 2 shows the overall schematic of the porous tip interface and exemplifies the uniform spray that is formed using porous tip design. A sharp tip, good electrical connections, and minimal bubble formation are the main reasons for the uniformity of the electrospray plume under porous tip design. An additional advantage of the porous tip is that the inlet and outlet inner diameters are the same, which reduces the chance of the tip getting clogged since any particulate that gets into the porous tip capillary will exit from the other end of the capillary. This is in contrast to the use of a nanospray interface using pulled

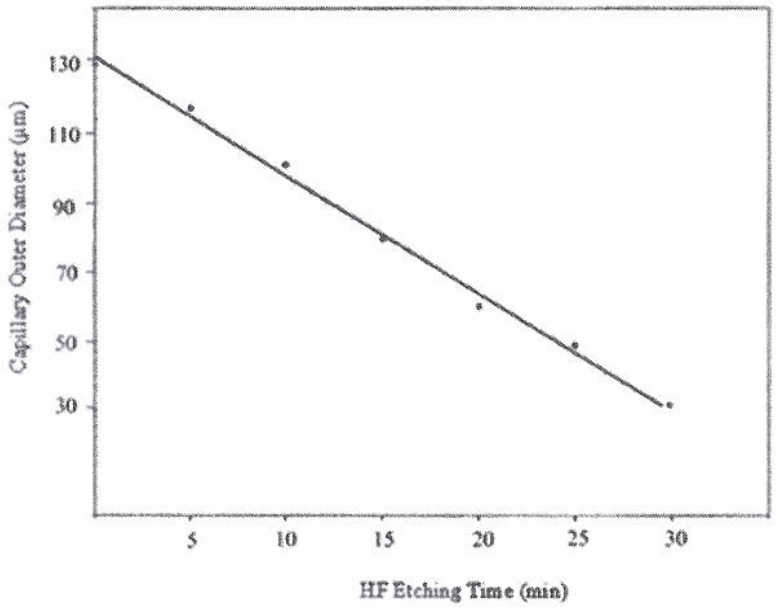

Fig. 1. Etching rate for a nominal 150-μm-o.d. fused-silica capillary in 49% HF.

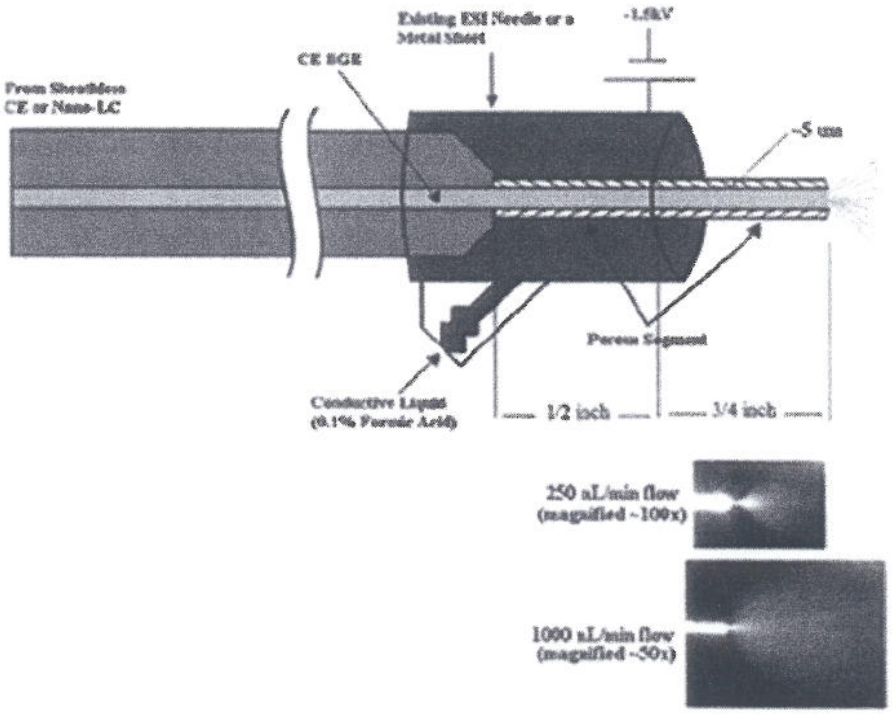

Fig. 2. Schematics of the porous tip. Insets, ESI plume from a nLC operating at 250 nL/min (magnified by ~100×) and 1 μL/min (magnified by ~50×) (reprinted with permission from (Moini, M. (2007) Simplifying CE-MS Operation. 2. Interfacing Low-Flow Separation Techniques to Mass Spectrometry Using a Porous Tip, *Anal. Chem.* 79, 4241–4246). Copyright (2007) American Chemical Society).

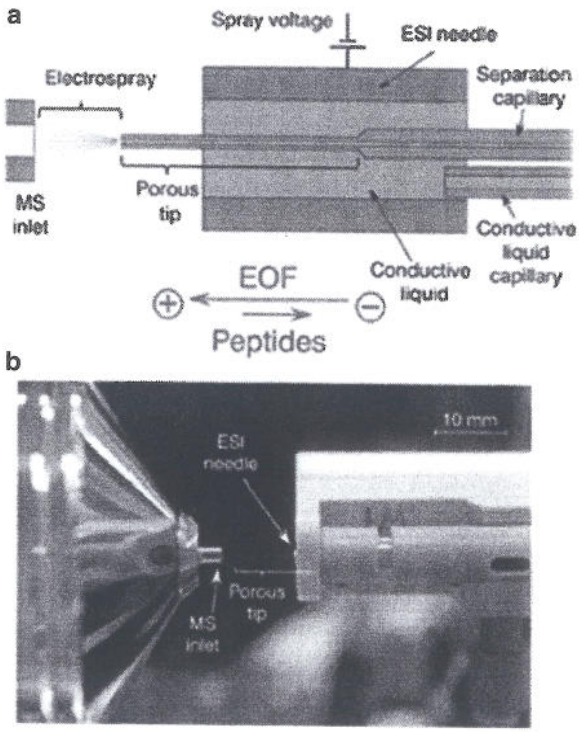

Fig. 3. The high-sensitivity porous sprayer interface (a) schematic and (b) photograph of the prototype interface ref 2 (reprinted with permission from (Faserl, K., Sarg, B., Kremser, L., Lindner, H. (2011) Optimization and Evaluation of a Sheathless Capillary Electrophoresis–Electrospray Ionization Mass Spectrometry Platform for Peptide Analysis: Comparison to Liquid Chromatography–Electrospray Ionization Mass Spectrometry, *Anal. Chem.* 83, 7297–7305). Copyright (2011) American Chemical Society).

tips, as its tip is usually drawn to less than 10 μm i.d., which can trap particulates with diameters larger than the capillary opening at the inlet. Since in the pull-tip design, only the tip is sharp, tip clogging will result in the loss of the spray tip and the analysis. Damage to a porous tip, however, can be easily repaired by etching away 1–2 mm of the tip off using the HF solution. Because the porous tip has about the same o.d. and i.d. for the ~4-cm porous tip, removing 2 mm of the tip will not affect spray performance. Figure 3 shows the prototype of a commercial version of this interface design (2).

Another advantage of the porous tip design is that it can be applied to a variety of capillary o.d.s and i.d.s, as demonstrated in Figs. 4 and 5, where an enolase digest has been analyzed using capillaries with the same inner diameters but different outer diameters (Fig. 4) and with the same capillary o.d. but different i.d.s and lengths (Fig. 5).

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2. Interfacing
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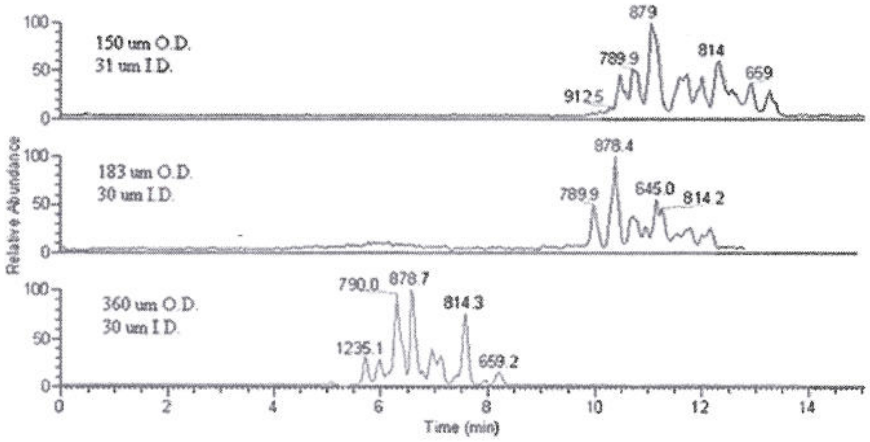

Fig. 4. CE-MS of enolase digest utilizing a porous tip on different capillary o.d.s.

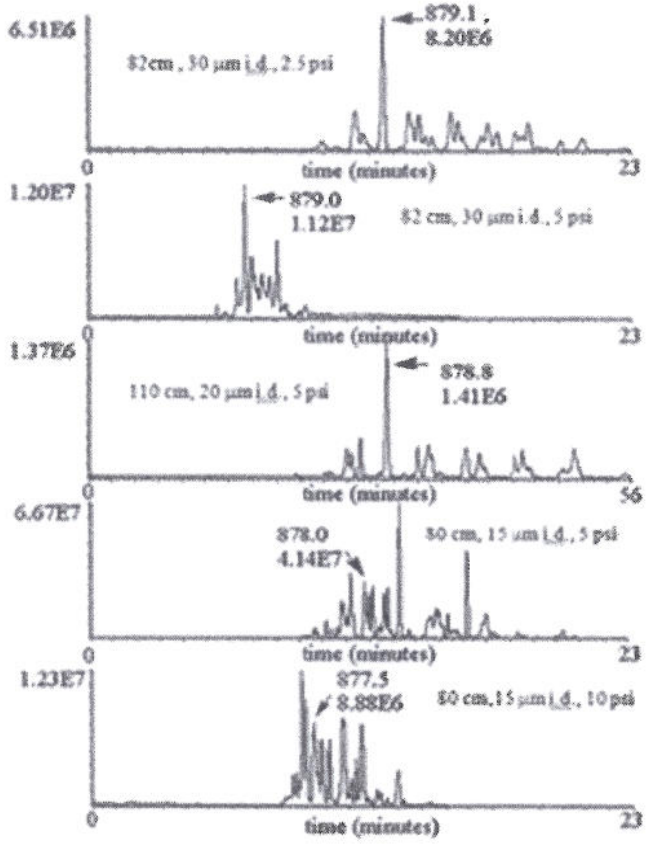

Fig. 5. CE-MS of enolase digest utilizing a porous tip on capillaries with the same o.d.s. (150 μm) but different i.d.s.

2. Applications of CE-MS

In the past decade we have applied CE-MS to the analysis of a wide range of biological mixtures from amino acid mixtures to the analysis of the chemical contents of a single cell. The idea is to develop sensitive, fast, and simple CE-MS methods that do not require analyte or capillary derivatization, which are both time-consuming and expensive. To achieve our goal we mainly rely on the addition of additives to the CE BGE that, for example, minimize analyte–wall interaction or enhance sensitivity and separation.

2.1. Analysis of Underivatized Amino Acid

CE-MS is an ideal analytical technique for the analysis of underivatized amino acid analysis. This is because gas chromatography–mass spectrometry is unable to analyze underivatized amino acids, which are thermally labile. Moreover, except for larger amino acids with hydrophobic side chain, reverse-phase HPLC-MS with conventional ion-pairing reagents (0.1% TFA and 0.1% formic acid) cannot analyze most amino acids because under these conditions amino acids are positively charged and they are co-eluted with void volume. However, CE-MS can separate and detect underivatized amino acids with ease. Figure 6 shows the CE/ESI-MS electropherogram of the separation of the 20 standard protein amino acids using a 130-cm-long, underivatized CE capillary in conjunction with 1 M formic acid as the BGE. For this experiment, ~0.4 nL of the 20 amino acid standard solution (~400 fmol of each amino acid) was injected (15). The use of an underivatized capillary is a big advantage since capillary derivatization techniques are usually long, labor-intensive, expensive, and short-lived. While under 1 M formic acid as the BGE, all amino acids were separated and detected, however, their relative intensities varied almost 3 orders of magnitude, even though an equimolar solution of amino acids had been used. The variation in relative intensities of amino acids is due to variation in ionization efficiencies of amino acids under ESI (16, 17).

2.1.1. Enhanced Sensitivity of Amino Acids by Adding 18-Crown-6 to the BGE During Amino Acid Analysis

To enhance the detection sensitivity of the amino acids that showed low sensitivity with 1 M formic acid as the BGE (Fig. 6) as well as to remedy the disparity between detection limits of amino acids, we have used 18-crown-6 (18-C-6) with a concentration between 20 and 30 mM as an additive and complexation reagent to the BGE. Once added to the BGE, 18-C-6 formed stable complexes with amino acids, which exhibited high ionization efficiencies and low (attomole levels) detection limit for most amino acids (Fig. 7).

