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Capillary electrophoresis using a surfactant-treated capillary coupled with offline matrix-assisted laser desorption ionization mass spectrometry for high efficiency and sensitivity detection of proteins

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Abstract

A method of combining capillary electrophoresis (CE) using a surfactant-modified capillary with matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) is described for protein analysis. The CE–MALDI-MS coupling is based on CE fraction collection of nanoliter volume samples in less than 5 μl of dilute acid. This offline coupling does not require any special instrumentation and can be readily performed with commercial instruments. Protein adsorption during CE separation is prevented by coating the capillary with the surfactant didodecyldimethylammonium bromide. This surfactant binds strongly with the capillary wall, hence it does not desorb significantly to interfere with subsequent MALDI-MS analysis. It is shown that the use of a dilute acid for CE fraction collection is advantageous in lowering the detection limit of MALDI-MS compared to using an electrophoretic buffer. The detection limit for proteins such as cytochrome *c* is 23 fmol injected for CE, or 1.2 fmol spotted for MALDI-MS. This sensitivity is comparable to alternative CE–MALDI-MS coupling techniques using direct CE sample deposition on the MALDI target. In addition, the fraction collection approach has the advantage of allowing multiple reactions to be carried out on the fractioned sample. These reactions are very important in protein identification and structure analysis. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Mass spectrometry (MS) is playing an important role for protein identification and characterization in proteomics. Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) MS are commonly used. ESI generates multiply charged ions that are particularly useful for tandem MS analysis. In addition, ESI can be readily interfaced with liquid phase separations such as liquid chromatography (LC) and capillary electrophoresis (CE). MALDI, on the other hand, has a higher tolerance to impurities. It produces simple mass spectra dominated by singly and doubly charged ions. Most importantly, it generally offers higher sensitivity for protein analysis compared to ESI.

There are several reports of combining liquid separation with MALDI-MS. In addition to the continuous flow probe method for LC–MALDI coupling [1–3], other on-line approaches have also been described [4]. However, on-line LC or CE–MALDI-MS has not been widely used for real-world

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applications due to current technical challenges, which lead to less than desirable performance for peptide and protein analysis [3,5]. Alternatively, offline coupling between LC and MALDI-MS can be performed by fraction collection. The offline coupling of MALDI-MS with CE, however, is less straightforward as the sample size in CE can be as low as a few nanoliters. In addition, electrical contact must be maintained during the electrophoretic separation. Methods reported for coupling CE and MALDI-MS generally involve either direct sample deposition from the CE capillary onto a MALDI target [6] or CE fraction collection followed by subsequent MALDI-MS analysis [7].

Direct sample deposition for offline coupling of CE with MALDI-MS results in excellent sensitivity. The sample is deposited onto targets pre-coated with MALDI matrix. All of the sample injected in CE is deposited with minimum handling. Sample deposition can be performed on a stationary MALDI plate in which the sample is spotted as droplets [8–10]. Alternatively, sample can be deposited as a trace on a moving MALDI target, such as a tape or a wheel [11–14]. The lowest detection limits reported for peptides and proteins using direct sample deposition are 50 amol and 2 fmol, respectively [12]. These numbers refer to the quantities injected into CE, and they are very close to the detection limits of the current MALDI mass spectrometers. While direct sample deposition offers low sensitivity, it does require special instrumentation alteration, which is not easily accessible by other users at present.

Fraction collection does not require special instrumental alteration, and it can be readily performed with commercial instruments. In addition, the fractionated samples can be potentially divided into smaller portions for down-stream chemical or enzymatic processing. The latter is crucial for protein identification and analysis of post-translational modifications. Multiple chemical or enzymatic reactions are necessary for achieving high amino acid coverage of a protein, thus increasing the confidence of identification or providing structural information on the modification sites. To perform fraction collection in CE, the electrophoretic separation is interrupted just before an analyte band reaches the outlet of capillary. The analyte band is then collected either hydrodynamically or electrokinetically [7]. When hydrodynamic fractionation is used, a low pressure is applied at the inlet to push the sample band off the capillary into a micro vial. However, significant band broadening is caused by the pressure-induced laminar flow during fraction collection. More importantly, the mobilities of the analytes remaining in the capillary are interrupted, and thus subsequent fraction collection is not accurate.