2.1.2. Separation of Amino Acid Enantiomers Using (+) 18-Crown-6-Tetracarboxylic Acid (18-C-6-TCA)

While the use of 18-C-6 enhanced the sensitivity of detection of most amino acids, it was unable to separate amino acid enantiomers because it lacks a chiral center. To achieve high sensitivity of detection for amino acid and to achieve amino acid enantiomer separation, enantiomeric pure (+) 18-C-6-TCA instead of 18-C-6

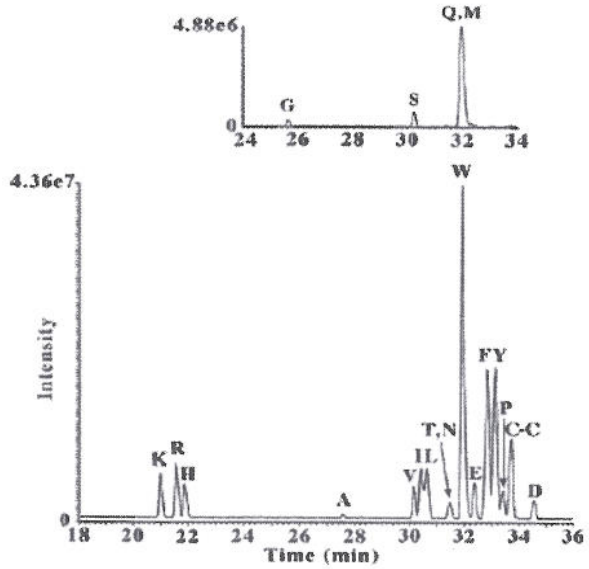

Fig. 6. CE-MS analysis of underivatized amino acids using 1 M formic acid as the BGE (reprinted with permission from (Schultz, C. L., Moini, M. (2003) The analysis of underivatized amino acids and their D/L enantiomers using sheathless CE-MS, *Anal. Chem.* 75, 1508–1513). Copyright (2003) American Chemical Society).

2.1.3. Amino Acid Racemization (AAR) as a Biological Clock

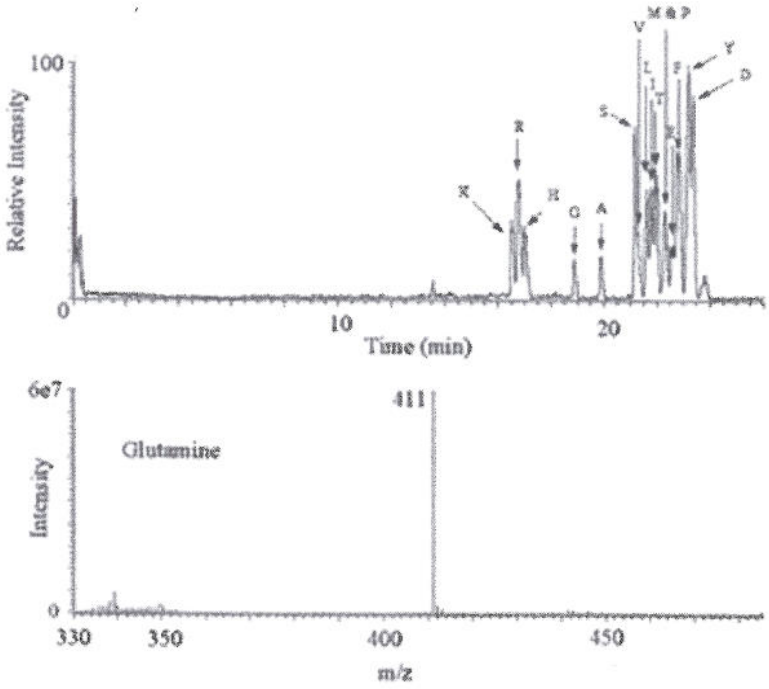

Fig. 7. CE-MS of amino acids using 18-crown-6 as the BGE additive (*top panel*). Mass spectrum of the protonated glutamine (MW 146)/18-crown-6 (MW 264) complex (*bottom panel*).

was utilized as the background electrolyte and used in conjunction with an underivatized, 130-cm-long, 20-μm-i.d., 150-μm-o.d. fused-silica capillary (18). During the enantiomer separation, while interaction between the protonated amine and the oxygens of the ethylene bridge of 18-C-6-TCA is responsible for complex formation, it is apparently the carboxyl group substituents of 18-C-6-TCA that allow for enantiorecognition (18). Both D and L enantiomers form stable complexes with 18-C-6-TCA; however, in most cases, the D enantiomers separate from the L enantiomers because D enantiomers spend more time with 18-C-6-TCA (except for serine and threonine, in which the L enantiomers migrated last). By monitoring the m/z range of the amino acid/18-C-6-TCA complexes (m/z 515–700), most of the standard amino acids and many of their enantiomers were separated and detected with high separation efficiency and high sensitivity (nanomolar concentration detection limits) in one run (Fig. 8). The solutions of 18-C-6-TCA also worked well as the CE/ESI-MS BGE for low-level detection of several neurotransmitters and some of their D/L enantiomers as well as for the analysis of amino acids at endogenous levels in lysed red blood cells.

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2.1.3. Amino Acid Racemization (AAR) as a Biological Clock

AAR is a posttranslational modification (i.e., modification of the protein structure after its initial synthesis), which stems from the intrinsic instability of certain AA residues that leads to racemization. On earth, proteins are synthesized from L-amino acids. However, after incorporation into proteins, bound L-AAs start to racemize to their D-form under a reversible first-order kinetic reaction until equilibrium is reached (19). Each amino acid has a different intrinsic AAR rate, which is a function of the physical, chemical, and biological (protein sequence and its secondary, tertiary, and quaternary structures) states of the specimen, and acts as an independent biological clock. To measure the rate of conversion of L- to D-amino acids in proteins, the proteinaceous specimens are digested by hydrochloric acid to free amino acids, D- and L-amino acids are separated by CE-MS, and the ratio of the D/L is measured to estimate the age of the specimen (20). Among AAR rates, the aspartic acid racemization rate is fast enough to be used for more recent specimens (<2,500 years); however, the AAR rates of other amino acids are used to date older specimens (21). The CE-MS technique discussed in Subheading 2.1.2 can separate and detect amino acids and their D/L enantiomers in one run, in ~20 min, and using only ~1 nL of sample. Unlike the analysis of amino acids by gas chromatography or HPLC with UV detectors, CE-MS uses underivatized amino acids and requires no sample preparation, which significantly reduces analysis time.

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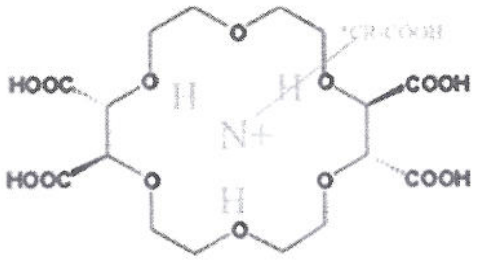

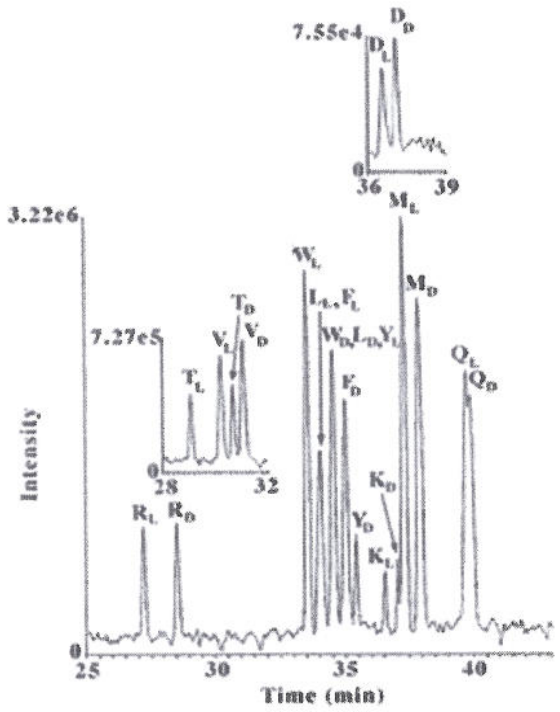

Fig. 8. CE-MS of 11 amino acid enantiomers in one run using a 30-mM aqueous solution of 18-C-6-TCA as the CE BGE (reprinted with permission from (Schultz, C. L., Moini, M. (2003) The analysis of underivatized amino acids and their D/L enantiomers using sheathless CE-MS, *Anal. Chem.* 75, 1508–1513). Copyright (2003) American Chemical Society).

2.1.4. Application of Amino Acid Racemization in Dating Museums' Proteinaceous Specimens

Because of the existence of a large number of precious proteinaceous specimens such as silk and wool textiles, leather and animal gut objects, bone and tissues, ink, paper, paint, coatings, binders (and associated adhesives), and paleo-organic matter in museum collections, the identification of the nature of the degradative state of such historic objects is often critical to their preservation. To preserve these objects it is desirable to understand the fundamental factors that cause their degradation, to identify the deterioration markers that determine their degradation stage and their age, and

2.1.5. Effect of Environmental Fact on Racemization

3. Application of CE-MS to the Analysis of Peptides and Proteins

to study the environmental factors that affect their deterioration. In most cases, however, there is insufficient uniform material available from museums' samples for accurate ^{14}C dating since this technique usually requires a few milligrams of specimen. For precious objects, even a few milligrams are a relatively large amount of material, and in most cases it is almost impossible to obtain (22). In this area our goal was to develop a high-throughput CE-MS technique for dating proteinaceous specimens while consuming minimal amount of samples. As a proof of concept, we applied this technique for dating silk textiles fabricated from the cocoons of the silkworm Bombyx mori by measuring the D/L ratio of the aspartic acid for several well-dated textiles ranging in age from the present to ~2,500 years ago (20). The silk samples included fresh silk (2010); untreated silk from the Oka Studio, Freer–Sackler Museum, Smithsonian Institution (SI), Washington, DC (~1990); Sheridan flag, National Museum of American History, SI (1883–1888); Mexican War flag, National Museum of American History, SI (1845–1846); a man's suit coat, Museum of the City of New York (1740); a silk textile from Istanbul, Textile Museum, Washington, DC (1551–1599); a silk tapestry from the Fontainebleau Series, Kunsthistorisches Museum, Vienna, Austria (1540s); tiraz silk yarns from Egypt, Textile Museum, Washington, DC (993); and silks from the Warring States Period, China, brown (top) and red (bottom), Metropolitan Museum of Art, New York City (475–221 b.c.). The results are shown in Fig. 9, with an insert showing the relative increase of the D vs. L enantiomer of aspartic acid/18-C-6-TCA complex (m/z 574). From Fig. 9, it is clear that the data points fit very well with a theoretical curve obtained from reversible first-order chemical kinetics (solid curve).

2.1.5. Effect of Environmental Factors on Racemization

The effect of UV radiation on modern degummed silk, which was UV-radiated from 0 to 1,920 h, was also investigated (Fig. 10). It was observed that the D/L ratio increased from ~1.5 to ~3.5%. The results indicate that after ~2,000 h of radiation, D/L ratio change is equivalent to natural aging of ~100 years. As shown, UV radiation has a direct effect on silk aging.

3. Application of CE-MS to the Analysis of Peptides and Proteins

The most important factor for successful CE-MS analysis of peptides and proteins is prevention of peptides and protein adsorption on fused-silica inner wall (23). Fused silica is the most commonly used capillary material in CE due to its good thermal conductivity, ultraviolet transparency, and uniform inner diameter (24). However, the use of bare fused-silica capillary in CE for peptide and protein analysis is problematic because of the chemical composition of the

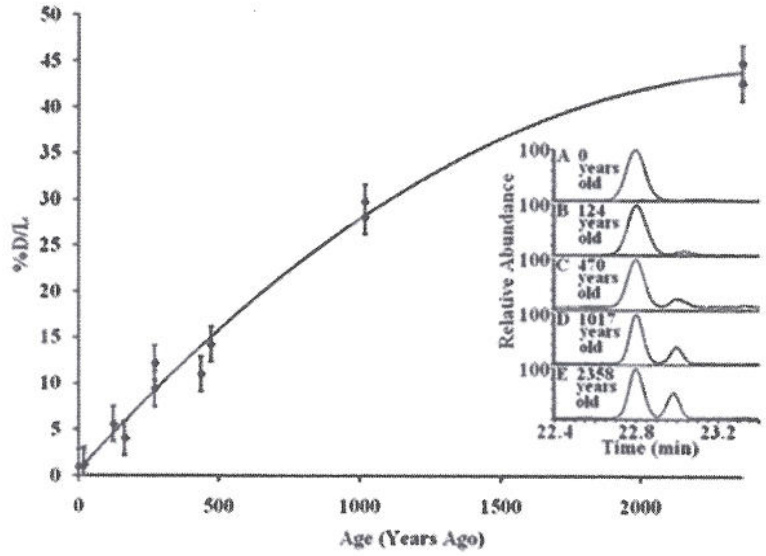

Fig. 9. Ratios of D- vs. l-aspartic acid for several well-dated museums' specimens. *X*-axis on the inset is relative intensity (reprinted with permission from (Moini, M., Klauenberg, K., Ballard, M. (2011) Dating Silk By Capillary Electrophoresis Mass Spectrometry, *Anal Chem.* 83, 7577–7581). Copyright (2011) American Chemical Society).

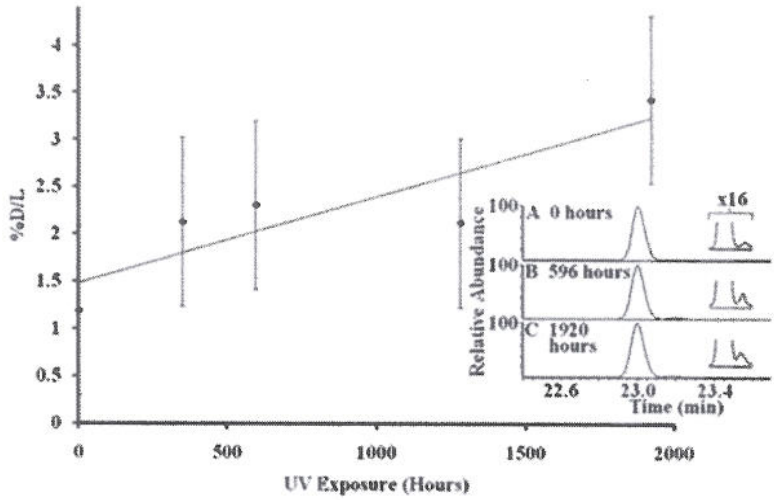

Fig. 10. Effect of UV radiation on the ratio of D- vs. l-aspartic acid of a modern silk sample (reprinted with permission from (Moini, M., Klauenberg, K., Ballard, M. (2011) Dating Silk By Capillary Electrophoresis Mass Spectrometry, *Anal Chem.* 83, 7577–7581). Copyright (2011) American Chemical Society).

inner wall of the capillary. It exposes a hydrophobic, negatively charged, and pH-dependent surface that can interact with the positively charged analyte (25). The analyte–wall interactions lead to irreproducibility, impaired efficiencies, and peak broadening. Because of the heterogeneity of silanol groups on the inner walls of the fused-silica capillary, there exists a spectrum of micro p*K*a values within the capillary centered on 6.3 (Fig. 11) (26). Since the

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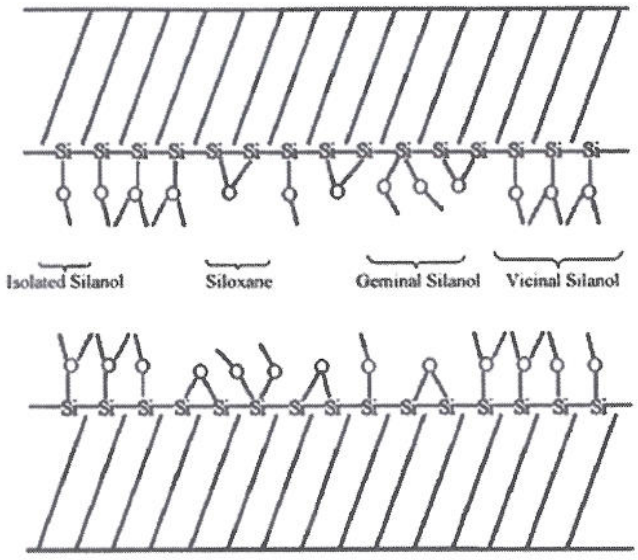

Fig. 11. Schematic diagram of the inner wall of an underivatized fused-silica capillary with silanol groups exposed (reprinted with permission from (Garza, S., Chang, S., Moini, M. (2007) Simplifying capillary electrophoresis–mass spectrometry operation: Eliminating capillary derivatization by using self-coating background electrolytes, *J. of Chromatogr. A* 1159, 14–21). Copyright (2007) Elsevier).

acid–base ionization constant (p*K*a) of silanol groups is 2–9 (based on change of electroosmotic flow (EOF) versus pH experiment), an aqueous BGE with a pH > p*K*a will impose a net negative charge on the silanol groups. Acidic BGEs with pH < 2 have been used to protonate these silanol groups to prevent analyte–wall interaction (4). However, highly acidic BGEs have disadvantages such as producing a high CE current which can cause electrical discharge through the capillary wall leading to capillary breakage, especially for thin-wall capillaries (e.g., 150 μm o.d.). In addition, highly acidic conditions are not suitable for analyzing protein complexes and other higher-order structures. To minimize analyte–wall interaction without the use of highly acidic BGE, a series of derivatization techniques have been developed to positively charge the capillary inner wall under mildly acidic BGE conditions suitable for ESI. Two general derivatization techniques are used: covalent and dynamic coating of the fused-silica capillary inner wall. Covalent coatings involve chemical reactions between the silanol groups on the capillary wall and the coating material (27). An example of a covalent coating is the trimethoxyaminopropylsilane (APS) treatment of the capillary. In the APS derivatization technique, the methoxy groups of APS and the –OH silanol groups attach to the inner wall and react together to leave positively charged amino groups inside the capillary wall under acidic conditions, which in turn reverses the EOF. Although covalent derivatization provides optimum separation for proteins and peptides under mildly acidic

BGE, the derivatization process is time-consuming, labor-intensive, and short-lived (a few runs). Moreover, the derivatization mostly fails to give complete coverage of the silica inner surface and is usually unstable at high pH. Furthermore, covalent coatings typically require in situ reaction steps that are hard to control and affect the homogeneity and reproducibility of the coated capillaries. Another limitation is that in situ derivatization can plug the capillary by producing a very viscous polymer solution that sometimes cannot be completely washed out. Alternatively, dynamic coatings require the temporary physical adsorption of the polymer to the capillary wall. Dynamic coatings consist of treating the capillary wall with a solution containing the coating agent, performing a few separations, removing the coating using a solution of NaOH, rinsing with water, and recoating the capillary again once the wall coating degrades. This type of coating can be divided into two general categories: adsorption of the polymer coating to the capillary wall prior to the CE analysis (precoating) or using the polymer reagent as an additive in the BGE (self-coating). The main disadvantage of precoating techniques is their gradual degradation. While multilayer dynamic coatings have enhanced the coating stability (28), the main advantage of the self-coating BGEs is their ease of use. The use of polymers as dynamic coatings for CE-UV analysis has recently been reviewed (29).

3.1. Simplifying CE-MS Operation by Using Self-Coating Background Electrolytes

Polybrene (PB) and polyE 323 (PE) are cationic polymers that are able to non-covalently attach to the negatively charged wall of fused-silica capillary. The excess positive charges on the newly created surface generate a stable anodic EOF that is independent of pH within the pH range of 4–8. The molecular structures of both PB and PE are shown in Fig. 12. The structure of PE is purposely constructed to contain mixed bonding characteristics. For example, the nitrogen atoms in the backbone of PE are separated by three-atom length, whereas nitrogen atoms in PB are separated by six carbon atoms. The length of the spacer arm between the nitrogen atoms in the backbone can affect the polymer's flexibility and hydrophobicity. Also, hydroxyl groups in PE increase polymer immobilization on the capillary wall by hydrogen bonding (30). PB and PE are very effective in reversing the charge on the capillary wall, which prevents peptide and protein attachment to the capillary wall, thereby significantly improving separation efficiency. Figure 13 shows the comparison of CE-MS analyses of peptide standard using an uncoated capillary (top panel), PB and PE precoated capillary, and self-coated capillary using 33–660-nM PB and PE (from top to bottom). Figures 14 and 15 show the separation efficiency for more complex mixtures including a mixture of 6-protein digest (Fig. 14) and for a tryptic digest of 55 ribosomal proteins. As shown both PB and PE are

3.2. High Sequence Coverage Proteomic: Using (CE-MS/MS)^n

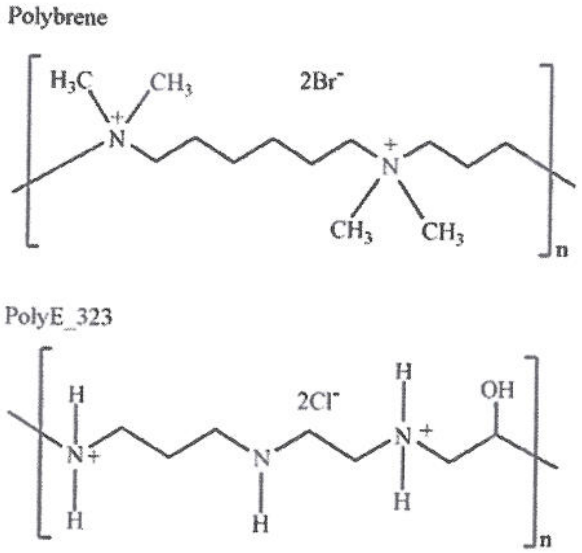

Fig. 12. Molecular structures of polybrene (PB) and polyE 323 (PE) (reprinted with permission from (Garza, S., Chang, S., Moini, M. (2007) Simplifying capillary electrophoresis–mass spectrometry operation: Eliminating capillary derivatization by using self-coating background electrolytes, *J. of Chromatogr. A* 1159, 14–21). Copyright (2007) Elsevier).

excellent additives as a self-coating BGE for the CE/ESI-MS analysis of peptides and proteins, and they can significantly simplify CE-MS operations (23, 31, 32).

3.2. High Sequence Coverage Proteomics Using (CE-MS/MS)^n

Capillary electrophoresis coupled with tandem mass spectrometry (CE-MS/MS) has been used to successfully identify proteins from a variety of organisms and can provide high sequence coverage of a protein digest, often approaching total coverage for a single protein digest (33–38). However, application of CE-MS/MS to the analysis of a digest of a complex protein mixture has been limited. This is because even under CE's high separation efficiency, each CE peak still contains several peptides. Since the number of peptides analyzed by MS under full scan and MS/MS modes is limited by the mass spectrometer's rate of switching between these two modes, sharp electrophoretic peaks containing several peptides result in undersampling; that is, only a fraction of the peptides are analyzed under MS/MS mode. In addition, due to the electrospray ionization mechanism, when manipulating experimental conditions, such as pH and composition of the CE BGE, only a specific number of peptides will have ion intensities detectable by the mass spectrometer. For example, when a protein digest of a complex protein mixture of *E. coli* ribosomal proteins was analyzed by CE-MS, a single electrophoretic peak of 6 s wide contained more than 20 peptides with a wide range of intensities (Fig. 15). This usually results in low sequence coverage since many

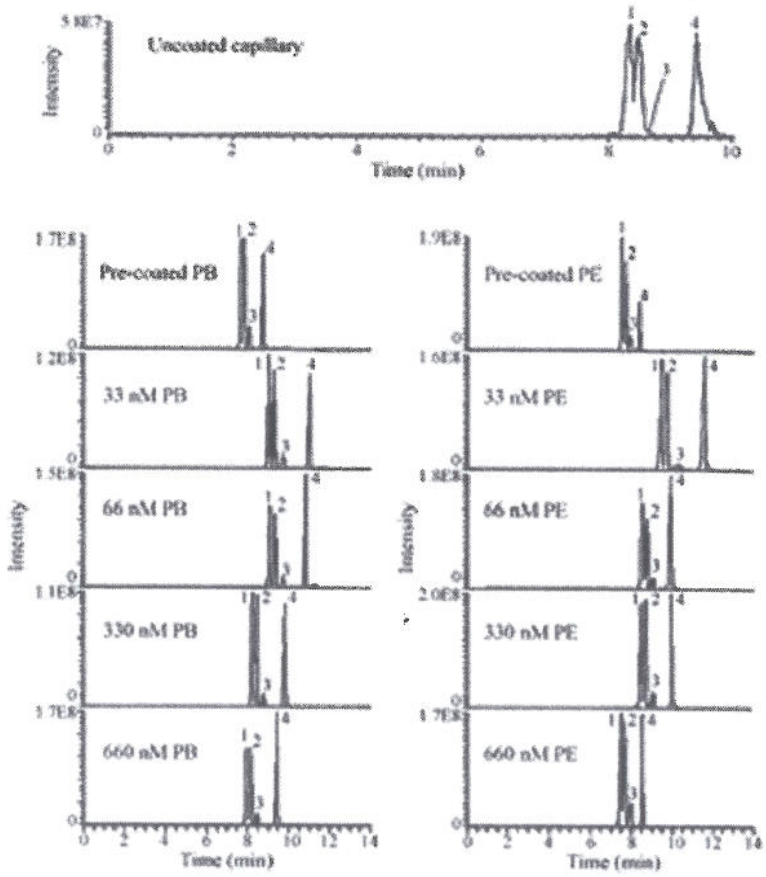

Fig. 13. Comparison of CE-MS analyses of peptide standard using an uncoated capillary (*top panel*), PB and PE precoated capillary, and self-coated capillary using 33–660-nM PB and PE (from *top* to *bottom*) (reprinted with permission from (Garza, S., Chang, S., Moini, M. (2007) Simplifying capillary electrophoresis–mass spectrometry operation: Eliminating capillary derivatization by using self-coating background electrolytes, *J. of Chromatogr. A* 1159, 14–21). Copyright (2007) Elsevier).

peptides are not sequenced by MS/MS. There are several reasons for the low coverage including the following: (1) undersampling (the existence of a large number of peptides that must be analyzed by MS/MS and the limited rate at which the mass spectrometer is capable of switching between MS and MS/MS modes); (2) wide concentration dynamic range of the proteins in a complex protein mixture, in which low-level peptides may go undetected by MS/MS; and (3) wide range of electrospray ionization efficiency of peptides under each mobile-phase composition, resulting in a wide variation in ion intensities, even for peptides with equimolar concentration. Under this condition, the mass spectrometer usually does not have sufficient time to analyze all the peptides by MS/MS, which results in undersampling and low protein coverage.