Alternatively, collection of the analyte band can be performed with an applied voltage. In electrokinetic fraction collection, high separation efficiency can be maintained. Nevertheless, electrokinetic fractionation is less straightforward compared to hydrodynamic fractionation. During fraction collection, an electrolyte is required to maintain the electrical current between the electrode and the capillary outlet. The electrophoretic buffer is generally used as the electrolyte. However, metal cations from the buffer, such as sodium and potassium, can drastically suppress the MALDI-MS signal of proteins. It is particularly problematic when analyzing low concentration proteins or peptides [15]. For this reason, fraction collection for CE–MALDI-MS is usually performed hydrodynamically, e.g. in the analyses of glycoproteins [16,17]. The use of dilute acid as the fraction collection electrolyte has been reported to improve MALDI signals [18]. We illustrate herein that CE fractionation with a dilute acid can also be performed electrokinetically in an easy and reliable manner. This technique allows multiple consecutive fraction collections in one CE separation.

Another challenge in the CE–MALDI-MS analysis of proteins is the adsorption of proteins onto the capillary wall. Protein adsorption can lead to serious band broadening, sample loss and poor reproducibility. A common approach to preventing protein adsorption is to form an inner coating in the capillary [19]. The capillary surfaces can be chemically modified with polymers to form permanent coatings [20]. Alternatively, dynamic coatings can be formed through the adsorption of surfactants [21]. The coating procedures for permanent coatings are usually long and tedious. Commercial capillaries with permanent coatings are available, but they are expensive and have limited lifetimes and operating conditions. Dynamic coatings, on the other hand, are inexpensive and easy to form, i.e. simply by adding surfactants in the electrophoretic buffer. More im-

portantly, superior performance has been reported in the prevention of protein adsorption with surfactant-modified capillaries. However, for coupling CE with MS, the use of surfactants has been traditionally avoided due to their incompatibility with MS detection. Surfactants form adducts with proteins and suppress sample ionization [22]. Hence CE–MALDI-MS has always been performed in permanently coated capillaries.

The use of a semi-permanent capillary wall coating formed by a two-tailed cationic surfactant, didodecyldimethylammonium bromide (DDAB), was recently reported by Melanson et al. [23]. Similar to other cationic surfactants, DDAB coating formation takes place by rinsing the capillary with a surfactant solution. However, the two-tail nature of DDAB results in a very strong hydrophobic effect, such that the coating remains after flushing out the DDAB solution. The coated capillary is then filled with a surfactant-free buffer, in which electrophoresis takes place. This potentially eliminates the presence of surfactant in the bulk solution inside the capillary, thus the surfactant should not interfere with the MALDI-MS measurement. The first application of surfactant-modified capillaries in CE–MALDI-MS is demonstrated and studied in this work.

2. Experimental

2.1. Apparatus

An Agilent ^{3D}CE instrument (Palo Alto, CA, USA) equipped with an ultraviolet–visible diode array detector was used to perform all CE separations and fraction collection. Fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with an inner diameter of 50 μm and an outer diameter of 365 μm were used. The total capillary length was 30.5 cm, and the length to detector was 22.3 cm. The capillary was thermostated at 25°C in all experiments. ChemStation software (Rev. A.06.03, Agilent) was used for data acquisition and instrument control on a Pentium-based microcomputer. Detection of all proteins was performed by direct UV absorption at 192 nm. Hydrodynamic sample injection was performed at a constant pressure of 5 kPa.

Mass spectral measurements were conducted with a time-lag focusing (TLF) MALDI-time-of-flight (TOF) mass spectrometer constructed in the laboratory [24,25]. Mass spectral data acquisition and processing was performed with Hewlett-Packard supporting software HP DADA-601 (S.02.99.1101). Data were reprocessed using IGOR Pro (Version 3.13, Wavemetrics, Lake Oswego, OR, USA).

2.2. Chemicals

Nanopure 18 MΩ water (Barnstead) was used in the preparation of all solutions. Reagent grade orthophosphoric acid (Mallinckrodt, Paris, KY, USA), acetic acid (Anachemia, Rouses Point, NY, USA), or formic acid (Fisher) was used to prepare the CE buffers. The pH was adjusted with reagent grade sodium hydroxide (Fisher). Hydrochloric acid used in fraction collection was purchased from Anachemia. The cationic surfactant DDAB (Aldrich) was used as received. MALDI matrix α-cyano-4-hydroxycinnamic acid (HCCA) was purchased from Aldrich and purified by recrystallization from ethanol prior to use. Standard proteins cytochrome *c* (bovine heart, C3131), cytochrome *c* (horse heart, C7752), and lysozyme (chicken egg white, L6876) were used as received from Sigma.

2.3. CE separation for proteins

Capillary pre-conditioning was performed with a high-pressure (100 kPa) rinse with NaOH for 5 min followed by distilled water for 1 min. New capillaries were used for each buffer system to avoid cross contamination. Capillary coating with DDAB was performed with a 5-min rinse (100 kPa) with 0.1 m*M* DDAB in water [23]. A 1-min rinse (100 kPa) with the electrophoretic buffer was used to remove the excess surfactant. Between subsequent separations, the capillary was reconditioned (100 kPa) for 2 min with 0.1 m*M* DDAB and for 1 min with the electrophoretic buffer.

For the fraction collection solution study, samples of 0.2 μg/μl horse heart cytochrome *c* in water were injected hydrodynamically at 5 kPa for 2.0 s in all cases. Phosphate, acetate, or formate, 25 m*M* at pH 4.00, were used as the electrophoretic buffers, and the applied voltage was −6 kV. In the detection limit

study, 0.2 μg/μl horse heart cytochrome *c* was injected at 5 kPa for 1.0 s. A phosphate buffer, 25 m*M* at pH 4.00, was used and the applied voltage was −6 kV.

In the separation and fraction collection of standard protein mixtures, a mixture of bovine heart cytochrome *c*, horse heart cytochrome *c*, and lysozyme (0.2 μg/μl each) was injected at 5 kPa for 2.0 s. The applied voltage in this case was −12 kV, and a 10 m*M* phosphate buffer at pH 7.00 was used. Efficiencies of all CE peaks were determined at the baseline using the tangent method.

2.4. CE fraction collection

Collection of an analyte band at the outlet of the capillary was performed electrokinetically into a polypropylene micro insert (Agilent, part no. 5182-0549). In the fractionation solution study, various buffers and dilute acid were placed in the insert for electrokinetic fraction collection. To minimize dilution, the volume of the fractionation solution was limited to 5 μl or less. This 5-μl volume resulted in a solution depth of ca. 2 mm in the micro insert. To provide electrical contact between the outlet electrode and the fractionation solution, the top of the micro insert was trimmed by 5 mm in order to raise the position of the insert closer to the electrode and capillary.

The fraction collection function programmed in the ChemStation software (Agilent) was not used in our experiment because it did not account for the vial exchange time (5–10 s) during fraction collection. This led to erroneous fraction collection timing, particularly during short fractionations (<30 s) for the highly efficient protein peaks. As a result, the fraction collection was performed manually.

The timing of fraction collection is calculated assuming that the mobility of the analytes is constant during the separation. That is, the time at which an analyte band reaches the outlet of the capillary (t_{outlet}) is obtained by multiplying the recorded migration time (t_m) with the ratio of total capillary length (L_t) to the length from inlet to detector (L_d):

$$t_{outlet} = t_m \cdot \frac{L_t}{L_d} \tag{1}$$

At t_{outlet}, the CE separation is interrupted by switching off the applied field. The electrophoretic buffer vial at the capillary outlet is removed, and the fractionation micro insert is placed at the outlet. The applied field is then resumed to perform electrokinetic fraction collection. The time length of electrokinetic fraction collection is determined by the baseline width of the peak at the capillary outlet ($W_{b,outlet}$), which is calculated from the baseline width recorded at the detector ($W_{b,detector}$):

$$W_{b,outlet} = W_{b,detector} \cdot \frac{L_t}{L_d} \tag{2}$$

When needed, consecutive fraction collections can be performed by placing new micro inserts for fractionation. Otherwise, the electrophoretic buffer vial can be repositioned at the outlet to resume the CE separation.

2.5. Sample deposition and MALDI-MS measurement for protein standards

A three-layer sample deposition method was used for MALDI-MS protein analysis. This method generated reproducible mass spectra from a given sample. In this method, 1 μl of 10 mg/ml HCCA in methanol–acetone (20:80, v/v) was first deposited on the MALDI target to form a thin layer of fine crystals. Then 0.25 μl of a saturated HCCA solution in methanol/water (33:67, v/v) was deposited on top of the first layer and allowed to dry. Finally a 0.25 μl aliquot of the sample was spotted as a third layer and allowed to dry prior to measurement. Each analysis was repeated in triplicate, with an average of 75 laser shots summed for each mass spectrum.