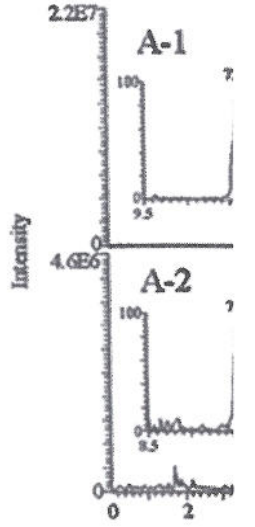

Fig. 14. Separation efficier
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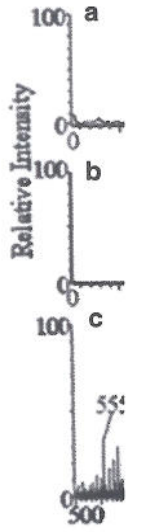

Fig. 15. Panel **a**: CE-MS/M
cal CE peak. Panel **c**: ma
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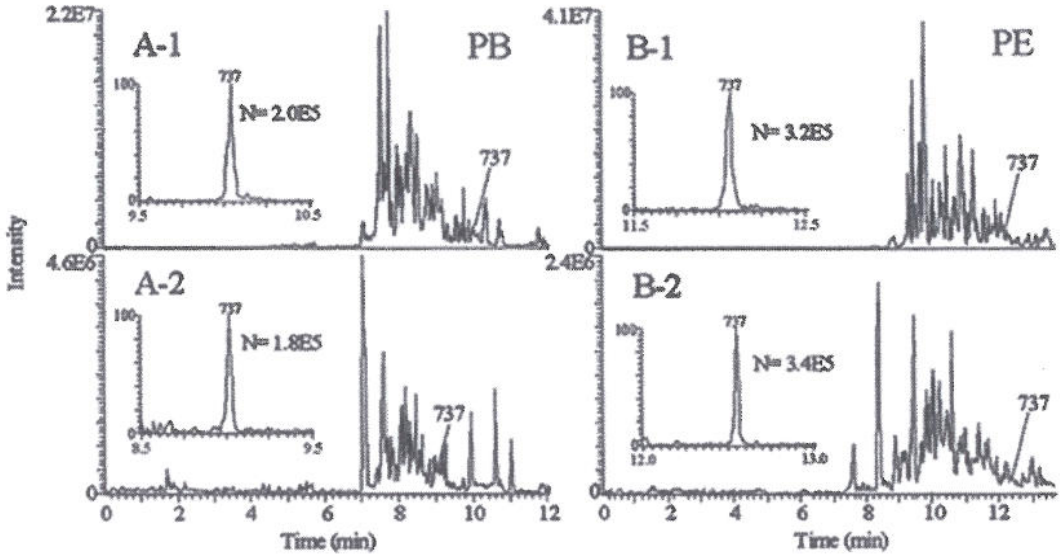

Fig. 14. Separation efficiency of CE-MS of 6-protein mix digest (reprinted with permission from (Garza, S., Chang, S., Moini, M. (2007) Simplifying capillary electrophoresis–mass spectrometry operation: Eliminating capillary derivatization by using self-coating background electrolytes, *J. of Chromatogr. A* 1159, 14–21). Copyright (2007) Elsevier).

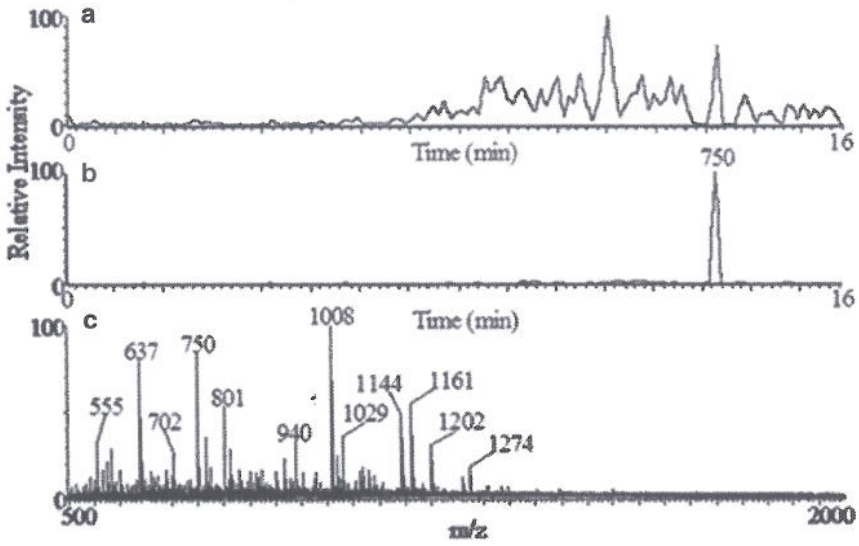

Fig. 15. Panel **a**: CE-MS/MS analysis of the tryptic digest of 55 ribosomal proteins. Panel **b**: separation efficiency for a typical CE peak. Panel **c**: mass spectrum of the peak from panel **b** (reprinted with permission from (Garza, S., Moini, M. (2006) Analysis of Complex Protein Mixtures with Improved Sequence Coverage Using (CE–MS/MS)^n, *Anal. Chem.* 78, 7309–7316). Copyright (2006) American Chemical Society).

Protein fractionation prior to MS analysis is yet another technique that can be used to solve the undersampling issue. In this technique, a complex protein mixture is first fractionated into several simpler mixtures using solubility or isoelectric point properties of the proteins. If the protein fractions are simple enough, they can

be dissociated directly in the gas phase via a top-down technique such as electron-capture dissociation (39) or electron-transfer dissociation (40). More complex protein fractions are usually further separated; for example, by using SDS gels, individual bands are chemically or enzymatically digested and then analyzed by nLC-MS/MS (41, 42). Because these bands usually contain ~1 pmol or less of sample, to achieve a sensitive analysis, it is often necessary to inject the entire sample followed by analysis utilizing nano-LC-MS or nLC-MS/MS (1, 41, 42). Under this condition, the multiple-injection technique under the same nLC-MS/MS is impractical. In addition, since each LC separation takes ~1 h for complex protein mixtures, analysis would be very time-consuming. Multiple sample injections followed by MS/MS analysis, however, are an ideal match for CE-MS/MS analysis. This is because each CE-MS/MS analysis consumes only nanoliters of sample (~1/100 of a nano-HPLC run), and each analysis takes ~10 min (<1/6 of the nLC-MS/MS) (1). Because the sample consumption associated with CE is insignificant and analysis time is short, an experiment can be designed in which all CE and MS parameters are considered variables that can be manipulated after each injection followed by CE-MS/MS analysis to provide maximum sequence coverage (1). To address this low sequence coverage, we introduce a novel technique, $(CE\text{-}MS/MS)^n$, which utilizes the most significant advantages of CE-MS/MS, including economy of sample size, fast analysis time, and high separation efficiency, to increase the sequence coverage of a complex protein mixture. Based on these characteristics, $(CE\text{-}MS/MS)^n$ can be performed in which multiple CE-MS/MS subanalyses (injections followed by analyses) are analyzed and experimental variables are manipulated during each CE-MS/MS subanalysis in order to maximize sequence coverage. $(CE\text{-}MS/MS)^n$ is a practical technique since each CE-MS/MS subanalysis consumes <10 nL, and each CE-MS/MS subanalysis takes ~10 min; therefore, several subanalyses can be performed in ~1 h consuming only nanoliters of the sample. Two techniques have been introduced to address the undersampling: (1) $(CE\text{-}MS/MS)^n$ using dynamic exclusion (in this technique, several CE-MS/MS analyses (injection followed by separation) were performed in one run using the dynamic exclusion capability of the mass spectrometer until all peptide peaks were analyzed by MS/MS) and (2) gas-phase fractionation (in this technique, $(CE\text{-}MS/MS)^n$ is performed by scanning a narrow mass range (every ~100 m/z) during each CE-MS/MS subanalysis without using dynamic exclusion). Under this condition, in each subanalysis, the number of peptides available for MS/MS analysis is significantly reduced, and peptides with the same nominal masses are analyzed, thereby increasing sequence coverage. Additionally, to address the lack of detection of low-level peptides in a mixture containing a wide concentration dynamic range, the

3.3. CE-MS Analysis of Intact Proteins of Whole-Cell Lysate and Subcellular Proteins

Table 1
Sequence coverage achieved by nano-LC vs. (CE-MS/MS)^6 for 6-protein digest

Protein	Nano-LC-MS coverage (%)	(CE-MS/MS)^6 coverage (%)
Carbonic anhydrase I (human)	31	81
Bovine serum albumin (bovine)	25	59
Phosphorylase B (rat)	23	54
Cathepsin C (bovine)	41	83
Carbonic anhydrase II (bovine)	12	59
Trypsinogen (bovine)	43	79

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concentration of the sample was systematically increased in each subanalysis (while utilizing dynamic exclusion) so that low-intensity peptides would rise above the mass spectrometer threshold and, consequently, undergo MS/MS analysis. Moreover, to alter the ionization efficiency of peptides with low electrospray ionization efficiency and to change the migration behavior of comigrating peptides under a specific liquid composition, the CE background electrolyte was modified in several subanalyses to further improve sequence coverage. The (CE-MS/MS)^6 was applied to the analysis of the tryptic digests of the six-protein mixture (Table 1), and the results were compared with the analysis of the same mixture using a nano-HPLC-MS (Fig. 16). The sequence coverage achieved with the nano-HPLC and the (CE-MS/MS)^6 is summarized in Table 1. As shown in Table 1, (CE-MS/MS)^6 produced much better sequence coverage using only a few nanoliters of the protein digest, while the HPLC analysis used 1 µL of the same solution. The results clearly show the advantage of the (CE-MS/MS)^n for high sequence coverage proteomics while consuming a minimal amount of the samples.

3.3. CE-MS Analysis of Intact Proteins of Whole-Cell Lysate and Subcellular Proteins

Perhaps one of the most important and underutilized advantages of the CE-MS is its capability to analyze intact proteins and protein complexes. While most hyphenated chromatography–mass spectrometry techniques have a hard time analyzing complex protein mixtures, the dynamically coated capillaries discussed above have no problem analyzing proteins and protein complexes. Separation and MS analysis of intact proteins are especially useful for top-down proteomics (43), in which intact proteins in complex mixtures are

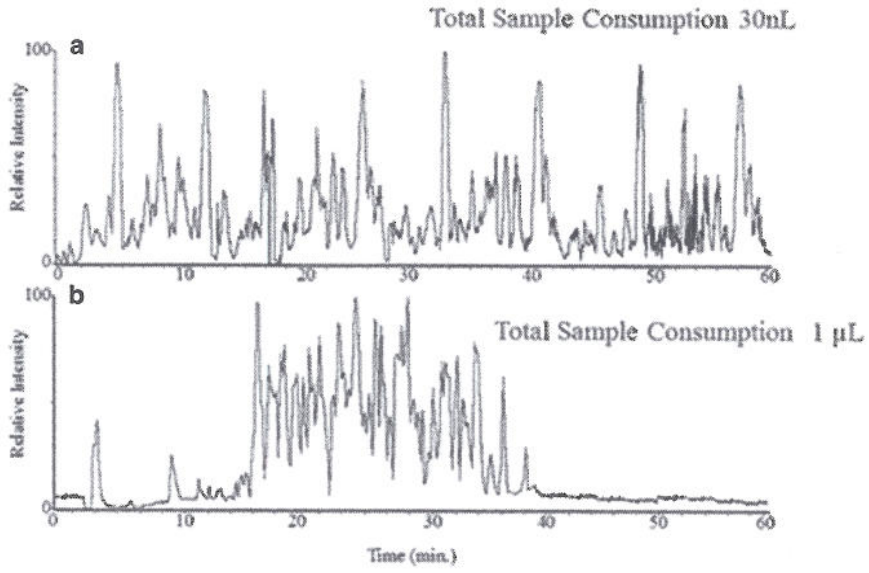

Fig. 16. Comparison between (CE-MS/MS)^6 and nano-HPLC-MS analyses of the tryptic digests of a six-protein mixture (reprinted with permission from (Garza, S., Moini, M. (2006) Analysis of Complex Protein Mixtures with Improved Sequence Coverage Using (CE–MS/MS)^n, *Anal. Chem.* 78, 7309–7316). Copyright (2006) American Chemical Society).