3. Results and discussion

3.1. CE fraction collection and MALDI-MS measurement

CE separations were performed in three buffer systems: 25 m*M* phosphate, formate and acetate, all at pH 4.00. Cytochrome *c* (horse heart) was used as a test protein. Fused silica capillaries were pretreated with DDAB to prevent protein adsorption onto the capillary wall [23]. Injection was performed at 5 kPa for 2.0 s with a 0.2 μg/μl cytochrome *c* (horse

heart) solution in water. The amount of cytochrome *c* injected was ca. 92 fmol in each run. The electropherograms obtained with the different buffers are shown in Fig. 1A–C, respectively. The peak efficiencies are 600 000 plates/m in phosphate (Fig. 1A), 300 000 plates/m in formate (Fig. 1B) and 200 000 plates/m in acetate (Fig. 1C). The measured efficiencies are similar to those reported previously by Melanson et al. [23].

The migration times of cytochrome *c* in all three buffers are relatively fast, from 2.0 min in formate to 4.4 min in phosphate. The fast migration times are caused by the fast electroosmotic flow (EOF) generated in the DDAB modified capillary. Most of the available coatings, either dynamic or permanent, are neutral in charge. The resulting EOF is therefore either greatly suppressed or near zero. The mobilities of analytes are solely driven by their own charges in the absence of the EOF. Slightly charged or near neutral proteins therefore migrate very slowly, resulting in long analysis times. DDAB, on the other hand, is a cationic surfactant that generates a highly cationic capillary wall coating, and thus a fast anodic EOF. Although cationic proteins migrate in the opposite direction as the anodic EOF, the magnitude of the EOF is sufficient to sweep the counter-migrating proteins to the detector. Such fast EOF is useful in carrying proteins with low or zero mobility to the detector, enabling their detection that would not be possible otherwise. The variation in the migration time among different buffers (Fig. 1) is due to the different magnitude of EOF in various buffers [26,27].

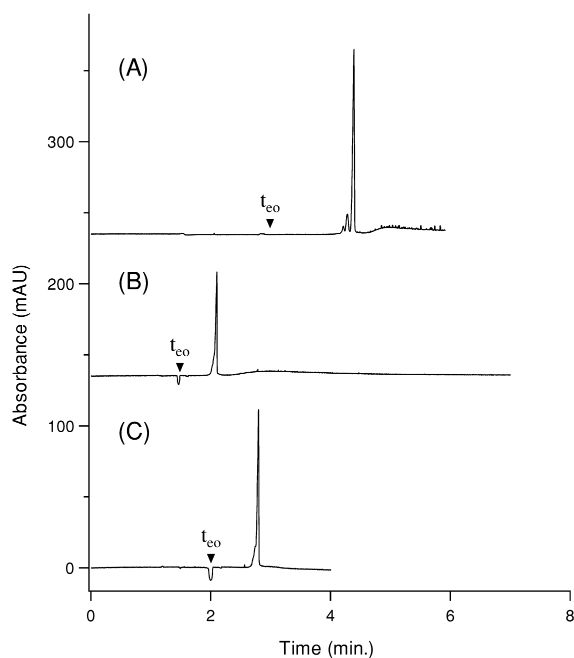

Fig. 1. CE electrophorograms of horse heart cytochrome *c* in (A) phosphate, (B) formate, and (C) acetate. Experiment conditions: capillary lengths, 22.3 cm (to detector) and 30.5 cm (total); separation voltage, −6 kV. The EOF is determined by injection of acetonitrile (A) or water (B and C).

Micro fraction collection was performed as outlined in the Experimental section. The measured peak width at baseline is ca. 6.5 s for cytochrome *c* in all three buffers. Taking account for the detector-to-total length ratio, this translates to an electrokinetic fraction collection interval of 9.0 s based on Eq. (2). For each of the three buffer systems (Fig. 1A–C), fraction collection was performed first in 5 μl of the electrophoretic buffer and then in 5 μl of dilute (10 ppm) HCl solution. The purpose of using a dilute HCl solution is to maintain an almost sodium-free electrolyte condition that will not significantly affect the performance of the subsequent MALDI-MS experiment (see below).

The mass spectra of the fractions collected in 5 μl of electrophoretic buffer are presented in Figs. 2A, 3A and 4A for phosphate, formate, and acetate, respectively. The corresponding mass spectra of the cytochrome *c* fractions collected in HCl are shown in Figs. 2B, 3B and 4B. As a control experiment, the mass spectrum of a 20 n*M* cytochrome *c* solution in water was also recorded. The 20 n*M* concentration was selected to approximate the 92 fmol of protein collected in 5 μl. The mass spectra of this solution represent the optimal signal intensity of cytochrome *c* in the absence of any signal suppression or sample loss during fraction collection. Since the MALDI-MS measurements for the three buffers were performed on three different days, a control measurement of 20 n*M* cytochrome *c* was performed on each day. They are shown in Figs. 2C, 3C and 4C. All spectra shown in Figs. 2–4 were collected in triplicate. The spectra shown represent the average of the triplicates.