analyzed by MS and then undergo collision-induced dissociation (CID), electron-capture dissociation (ECD), or electron-transfer dissociation (ETD) where protein identification can be achieved without enzymatic digestion (44). While perhaps the ultimate goal for CE-MS analysis of complex protein mixtures is to analyze whole-cell lysates, currently CE-MS analysis of protein mixtures is limited to analyzing subcellular protein contents such as ribosomal proteins. Limitation for the analysis of whole-cell lysate is due to (1) limited peak capacity of CE-MS which is currently ~100, (2) insolubility of a large number of whole-cell lysate proteins in the MS-friendly background electrolyte, (3) a wide concentration range of proteins in the cell, and (4) limited volume of the sample that can be injected into CE capillary. For example, when the whole-cell lysate of *E. coli* was injected into a capillary containing 0.1% acetic acid and 25–50% organic solvents, protein precipitation resulted in capillary blockage (45). While the use of a basic BGE (20 mM ammonium bicarbonate (NH_4HCO_3) in water/methanol (75/25 v/v) with pH 8 (using NH_4OH)) reduced precipitation, because of low sensitivity of detection of proteins under this BGE, only highly abundant proteins were detected. To increase the number of proteins detected, the whole-cell lysate was fractioned into acidic and basic fractions by dissolving the whole-cell lysate in the acidic BGE. The supernatant was then analyzed using the acidic BGE and the APS-derivatized capillary. The precipitate of the acidic cell lysate was then dissolved in the 20 mM ammonium

3.3.1. Application of CE-MS to the Analys of the Major Proteins of Red Blood Cells (RB Under Denaturing Condition

bicarbonate and analyzed by CE-MS, using the basic BGE and the HPMC-coated capillary. For each analysis, 1 nL of the acidic or basic crude lysate solution was injected. Figure 1d displays an example of the mass spectrum observed for each peak of the electropherogram. As shown, because of the large concentration dynamic range of proteins in *E. coli* cells and the limited injection volume of the CE, only 58 proteins were detected in these experiments. At the cell lysate concentration used, many low-level proteins either were below the CE-MS detection limit or were masked by comigrating proteins of larger quantity. Moreover, some proteins were not detected due to their poor solubility. The results clearly show that 1-D CE is not capable of completely analyzing such a complex protein solution with a large concentration dynamic range. Therefore, the utility of CE-MS in analyzing a less-complicated protein mixture was investigated using *E. coli* ribosomal proteins. Figure 17 shows the CE-MS electropherograms for the analysis of ribosomal proteins using 0.1% acetic acid in water/acetonitrile (50/50 v/v) (A) and 0.1% acetic acid (B) as the BGE, utilizing a 15-mm-i.d., 120-cm-long capillary. In each experiment, 1 nL of the protein solution (1.7 ng of total ribosomal protein) was injected. As shown in Fig. 17, respectively, 44 and 39 different ribosomal proteins were detected in each run. Some of the proteins not detected using the BGE containing 0.1% acetic acid in water/acetonitrile (50/50 v/v) were detected using 0.1% acetic acid. Overall 55 out of the 56 (45, 46) proteins were detected in two runs, consuming only 2 nL of the sample (3.4 ng of ribosomal protein). The results clearly show that CE-MS is a viable technique for top-down analysis in subcellular proteomics.

3.3.1. Application of CE-MS to the Analysis of the Major Proteins of Red Blood Cells (RBCs) Under Denaturing Condition

In addition to subcellular proteomics mentioned above, CE-MS is especially useful for the analysis of the major proteins of mammalian cells. Most mammalian cells are specialized cells and are usually packed with a few major proteins that are involved with the specific functions of that cell. One such cell is human RBC which is specialized in transport of oxygen and carbon dioxide. RBC contains three major proteins: hemoglobin (Hb), carbonic anhydrase I (CAI), and CAII. In its endogenous state, hemoglobin (Hb) exists as a tetramer (~450 amol/cell), which consists of two dimers, each composed of an α-chain (average MW 15 126) and a β-chain (average MW 15 865). Each chain contains a heme group (47). Carbonic anhydrase I (CAI, average MW 28 780) and carbonic anhydrase II (CAII, average MW 29 156) are the next most abundant proteins (48, 49), with the respective quantities of ~7 and ~0.8 amol in each adult RBC (50, 51). In addition to the common reasons for monitoring hemoglobin (e.g., for identification of variants), there is also a need for the identification of carbonic anhydrase isoforms. For example, a significant decrease in CAI levels could suggest hemolytic anemia or hyperthyroid Graves' disease (52, 53), CAII

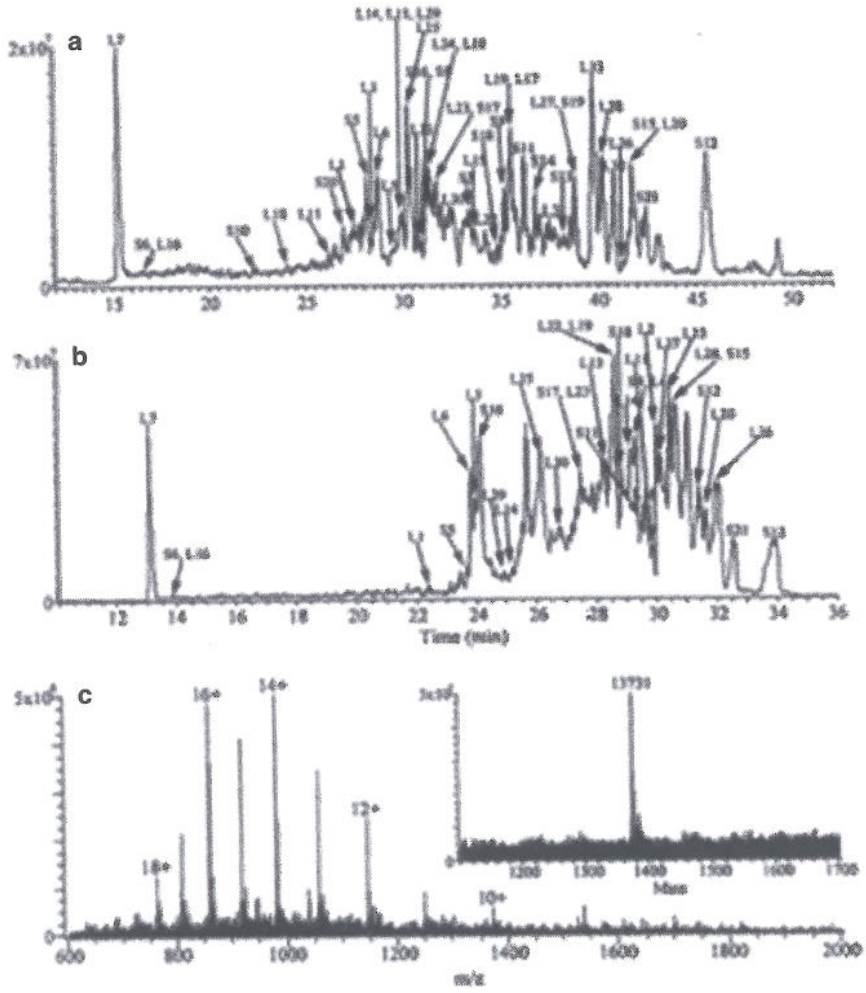

Fig. 17. (a) CE/ESI-MS base peak electropherogram of the *E. coli* ribosomal proteins using 0.1% acetic acid in 50% acetonitrile as the BGE. (b) Same as (a) except that 0.1% acetic acid in water was used as the BGE. Approximately 1.7 ng of total ribosomal proteins was injected in each analysis. (c) Mass spectrum of the S11 peak in (a). The inset shows the deconvoluted mass spectrum (13 731 Da) of the peak of (c) (reprinted with permission from (Moini, M., Huang, H. (2004) Application of capillary electrophoresis/electrospray ionization-mass spectrometry to subcellular proteomics of *Escherichia coli* ribosomal proteins, *Electrophoresis* 25, 1981–1987). Copyright (2004) John Wiley & Sons, Ltd).

deficiency results in renal tubular acidosis (54), while osteoporosis, symmetrical cerebral calcification, and mental retardation have been noted in individuals with very low levels of CAII in their RBCs (55). Moreover, a decrease in the ratio of CAI/CAII is observed in individuals deficient in glucose-6-phosphate dehydrogenase. Therefore, it is important to develop sensitive analytical techniques for the detection and identification of hemoglobin and carbonic anhydrase isoforms, preferably at single-cell levels. There are two ways to detect the chemical contents of RBCs: (1) under denaturing conditions, where protein–protein and protein–metal complexes are dissociated, and (2) under native

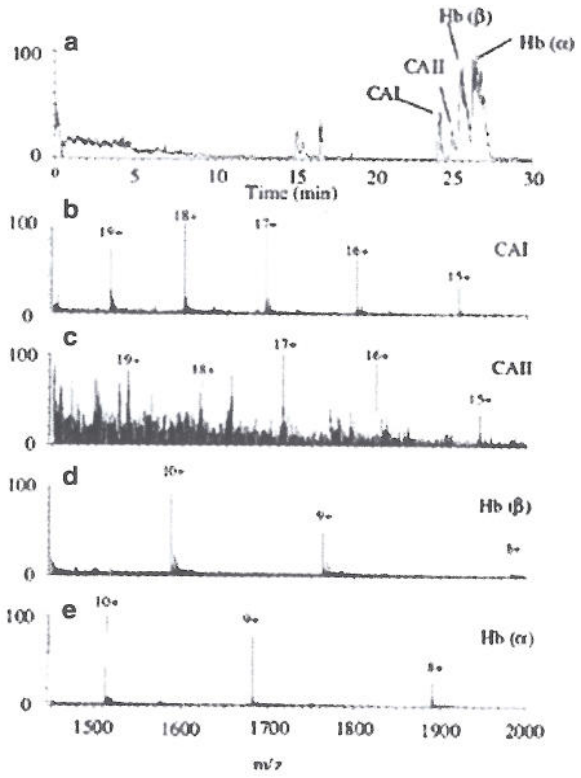

Fig. 18. (**a**) Base peak electropherogram of the lysed blood at a CAII concentration of ~88 amol/nL using the long column. Approximately 0.5 nL of lysed blood was injected. (**b**) Mass spectrum of the peak marked CAI of panel **a**. (**c**) Mass spectrum of the peak marked CAII of panel **a**. (**d**) Mass spectrum of the peak labeled Hb (β) of panel **a**. (**e**) Mass spectrum of the peak labeled Hb (α) of panel **a**. The 0.1% acetic acid solution containing 50% acetonitrile was used for this experiment. Unmarked peaks on panel **a** were not identified (reprinted with permission from (Moini, M., Demars, S.M., Huang, H. (2002) Analysis of Carbonic Anhydrase in Human Red Blood Cells Using Capillary Electrophoresis/ Electrospray Ionization-Mass Spectrometry, *Anal. Chem.* 74, 3772–3776). Copyright (2002) American Chemical Society).

conditions in which protein–protein and protein–metal complexes remain intact. In this section CE-MS analysis of RBCs under denaturing conditions will be discussed.

Under denaturing conditions, all four major proteins of RBCs are detectable by CE-MS (Fig. 18); however, since CAI (~7 amol/cell) and CAII (~0.8 amol/cell) are, respectively, ~100× and ~1,000× less than α-chain and β-chain of hemoglobin (~900 amol/cell), they must be well separated from hemoglobin before they can be detected (56). Otherwise, the background chemical noise associated with the ~1,000× molar excess of hemoglobin masks the CA signals. Because of this large molar excess ratio of hemoglobin, successful detection of CAI and CAII requires complete separation of these two proteins from hemoglobin. This was achieved by using

a long (125-cm) capillary and operating under reverse polarity mode (−30 kV at the inlet), which allowed CAI and CAII to migrate before hemoglobin chains and be detected. Under this condition, the detection limit of CAII in lysed blood was ~44 amol. On the other hand, the limit of detection for CAI, which was completely separated from Hb, was ~20 amol, which was limited by the LCQ MS sensitivity. For example, when a single intact RBC was analyzed with this capillary, CAI (~7 amol/RBC) was not detected. This narrow dynamic range is a limiting factor in CE/ESI-MS analysis of samples containing components in a wide range of concentrations. At the single-cell level, a more sensitive mass spectrometer is required to detect components present in low quantities (<20 amol). At the multi-cell level, or when lysed cells are used, a long capillary is needed to separate the components of the mixture. However, it is important to note that this level of sensitivity was achieved by using an older generation of mass spectrometer. Modern mass spectrometers will probably be able to detect CAI at single-cell level. A drawback of using a long capillary is, however, longer analysis time. Off-line separation of the major constituents of samples prior to CE/ESI-MS is another alternative for the detection of minor components of cells. However, this technique is time-consuming and may result in loss of sample due to additional sample handling. The results demonstrate that CE/ESI-MS is a powerful tool for the analysis of the chemical contents of single cells at low attomole levels.