In all of the spectra recorded (Figs. 2–4), three main ion peaks are detected: $(M+H)^{+}$, $(M+2H)^{2+}$,

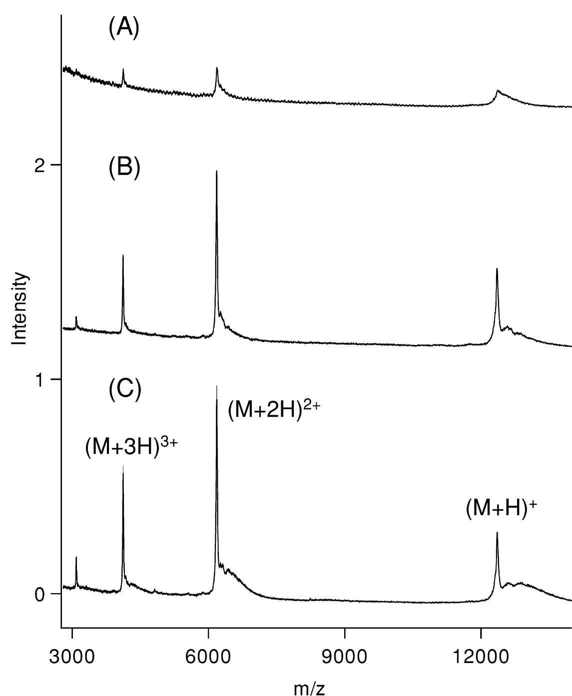

Fig. 2. MALDI mass spectra of cytochrome *c* from (A) CE fraction collected in electrophoretic buffer, (B) CE fraction collected in 10 ppm HCl, and (C) a 20 n*M* cytochrome *c* solution. CE separation was performed in 25 m*M* sodium phosphate at pH 4.0.

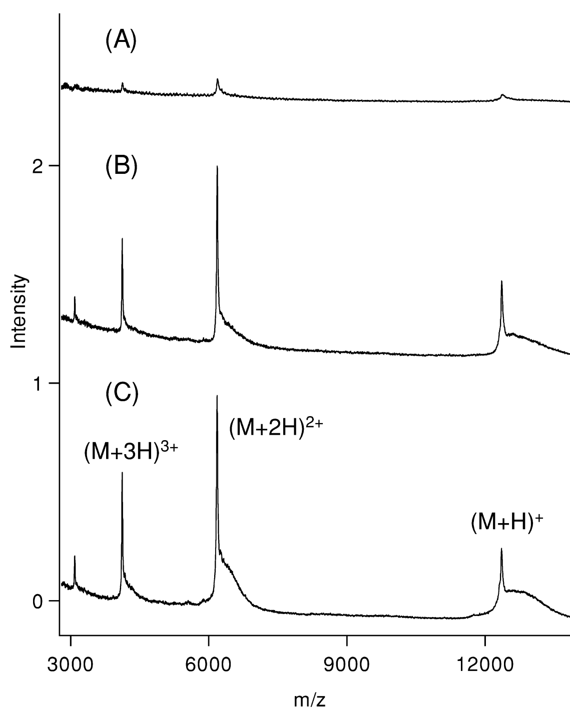

Fig. 3. MALDI mass spectra of cytochrome *c* from (A) CE fraction collected in electrophoretic buffer, (B) CE fraction collected in 10 ppm HCl, and (C) a 20 n*M* cytochrome *c* solution. CE separation was performed in 25 m*M* sodium formate at pH 4.0.

and $(M+3H)^{3+}$. A variation in signal intensity is observed between the different fractionation electrolytes. In all cases studied, the mass spectra of fractions collected in the electrophoretic buffer result in the lowest signal intensity (Figs. 2A, 3A and 4A). When the same fraction collection is performed in dilute HCl, peak intensities similar to those from the 20 n*M* cytochrome *c* standard solution are observed. This confirms that MS signal suppression occurs when the electrophoretic buffers (sodium phosphate, sodium formate and sodium acetate) are used as the fraction collection electrolyte. Such MS signal suppression can be prevented by using a dilute HCl solution for fraction collection.