3.4. Analysis of Protein Complexes by CE-MS

Analysis of the protein contents of cells under native conditions when proteins maintain their non-covalent association with other proteins, molecules, and metals is an important task. This is because the genome itself does not explain many cellular functions and processes. Proteins have specialized functions in the cellular environment and may non-covalently aggregate with DNA, RNA, cofactors, ligands, and other proteins to produce protein complexes. Protein complexes found inside and outside of biological cells take part in many biochemical pathways and perform many different functions. The effects of drugs and other ligands on a particular protein complex can also be investigated, which can become a powerful tool for the biotechnology industry. Mass spectrometry analysis of protein complexes in cells under native conditions is essential for their identification since the formation of the complex is largely affected by pH and other environmental factors. To date, MS analysis of a mixture of protein complexes is mostly achieved by off-line separation and purification of protein complexes followed by MS analysis using infusion electrospray ionization (ESI). By utilizing this technique, several types of protein complexes have been analyzed including the following: protein–DNA, protein–ligand, and protein–protein complexes (57–66). The main disadvantage of infusion ESI-MS for the analy-

3.4.1. Application of CE-MS to the Anal of Major Protein–Pro and Protein–Metal Complexes of Erythrc Directly from Cell Lys

sis of protein complexes is that unbound analytes and salts present in the solution can associate with the protein complexes during the ESI process, resulting in degradation of peak resolution when the resolving power of the instrument is not high enough to separate the individual components as well as shift to higher average MW for the protein complexes as a result of these nonspecific adducts. Moreover, separation and sample cleanup is labor-intensive and time-consuming.

Capillary electrophoresis is an ideal separation technique for the analysis of protein complexes. Two important advantages of CE that are essential for the analysis of protein complexes are the following: (1) compatibility with a variety of background electrolytes at a wide pH range, which allows separation of intact protein complexes under native condition (for example, by switching from 0.1% acetic acid solution (pH ~3.5) to ammonium acetate with pH ~7 as the background electrolyte, protein complexes were measured using the same capillary that is normally used for the analysis of intact proteins under denaturing conditions), and (2) separation of endogenous protein complexes from analytes not associated with the complex that could otherwise be attached to the complex during electrospray ionization process. By separating these nonspecific analytes, CE-MS provides a more accurate representation of the constituents of the complex without the use of hydrophobic media that can disintegrate protein complexes. In recent years, we have applied this technique to the analysis of the protein complexes of RBCs directly from cell lysates and for the measurement of the protein–metal stoichiometry.

3.4.1. Application of CE-MS to the Analysis of Major Protein–Protein and Protein–Metal Complexes of Erythrocytes Directly from Cell Lysate

In this section we discuss CE-MS analysis of the RBC under native conditions, in which protein–protein and protein–metal complexes remain intact (67). Under native conditions, hemoglobin (average MW 64446) is a protein complex in RBCs that exists as a tetramer (Hb-tetramer) consisting of four non-covalently bonded protein subunits: two α-chains and two β-chains with each chain attached to a heme group (MW 616). CAI and CAII are two other proteins in RBCs that are each complexed with one zinc cofactor. This Zn cofactor is very tightly bounded to both CAI (CAI-Zn, MW 28 845) and CAII (CAII-Zn, MW 29 221) at pH ~7. Figure 1 shows the electropherogram (panel a) of a lysed blood kept in ammonium acetate for 4 h before analysis and the corresponding mass spectra (panels b–d) of the CAII-Zn, CAI-Zn, and Hb-tetramer protein complexes of panel a, respectively. The order of migration, as shown in Fig. 1, is CAI-Zn, CAII-Zn, and Hb-tetramer. This order of migration is different from our previous CE-MS analysis of lysed RBCs (56) because of the major experimental differences between CE-MS analyses of RBC under denature condition compared with analysis under native conditions. To analyze intact protein complexes, we used ammonium acetate at pH~7 (the BGE

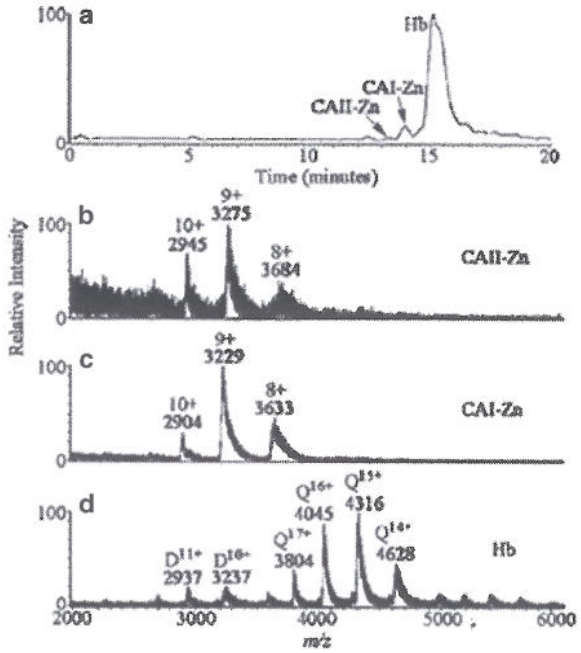

Fig. 19. (a) Base peak electropherogram of blood lysed and diluted 100× in 1.7 mM ammonium acetate (pH 7.4) and kept in the solution for 4 h before CE/ESI-MS analysis with 5 nL of sample injected. (b–d) Corresponding mass spectra of the peaks of panel a marked with CAII-Zn, CAI-Zn, and Hb. The Hb-dimer present in (d) is found to have 2 hemes attached (MW 32 322) (reprinted with permission from (Nguyen, A., Moini, M. (2008) Analysis of Major Protein–Protein and Protein–Metal Complexes of Erythrocytes Directly from Cell Lysate Utilizing Capillary Electrophoresis Mass Spectrometry, *Anal. Chem.* 80, 7169–7173). Copyright (2008) American Chemical Society).

3.4.2. Application of CE-MS to Metal Displacement and Stoichiometry of Protei Metal Complexes

also contained polybrene as a self-coating reagent, to prevent analyte–wall interaction.) In addition, the experiments were performed under forward polarity mode so the CE outlet electrode acted as the cathode with respect to the CE inlet electrode and acted as an anode with respect to ESI. By acting as both anode and cathode, the outlet electrode maintains a neutral pH at the end of the capillary, thereby preventing dissociation of the tetramer. In addition, under forward polarity mode, CAII-Zn and CAI-Zn migrated ahead of Hb-tetramer, avoiding being masked by, respectively, 562× and 64× molar excess of Hb-tetramer. Moreover, to achieve high separation efficiency, injection volume was limited to ~5 nL (of the 100× diluted lysed blood) resulting in high separation efficiency. Under these conditions, CAII-Zn, CAI-Zn, and Hb were almost baseline separated. Figure 19 shows the base peak electropherogram of blood lysed and diluted 100× in 1.7 mM ammonium acetate (pH 7.4) and kept in the solution for 4 h before CE/ESI-MS analysis (panel a). Panels b–d show the corresponding mass spectra of the peaks of panel a marked with CAII-Zn, CAI-Zn, and Hb. The Hb-dimer present in (D) is found to have 2 hemes attached (MW 32 322). The mass spectrum of panel d also

contained a small amount of (Hb-dimer + 2(heme)) as indicated by D^{10+} and D^{11+}, as well as monomers. However, the extracted ion electropherograms of the monomers and the dimer were almost identical to that of the electrophoretic peak profile of the Hb-tetramer, indicating that they were most likely from the dissociation of Hb-tetramer at the end of the CE capillary and during the desolvation/ionization process. No separate electrophoretic peak for Hb-dimer or Hb-monomer was observed, even when injecting 4× more samples (20 nL rather than 5 nL) and using a blood sample left in ammonium acetate for 6 h (Fig. 2). These results implied that, at physiological pH, the amount of Hb-dimer in lysed blood was too low to be detected by CE/ESI-MS. This is consistent with the binding constant (K) of the Hb-dimer ↔ Hb-tetramer, which is reported to be ~4×10^5 M^{-1} for fully oxygenated blood (K increases as less oxygen binds) at pH 7.4. In summary, the use of polybrene as a self-coating reagent in conjunction with ammonium acetate at pH ~7.4, narrow capillary for high separation efficiency, and forward polarity CE to avoid acid production at the tip of the capillary were overriding experimental factors for successful analysis of protein complexes. Diluting the lysed blood sample in ammonium acetate for a minimum of 6 h before injecting the sample into the CE was essential for obtaining the mass accuracy consistent with their theoretical average molecular weights. A separate electrophoretic peak for the Hb-dimer was only detected when the pH of the BGE was lowered from 7.4 to ~6.6. The tetramer was completely dissociated to dimer when the BGE pH was dropped to 5. At pH 5, the mass spectrum of the Hb-tetramer was similar to CE-MS analysis under reverse polarity mode, indicating that under reverse polarity mode the pH at the ESI tip had decreased from pH 7.4 to ~5, the pH at which Hb-tetramer completely dissociates to dimer (60).

3.4.2. Application of CE-MS to Metal Displacement and Stoichiometry of Protein–Metal Complexes

Another example of the application for CE-MS analysis to protein complexes is the study of metal displacement and stoichiometry of protein–metal complexes under native conditions. Currently, most MS analyses of protein complexes use direct-infusion ESI-MS. While this technique offers much higher sensitivity of detection compared to nuclear magnetic resonance (NMR) and X-ray diffraction techniques, since protein–metal complexes under native conditions usually are dissolved in salt solutions, their direct ESI-MS analysis requires off-line sample cleanup prior to MS analysis to avoid sample suppression during ESI. Moreover, direct infusion of the salty solution promotes nonspecific salt adduct formation by the protein–metal complexes under ESI-MS, which complicates the identification and stoichiometry measurements of the protein–metal complexes. Because of the high mass of protein–metal complexes and lack of sufficient resolution by most mass spectrometers to separate nonspecific from specific metal–protein

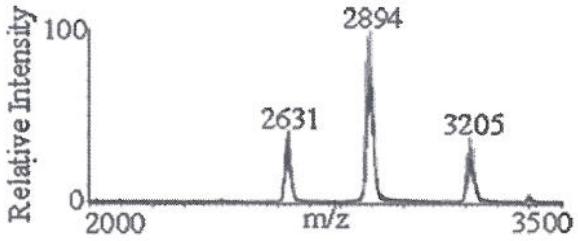

Fig. 20. Mass spectrum of the AiiA-$2Co^{2+}$ complex at pH 6.9 obtained using infusion ESI-MS (reprinted with permission from (Garza, S., Thomas, P.W., Fast, W., Moini, M. (2010) Metal displacement and stoichiometry of protein–metal complexes under native conditions using capillary electrophoresis/mass spectrometry, *Rapid Commun. Mass Spectrom.* 24, 2730–2734). Copyright (2010) John Wiley & Sons, Ltd).

complexes, accurate protein–metal stoichiometry measurements require some form of sample cleanup prior to ESI-MS analysis. CE-MS in conjunction with a medium-resolution (10,000) mass spectrometer is an efficient, sensitive, and fast method for the measurement of the stoichiometry of the protein–metal complexes under physiological conditions (pH ~7) without the complications of the infusion ESI-MS. To demonstrate this, recently we compared the metal displacement of Co^{2+} to Cd^{2+} using both ESI-MS and CE-ESI-MS using the procedure explained above for the analysis on intact protein–protein and protein–metal complexes of RBC directly from cell lysates (68). Co^{2+} and Cd^{2+} are two metal ions necessary for activation in the monomeric AHL lactonase produced by B. thuringiensis. The AiiA from B. thuringiensis (average MW 28633) is known to have quorum-quenching activity in the presence of two bound metal ions. The AiiA–metal complex is most stable at a pH ~7 in the presence of buffer salts; however, the presence of this buffer during the ESI process suppresses the ionization of the complex. CE/ESI-MS of the AiiA-$2Co^{2+}$ complex was performed using 10 mM ammonium acetate at pH values of 4.8 and 6.9; however, at pH of 4.8, the protein–metal complex dissociated and only the protein was detected. In contrast, at pH 6.9 the protein–metal complex remained intact. Similar to our RBC study, to obtain an accurate molecular weight free from the nonspecific salt adduct, it was necessary to dilute the sample in a solution of 10 mM ammonium acetate (pH 6.9) before performing CE/MS analysis. Figure 20 shows ESI-MS of AiiA-Co^{2+} using infusion ESI. As shown, even though the complex was diluted in ammonium acetate solution before infusion ESI-MS, the mass spectrum of the protein–metal complex still contains a number of unresolved peaks caused by multiple nonspecific metal (mostly sodium) adductions. Since the resolution of a typical TOF mass analyzer is usually not high enough to differentiate the isotope clusters of protein–metal complexes from proteins bound to

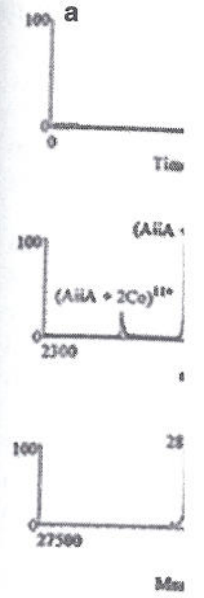

Fig. 21. The electroph
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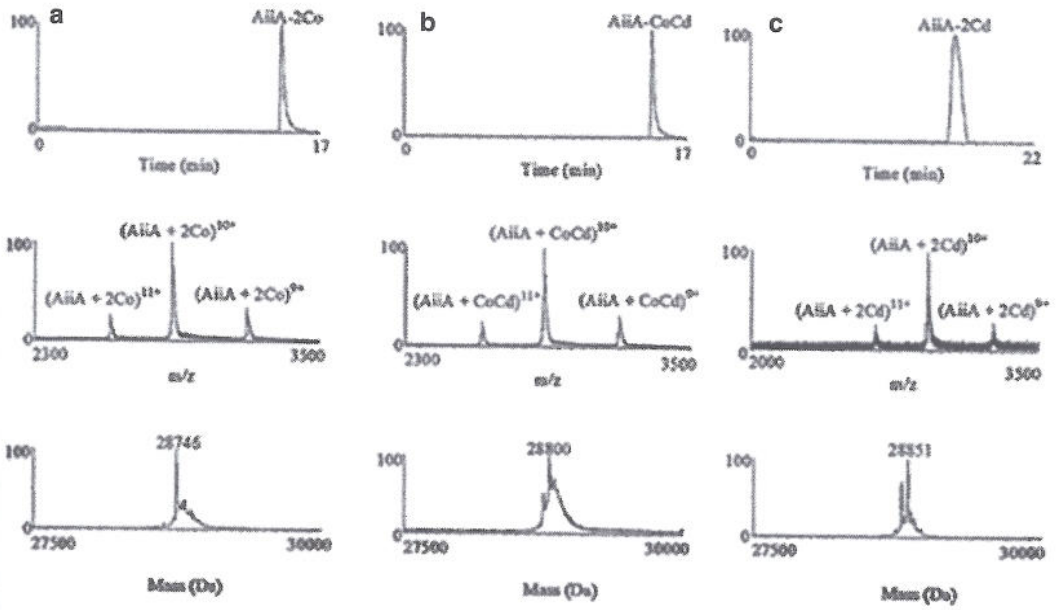

Fig. 21. The electropherograms (*top panels*), mass spectra (*middle panels*), and deconvoluted mass spectra (*bottom panels*) of AiiA–metal complexes when a 1:1 ratio (**a**), a 10:1 ratio (**b**), and a 100:1 ratio (**c**) of Cd^{2+}:Co^{2+} solutions were added to the protein solution (reprinted with permission from (Garza, S., Thomas, P.W., Fast, W., Moini, M. (2010) Metal displacement and stoichiometry of protein–metal complexes under native conditions using capillary electrophoresis/mass spectrometry, *Rapid Commun. Mass Spectrom.* 24, 2730–2734). Copyright (2010) John Wiley & Sons, Ltd).

nonspecific and specific metal ions, it becomes difficult to accurately identify the composition and stoichiometry of the protein–metal complexes. Therefore, to utilize direct-infusion MS, it is necessary to significantly, but not completely, reduce the salt content by using off-line salt-removal techniques. In contrast, under CE/MS conditions, the mass spectrum of the electrophoretic peak of the same sample contained sharp peaks (Fig. 21, panel a), corresponding to the m/z value of the protein–metal complex within the experimental error of ~0.02%.

The AiiA protein was originally grown in a medium containing $CoCl_2$. The mass spectrum of the protein complex shows that the AiiA is binuclear, with two cobalt atoms bound to the protein. To determine the relative binding stoichiometry of the Cd^{2+}, different ratios of $CdCl_2$ (1:1, 10:1, and 100:1) were added to the AiiA-Co^{2+} complex in order to substitute the two Co^{2+} ions with two Cd^{2+} ions. In Fig. 21, panels a–c show the CE/MS analyses of these three ratios, respectively. The top panels illustrate the electropherogram of the protein–metal complexes, the middle panels show the corresponding mass spectra, and the bottom panels show the deconvoluted mass spectra. From Fig. 21, the relative binding stoichiometry of each metal can be determined. The results indicate that, at a 1:1 ratio of Co^{2+} to Cd^{2+}, both Co^{2+} ions are tightly bound to the AiiA protein. However, as more Cd^{2+} is added (10:1 ratio),

one Co^{2+} ion is completely replaced by one Cd^{2+} ion. At a 100:1 ratio of Cd^{2+} to Co^{2+}, both Co^{2+} ions are replaced by two Cd^{2+} ions. Because the results were obtained by CE/MS, all free metal ions were separated from the protein–metal complex and did not participate in the complex formation during ESI, allowing a more accurate determination of the ratios of the metal concentration needed for metal displacement.

As shown in this section, the analysis of intact protein–protein and protein–metal complexes was accomplished using CE/ESI-MS. Intact protein complexes were analyzed with no prior sample preparation. Accurate MWs (~0.01% error) were obtained for all major protein–protein and protein–metal complexes. It was observed that more accurate MW was obtained when protein complexes were diluted in ammonium acetate buffer and left in that solution for a few hours. Ion exchange between ammonium ions in the solvent and nonspecific sodium adducts of the protein–protein and protein–metal complexes slightly unraveled the complexes. CE/ESI-MS further enhanced this charge exchange and separated the nonspecific salt adducts from protein complexes, resulting in formation of higher charge-state protein complexes free from nonspecific salt adducts. Deconvolution of these higher charge-state ions provided accurate (~0.01% error) average MW of intact protein–protein and protein–metal complexes for better identification of the constituents of the complexes. Operation of the CE under forward polarity mode was essential for detection of intact protein–protein and protein–metal complexes. This was because of the electrochemical nature of CE and ESI processes. These results demonstrate that CE/ESI-MS is an effective tool for the analysis of the mixture of protein complexes and can accurately provide average MW for unambiguous identification of small (<100,000 Da) protein complexes.

4. High-Throughput CE-MS Analysis Using a CE with Eight Capillaries in Conjunction with a Mass Spectrometer with Eight Inlets

High-throughput analysis of complex mixtures has been a primary need for industries such as pharmaceutical, biotechnology, and combinatorial chemistry, where a large number of reaction by-products must be analyzed. In recent years, the effort to increase throughput using separation in conjunction with mass spectrometry has been focused on reducing the separation time. In CE-MS, shorter analysis time is also achieved by using ultrafast CE-MS (UFCE-MS) by reducing capillary inner diameter (i.d.) (<10 μm) and capillary length (<30 cm) while increasing separation voltage (>1,000 V/cm) (69). To increase throughput beyond this technique, we recently introduce multi-CE capillaries in

conjunction with gated multi-inlet mass spectrometry (70). In this technique the MS atmospheric pressure sampling inlet (nozzle, bore, sampling orifice, or heated capillary) is modified by replacing its single inlet with multiple atmospheric pressure inlets. This allowed for the parallel introduction of multiple liquid streams into the MS using one electrosprayer for each inlet so that the chemical contents of all liquid streams are analyzed concurrently using a single MS. In our original multi-sprayer, multi-inlet design (71, 72), all inlets were open to the atmosphere at all times and the ESI sprayers either sprayed continuously or were turned on one at a time using a high-voltage switch. Since all of the inlets were open to the atmosphere at all times, the pressure in the first stage of the vacuum system was too high for the original pumping system to evacuate when multiple inlets were used. To maintain operating pressure within the MS, the inlet i.d. was reduced and multiple pumps were used. In order to maintain the original inlet i.d. and pumping system while still maintaining the original sensitivity (per inlet), a gated multi-sprayer, multi-inlet design was developed. As a proof of concept we used a CE with 8 parallel CE capillaries in conjunction with a mass spectrometer equipped with 8 sprayers and 8 inlets for high-throughput and concurrent analysis of 8 complex mixtures.

For multi-CE, multi-inlet TOF-MS analysis, an in-house fabricated CE instrument capable of handling eight samples and eight buffer vials was used (Fig. 22). The CE instrument was capable of pressure programming during a voltage separation. Pressure and voltage were supplied by a commercial CE instrument (Beckman Coulters, Fullerton, CA, USA). Eight 50-μm-i.d., 150-μm-o.d. CE capillaries, ranging between 50 and 80 cm in length, were interfaced to the multi-inlet MS using a sheathless CE/ESI-MS interface. Approximately 5 nL of the peptide standard was injected into each capillary using the pressure injection mode. The CE was operated in reverse polarity mode (−15 kV applied at the CE inlet) and a 0.1% acetic acid solution was used as the BGE. Figure 23 shows the schematic of the gated eight-sprayer, eight-inlet TOF-MS. For proof of concept a peptide mixture containing 5 peptides was injected into all eight-CE capillaries. Figure 24 (top panel) shows the total ion electropherogram of the analysis of the peptide standard using eight-CE capillaries. Figure 24 (bottom panel) shows the reconstructed total ion electropherogram for one of the capillaries. The low resolution achieved in this electropherogram was because of the wider i.d. (50 μm) capillary in conjunction with low separation voltage (15 kV) used in this experiment. The use of a wider capillary and lower separation voltage was necessary to obtain peak widths that were compatible with the acquisition time (4.8-s turnaround time) of our current multi-inlet instrument. This preliminary result demonstrates that gated multi-inlet mass

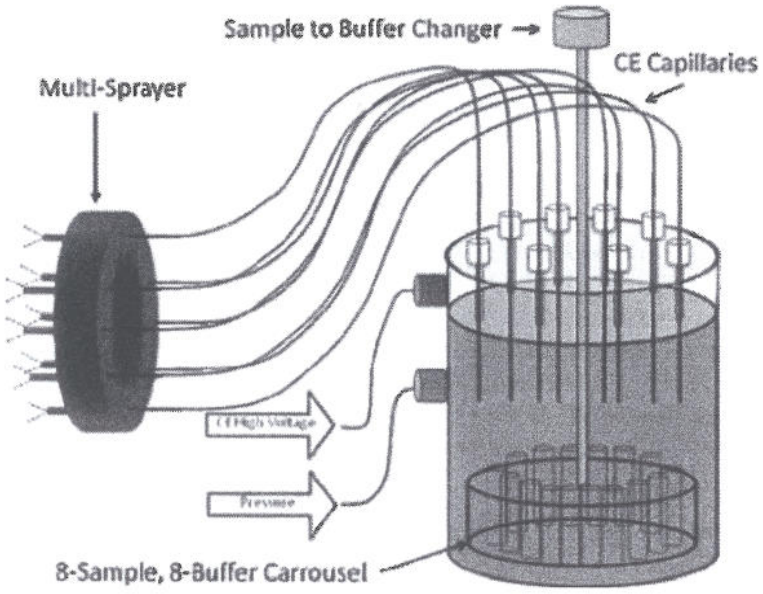

Fig. 22. Schematic of the eight-capillary CE instrument capable of providing pressure programming under voltage separation (reprinted with permission from (Moini, M., Jiang, L., Bootwala, S. (2011) High-throughput analysis using gated multi-inlet mass spectrometry, *Rapid Commun. Mass Spectrom.* 25, 789–794). Copyright (2011) John Wiley & Sons, Ltd).

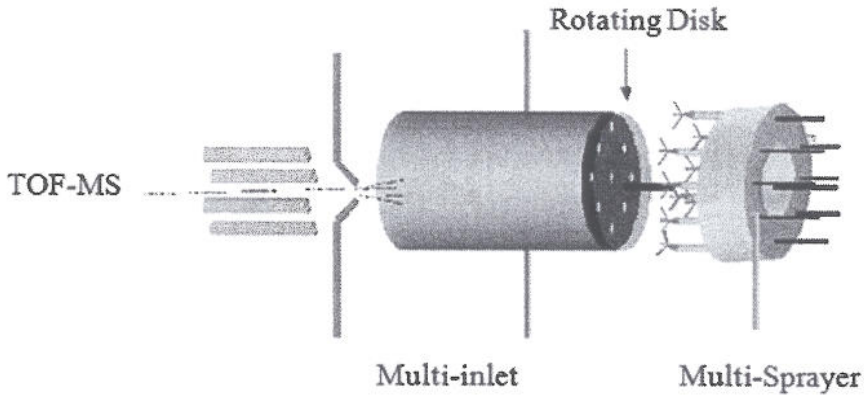

Fig. 23. Schematic of the gated eight-sprayer, eight-inlet TOF-MS (reprinted with permission from (Moini, M., Jiang, L., Bootwala, S. (2011) High-throughput analysis using gated multi-inlet mass spectrometry, *Rapid Commun. Mass Spectrom.* 25, 789–794). Copyright (2011) John Wiley & Sons, Ltd).

spectrometry is a viable technique for high-throughput analysis, where multiple streams of liquids from several CE capillaries can be analyzed using a single mass spectrometer. Up to eight peptide mixtures from up to eight CE capillaries were analyzed in about 30 min.