A potential problem in using a non-buffered electrolyte, such as dilute HCl, for electrokinetic fraction collection is electrolysis. Electrolysis at the capillary outlet can cause a pH change, which in turn may alter the protein mobility. We believe that the use of a DDAB-modified capillary helped to reduce the effect of the potential electrolysis. A fast, forward-moving EOF is generated in DDAB capillaries. This EOF may help preventing the fraction collection electrolyte from entering the capillary at the outlet. In addition, a reversed polarity setting is used with a DDAB capillary, i.e. the anode is at the capillary outlet. If electrolysis takes place, the pH of the fraction collection electrolyte will be lowered by oxidation. Normally CE separation of proteins is performed in acidic buffers, such as pH 4 in this work. Fortunately, the ionization of proteins is less sensitive to pH changes in the acidic direction, as these proteins are close to being fully protonated at pH 4. On the other hand, normal polarity must be used for most permanently derivatized capillaries due to the suppressed EOF. Reduction takes place at the capillary outlet and increases the buffer pH. This can cause significant changes in the proteins' ionization and thus mobilities.

Although DDAB is useful in achieving fast analy-

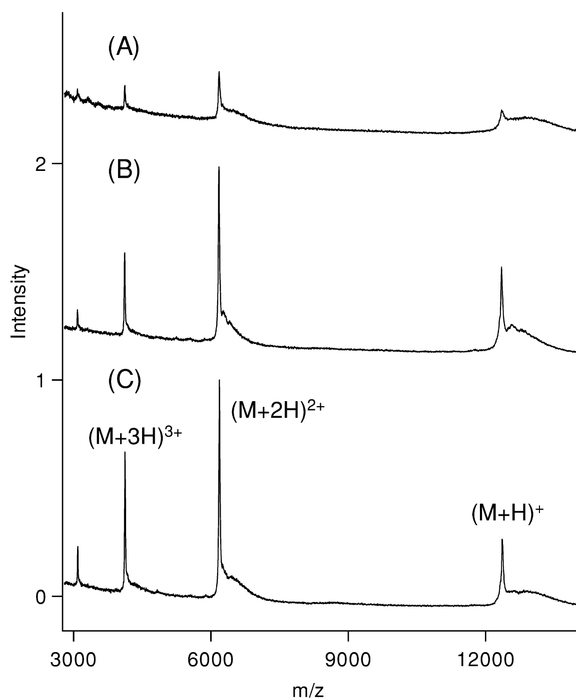

Fig. 4. MALDI mass spectra of cytochrome *c* from (A) CE fraction collected in electrophoretic buffer, (B) CE fraction collected in 10 ppm HCl, and (C) a 20 n*M* cytochrome *c* solution. CE separation was performed in 25 m*M* sodium acetate at pH 4.0.

sis times and allows the use of non-buffered fraction collection electrolytes, it is important to make sure that the DDAB from the capillary coating does not cause interference in MALDI-MS. The MALDI spectra in panel B of Figs. 2–4 are recorded from fractions collected from DDAB-coated capillaries. The intensities of the $(M+2H)^{2+}$ peaks are 80–90% of those observed in the spectra of 20 n*M* cytochrome *c* solution (panel C in Figs. 2–4). The small difference in intensity may reflect the different amount of cytochrome *c* used, 92 fmol from the CE fraction and 100 fmol from 20 n*M* solution. In addition, sample handling during fraction collection, such as solution transfer between vials, can also result in sample loss. Overall, significant interference caused by DDAB is not evident. The first coupling of CE and MALDI-MS with the use of a surfactant modified capillary is therefore successfully demonstrated. The use of permanently derivatized capillaries is no longer the only choice for CE–MALDI-MS.

3.2. Detection limit

To determine the detection limit of our MALDI-MS measurements on the CE fractions with 10 ppm HCl, we performed CE–MALDI-MS with different amounts of cytochrome *c* (horse heart) injected. The separation was performed in phosphate buffer (25 m*M*) at pH 4.00, and fraction collection was performed as previously described at −6 kV for 9.0 s. The signal-to-noise ratio (S/N) of the $(M+2H)^{2+}$ peak was recorded for each sample in four replicates. The lowest amount of cytochrome *c* (horse heart) injected that resulted in an MS signal of $S/N \geq 3$ in all four replicates is 23 fmol, with S/N ranging from 6 to 60. The wide range of signals observed from the replicate experiments indicates that this amount is close to the detection limit. Indeed, any lower amount of cytochrome *c* resulted in signals below the detection limit in at least one of the four replicates. The lowest detection limit reported in the literature for CE–MALDI-MS is 2 fmol for proteins injected in CE [12]. Our detection limit is about 10 times higher. Unlike the direct sample deposition used in Ref. [12], not all of the protein collected in each fraction is used for MALDI-MS in our case. Out of the 5 μl in each fraction, only 0.25 μl and thus only 1.2 fmol is actually spotted onto the MALDI probe for each measurement. The detection limit of our laboratory-built MALDI instrument is around 1 fmol for proteins such as cytochrome *c* [28]. In other words, the MALDI-MS detection limit is not affected by the CE separation and fraction collection.