5. Appendix 1. Standard Operating Procedure for Making Porous Capillary Using H

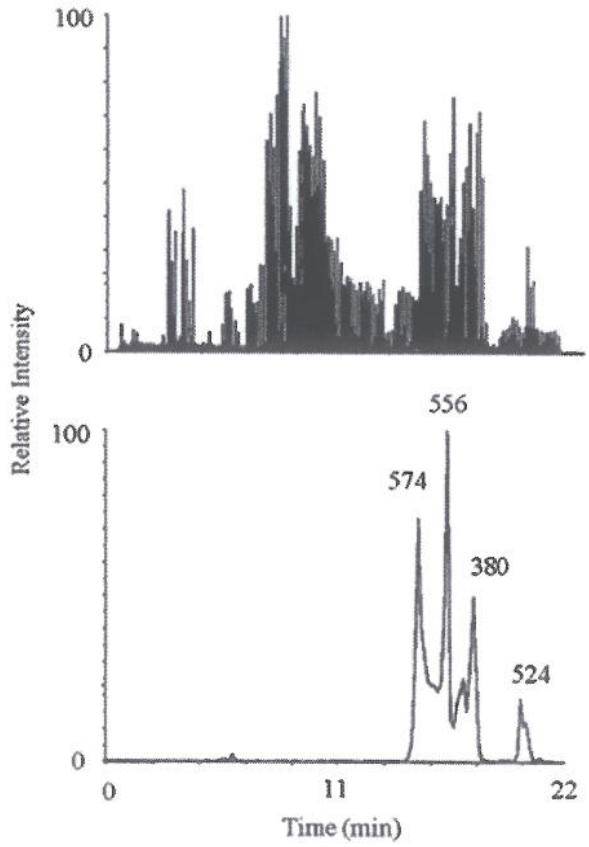

Fig. 24. Combined ion electropherogram (CIE) of the eight-CE capillary system (*top*). The same peptide standard was injected in each capillary. Extracted total ion electropherogram (TIE) of one capillary/inlet is shown at the *bottom panel*. The *m/z* 574 (1+), 556 (1+), 380 (1+), and 524 (2+) are, respectively, Met-enkephalin, Leu-enkephalin, Val-Tyr-Val, and angiotensin II (reprinted with permission from (Moini, M., Jiang, L., Bootwala, S. (2011) High-throughput analysis using gated multi-inlet mass spectrometry, *Rapid Commun. Mass Spectrom.* 25, 789–794). Copyright (2011) John Wiley & Sons, Ltd).

5. Appendix 1. Standard Operating Procedure for Making Porous Capillary Using HF

Substance: hydrogen fluoride, HF

Identification numbers: CAS number 7664-39-3

Main risks and target organs: Hydrogen fluoride is highly corrosive to all tissues. Skin: burns, necrosis; underlying bone may be decalcified. Eyes: burns

The course of the skin burns from hydrogen fluoride depends on concentration; more dilute solutions cause potentially serious but delayed (24 h) systemic effects. Absorption of the fluoride ion is a significant hazard mainly due to hypocalcaemia. Hydrogen fluoride burns to the eye are corrosive and require immediate flushing and ophthalmologic consultation.

5.1. Storage

Hydrogen fluoride should be stored in a cool, dry, well-ventilated area in tightly sealed containers. Containers of hydrogen fluoride should be protected from physical damage and should be stored separately from metals, concrete, glass, strong bases, sodium hydroxide, potassium hydroxide, and ceramics. Glass containers should not be used for storage as hydrogen fluoride will dissolve glass. Explicit warnings in appropriate languages and graphics should be maintained on all containers and work areas.

5.2. Personal Hygiene Procedures

If hydrogen fluoride contacts the skin, workers should flush the affected areas immediately with plenty of water for 15 min, followed by washing with soap and water.

Clothing contaminated with hydrogen fluoride should be removed immediately, and provisions should be made for the safe removal of the chemical from the clothing. Persons laundering the clothes should be informed of the hazardous properties of hydrogen fluoride, particularly its potential for causing irritation.

A worker who handles hydrogen fluoride should thoroughly wash hands, forearms, and face with soap and water before eating, using tobacco products, using toilet facilities, applying cosmetics, or taking medication.

Workers should not eat, drink, use tobacco products, apply cosmetics, or take medication in areas where hydrogen fluoride or a solution containing hydrogen fluoride is handled, processed, or stored.

5.2.1. Initial Medical Treatment

Thoroughly irrigate with water. Massage calcium gluconate gel into the burned skin for a minimum of 30 min and for as long as the pain persists; at times pain persists up to 4 h. If no gel is available, use a calcium (20 g Ca^{2+} in about 2 L of water) or magnesium solution for soaking.

5.2.2. Specific Preventive Measures

Appropriate latex or rubber gloves should be used when working with hydrogen fluoride. Frequent checks of glove integrity or changes of the gloves should be performed. Any suspected exposure should be immediately decontaminated. Eye goggles, aprons, sleeves, and other protective devices should be used in accord with the conditions of use. Concentrated and anhydrous hydrogen fluoride should only be used in the hood.

Use nitrile gloves, apron, face mask, and rubber gloves when working with HF solution and nitrile gloves and safety glasses when dealing with etched capillaries. Work under the hood when dealing with HF. Replace gloves each time your hands need to be outside the hood. Change gloves without touching skin.

5.2.3. Procedure for Making the Porous Tip Using the Teflon Container

1. Prepare a 500-ml saturated solution of calcium carbonate in a beaker and 500 mL of DI water in another beaker. These solutions will be used for neutralization of HF-contaminated glassware and fused-silica capillaries.

6. Appendix 2. Methods and Techniques for Analyzing Re Blood Cells

2. Fill Teflon container with fresh 48% HF, which is in a plastic container under a well-ventilated hood. To do this you may have to discard the old HF in the container by removing ~100 µL of HF solution from the HF container and slowly pouring it into the carbonate solution. Wash the container with DI water by filling the container with water and discard the water using disposable pipettes. Pour methanol into the empty Teflon container and discard it into the carbonate container.
3. Wait a few minutes for the leftover methanol in the HF container to evaporate.
4. Gradually (300–400 µL at a time) fill the dry Teflon container with fresh HF using a plastic disposable pipette (or a glass pipette if no plastic pipette is available). Fill the HF container to the top.
5. Replace gloves and etch a capillary by first burning about 4–5 cm of the capillary using a lighter. Second, remove the burnt debris at the capillary tip by using Chemwipes and methanol.
6. Attach the inlet of the capillary to the air hose in the hood using an Upchurch union.
7. Insert the bare fused-silica end of the capillary (the outlet) into the HF container all the way without touching the bottom of the container, and adjust it to prevent it from touching the wall of the HF container.
8. Wait appropriate amount of time for the outlet tip to become porous.
9. Using fresh gloves, remove the capillary outlet from the HF container and neutralize it by immersing it into the carbonate solution for a few seconds and then in water for a few seconds. Disconnect the fused-silica capillary from the air pressure and wash it under the faucet.
10. Replace the HF container top.
11. Decontaminate the area using the carbonated solution.

6. Appendix 2. Methods and Techniques for Analyzing Red Blood Cells

The chemical contents of RBCs were analyzed using three different sampling methods:

(1) A solution of lysed RBCs was injected into the capillary using the pressure injection mode. The solution was prepared by diluting 5 µL of fresh blood from a healthy adult male to 50 µL using saline solution, followed by centrifugation and removal

of the supernatant, leaving only intact cells without the plasma. After adding 50 µL of water to lyse the cells, the lysed cells were then diluted either 5× or 200× with water to final dilutions of 50× and 2,000×, respectively.

(2) Intact RBCs (one or three cells) were drawn into the capillary inlet using suction and a 200× microscope for observation.

(3) Intact RBCs suspended in a saline solution were injected into the CE capillary using the pressure injection mode of the CE instrument. The solution of suspended intact RBCs was prepared in a manner almost identical to method 1, except that saline solution was used instead of water to maintain intact cells. To homogenize the solution, the sample vial was hand-shaken immediately before sampling. It should be noted that it was necessary to use fresh blood to separate the Hb chains; when a 1-week-old blood sample was analyzed using the 0.1% acetic acid solution, only one peak with an average molecular weight of ~65,000 was observed. For intact cell analysis, best overall performance (separation and sensitivity) was achieved using the 0.1% acetic acid solution without any organic additives. However, when lysed blood was analyzed, the best overall performance was achieved using a 0.1% acetic acid solution containing 50% acetonitrile. Under these conditions, the presence of acetonitrile in the CE BGE reduced the EOF, and consequently, the four major proteins of RBCs were all separated (Fig. 18). Moreover, addition of acetonitrile also reduced the background chemical noise and adduct formation due to the incomplete desolvation of liquid droplets and, therefore, improved the S/N of CAI (Fig. 18, panel b). The improvement of S/N due to the latter BGE was further confirmed by analyzing a standard mixture of CAI and CAII (0.1 mg/mL) using a 0.1% acetic acid solution and a 0.1% acetic acid solution containing 50% acetonitrile. It was observed that while the mass spectra of these compounds had similar intensity on the absolute scale, both noise level and adduct formation were significantly lower for the BGE containing 50% acetonitrile. Other possible factors for the higher sensitivity of these proteins include better solubility and less adsorption to the capillary wall when the acetonitrile-containing BGE was used.

7. Appendix 3. Successful CE-MS analysis of protein complexes depends on three major factors

1. The use of ammonium acetate at pH 7.4. Ammonium acetate at pH ~7 was ideal for the analysis of protein complexes that we have studied. This is because it keeps the complexes intact and it is a mass-friendly background electrolyte. It was made by titrating a 0.01% acetic acid solution with a 0.01% ammonium

hydroxide solution until desired pHs were reached. At pH of 7.4 the concentration of the ammonium acetate (NH4OAc) buffer was ~1.7 mM. Polybrene (PB) was added to these buffers as the self-coating reagent 2 with a final concentration of PB at ~0.1%.

2. Soaking the protein complex in ammonium acetate for a few hours before CE-MS analysis. More accurate MWs (~0.01% error) were obtained for all major protein–protein and protein–metal complexes when they were diluted in ammonium acetate buffer and left in that solution for a few hours. The longer the protein complexes stayed in ammonium acetate solution before CE-MS analysis, the more intense and narrow the higher charge states of the protein complexes became. This was because longer times improved the ion exchange between the nonspecific, nonvolatile salts (mostly sodium) that were associated with the interior of the protein complexes under native conditions and the volatile ammonium ions of the BGE. Ion exchange between ammonium ions in the solvent and nonspecific sodium adducts of the protein–protein and protein–metal complexes slightly unraveled the complexes. CE/ESI-MS further enhanced this charge exchange and separated the nonspecific salt adducts from protein complexes, resulting in formation of higher charge-state protein complexes free from nonspecific salt adducts. Deconvolution of these higher charge-state ions provided more accurate (~0.01% error) average MW of intact protein–protein and protein–metal complexes for better identification of the constituents of the complexes.
3. Operation of the CE under forward polarity. Operation of the CE under forward polarity mode was essential for detection of intact protein–protein and protein–metal complexes. Intact protein complexes with minimal fragmentation can only be observed in forward polarity. This was because of the electrochemical nature of CE and ESI processes. As we have explained before, CE and ESI-MS represent two electrical circuits, each with two sets of electrodes, CE inlet and outlet electrodes, ESI emitter, and MS inlet electrodes. The CE/ESI-MS overlays these two separate circuits so that the CE outlet electrode and the ES emitter electrode are shared between the two circuits (CE outlet/ESI shared electrode). Therefore, under CE/ESI-MS, at the shared electrode two electrochemical reactions occur simultaneously. Depending on the polarity and magnitude of the voltage of the shared electrode compared with that of the CE inlet and MS inlet electrodes, electrochemical reactions at the shared electrode can be both reductive, both oxidative, or one reductive and the other oxidative. For example, under positive electrospray ionization mode, where +1.5 kV is applied to the shared electrode and 0–100 V is applied to the MS inlet electrode, two possibilities exist: (1) Under reverse

polarity CE, where −30 kV is applied to the CE inlet electrode, the shared electrode is anodic with respect to both CE inlet electrodes and the MS inlet electrode. (2) Under forward polarity CE, where +30 kV is applied to the CE electrode, the shared electrode is cathodic with respect to CE inlet electrode and anodic with respect to MS inlet electrode. At the anode, the major oxidation reaction for aqueous buffer solutions is typically the electrochemical oxidation of water ($2H_2O \leftrightarrow 4\ H^+ + O_2 + 4e^-$). The extent of the electrochemical reactions and therefore the pH change depends on the magnitude of the current that flows through each circuit. Under experimental conditions used here, CE current was ~1 μA, while ESI current was in the nanoampere range. Under reverse polarity mode in which the electrochemical reactions at the shared electrode are anodic for both circuits, the pH of the solution decreases enough to dissociate all the tetramer. The pH at the ESI tip under reverse polarity is estimated to decrease from pH 7.4 to 5, the pH at which some protein complexes dissociate to dimer. However, under forward polarity mode, the shared electrode is only anodic in the ESI circuit, which has a lower current, and its effect is mostly canceled by the cathodic reaction at this electrode in the CE circuit causing minimal dissociation of the tetramer. Since this dissociation (in both cases) is happening close to the outlet of the CE capillary, all dissociation products comigrate and show up as a single electrophoretic peak.

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