Because only 0.25 μl is consumed for each MALDI-MS measurement, we can, in principle, perform 20 measurements for each 5 μl CE fraction. Alternatively, the 5-μl fraction can be divided into several portions for various sample treatments such as enzymatic digestions and chemical reactions. We are currently developing and applying a nanoliter-volume sample handling technique to carry out post-separation sample treatments for protein identification and structure analysis.

When post-separation sample treatment is not required, we can reduce the amount of fractionation electrolyte to less than 5 μl in order to lower the detection limit. The smallest amount of fractionation electrolyte used was 2 μl. In theory this can lower

the detection limit from 23 to 9 fmol, which is closer to the low detection limit record of 2 fmol reported [12]. Nevertheless, the 2-μl fractionation electrolyte is so small that it occasionally does not result in a successful collection, presumably when it fails to maintain electrical contact between the electrode and the capillary outlet. Care must be exercised when using such small volumes of fractionation electrolyte.

An alternate approach has been attempted to increase the sensitivity, namely by concentrating the collected fraction prior to MALDI-MS measurements. A 5-μl fraction was transferred to a siliconized polypropylene vial (600 μl) and evaporated down to 1 μl by vacuum centrifugation. Unfortunately, such sample concentration was not successful as the protein was presumably lost due to adsorption onto the container. As a result, the "concentrated" samples actually resulted in lower MALDI-MS signals. Other sample concentration techniques, such as the use of reverse phase particles [29], will be investigated in the future.

3.3. Accuracy of fraction collection timing

Since the separation efficiency in this CE method is very high, a suitable means of collecting fractions for only a few seconds is required. The timing of our electrophoretic fraction collection is based on the extrapolation of the migration time recorded by an online detector. Errors may arise from any changes in analyte mobility during electrophoresis and fraction collection. Electrolysis can take place at the electrode to alter the pH, protein mobility and EOF. Additional band broadening or siphoning during vial transfers, as well as the uncertainty of the voltage control during ramping, can also give rise to erroneous timing during fraction collection.

To evaluate the accuracy of our fraction collection, we performed multiple consecutive fraction collections within one CE separation. The CE separation of a mixture of three proteins, cytochrome *c* (bovine heart), cytochrome *c* (horse heart) and lysozyme (chicken egg white), was performed at −12 kV in a pH 7.0 phosphate buffer (10 m*M*). The proteins and the experimental conditions were optimized to yield three closely resolved peaks (Fig. 5). Poor accuracy in the fractionation timing would

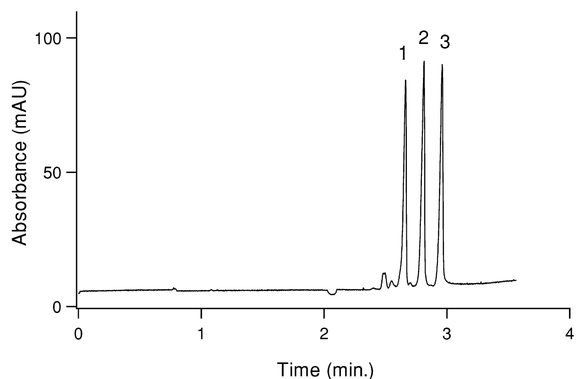

Fig. 5. CE separation of a mixture of bovine heart cytochrome *c* (1), horse heart cytochrome *c* (2), and lysozyme (3). Experiment conditions: buffer, 10 m*M* phosphate at pH 7.0; voltage, −12 kV; other conditions as stated in Fig. 1.

result in collection of unintended components, which would be reflected in the subsequent MALDI mass spectra. Based on the calculation from Eqs. (1) and (2), collection of the three protein peaks was performed as three consecutive fractions with intervals of 10–12 s at −12 kV. MALDI mass spectra were recorded for the fractions. Initially the MALDI signals appeared to be weaker than that recorded previously when the fractionation voltage was −6 kV. A similar observation has been reported by others [30]. It is caused by a loss of analytes when high voltage (−12 kV) is used in electrokinetic fraction collection. Sample binding or interaction with the outlet electrode occurs, resulting in incomplete collection. The problem can be solved by simply reducing the fractionation voltage and adjusting the collection time duration proportionally [7]. In this case, fractionation was performed at half the voltage (−6 kV) for twice the time duration (18 s). The intensity of the MS signal improved significantly with the reduced fractionation voltage.

The MALDI mass spectra of the cytochrome *c* (bovine heart), cytochrome *c* (horse heart) and lysozyme CE fractions are presented in panels Fig. 6A–C, respectively. In general, the major peaks observed in each mass spectrum correspond to the protein expected. In the first fraction (Fig. 6A), only signals from cytochrome *c* (bovine heart) are observed. In the second fraction (Fig. 6B), small quantities of lysozyme, $(M+2H)^{2+}$, is observed. Impurity from cytochrome *c* (bovine heart) may also

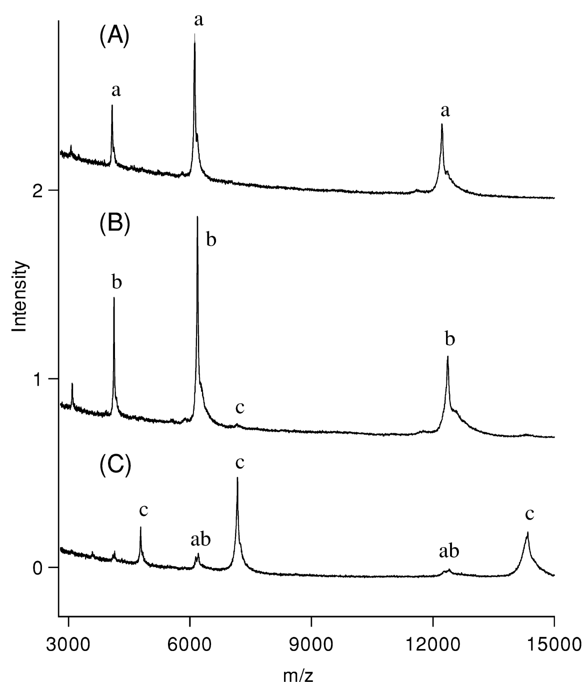

Fig. 6. MALDI mass spectra of CE fractions for peaks 1, 2 and 3 from Fig. 5 (A–C, respectively). The lower case letters refer to the peaks identified for bovine heart cytochrome *c* (a), horse heart cytochrome *c* (b), and lysozyme (c).

present in the second fraction, but the MS resolution is not sufficient to resolve the small mass difference between the two cytochrome *c*'s. Likewise, small signals of cytochrome *c* (bovine heart) and cytochrome *c* (horse heart) are detected in the third fraction (Fig. 6C). A small degree of sample carry-over from one fraction to another is suspected to be the cause of the contamination.

The above results show that multiple consecutive fraction collections can be done within a short collection time and collection of the intended component is evident in the MALDI-MS results. In fact, multiple consecutive fractionations can be performed throughout the entire separation and the application of this method for analyzing cell lysates will be reported elsewhere. Using dilute HCl as fraction collection electrolyte did not lead to any significant error in the collection timing. Thus the mobility of the proteins is not altered significantly by electrolysis during fractionation. Overall, our fraction collection is performed in a precisely controlled manner.

4. Conclusions

Micro fraction collection in CE offers simple offline coupling with MALDI-MS. The MALDI-MS detection limit is not significantly affected when analyzing proteins in CE fractions. The timing of the fraction collection is well controlled using electrokinetic fractionation. Collection of multiple consecutive fractions in one CE separation within a short time window was demonstrated. The use of capillaries treated with the two-tailed surfactant DDAB prevents adsorption of proteins and provides a fast EOF for short separations. Unlike other surfactants, DDAB forms a semi-permanent wall coating, and thus is compatible with subsequent MALDI-MS measurements. The choice of electrolyte for CE fraction collection is critical. Ions from buffers tend to interfere with the MS signals, so dilute acid (10 ppm HCl) should be used instead. It is shown that the use of dilute acid does not alter separation efficiency and fraction collection accuracy. To further develop this CE–MALDI-MS method into a powerful tool for proteomics application, future work will mainly focus on developing a means of performing chemical and enzymatic reactions on individual fractions for protein identification and structure analysis.

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