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Performance of a sheathless porous tip sprayer for capillary electrophoresis–electrospray ionization-mass spectrometry of intact proteins

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ABSTRACT

The performance of a prototype porous tip sprayer for sheathless capillary electrophoresis–mass spectrometry (CE–MS) of intact proteins was studied. Capillaries with a porous tip were inserted in a stainless steel needle filled with static conductive liquid and installed in a conventional electrospray ionization (ESI) source. Using a BGE of 100 mM acetic acid (pH 3.1) and a positively charged capillary coating, a highly reproducible and efficient separation of four model proteins (insulin, carbonic anhydrase II, ribonuclease A and lysozyme) was obtained. The protein mass spectra were of good quality allowing reliable mass determination of the proteins and some of their impurities. Sheath-liquid CE–MS using the same porous tip capillary and an isopropanol–water–acetic acid sheath liquid showed slightly lower to similar analyte responses. However, as noise levels increased with sheath-liquid CE–MS, detection limits were improved by a factor 6.5–20 with sheathless CE–MS. The analyte response in sheathless CE–MS could be enhanced using a nanoESI source and adding 5% isopropanol to the BGE, leading to improved detection limits by 50-fold to 140-fold as compared to sheath liquid interfacing using the same capillary – equivalent to sub-nM detection limits for three out of four proteins. Clearly, the sheathless porous tip sprayer provides high sensitivity CE–MS of intact proteins.

1. Introduction

In the fields of protein chemistry, biopharmaceutics, and biotechnology there is a growing need for sensitive and selective analytical tools for the determination of intact (i.e., non-digested, non-derivatized) proteins. Capillary electrophoresis (CE) shows attractive features for the highly efficient separations of intact proteins, and its combination with mass spectrometry (MS) would provide a very selective tool for protein characterization. Since the introduction of CE–MS using electrospray ionization (ESI) and the first exploratory experiments on its applicability by Smith et al. [1–6], CE–ESI-MS has proven to be a strong analytical tool with a wide applicability, including determination of e.g. drugs, metabolites and peptides, but also intact proteins [7–11].

Coupling of CE with ESI-MS is not straightforward as both the CE and ESI processes require closed electrical circuits having a common electrode at the capillary outlet. Generally, CE–MS interfacing via an ESI source can be performed in two different ways [12]. In the most frequently applied approach, the interface uses a sheath liquid that mixes with the CE effluent as it exits the separation capillary. The sheath flow serves to establish the electrical contact with the CE background electrolyte (BGE) and facilitates ESI using a conventional ion source applying a nebulization gas. The sheath liquid allows use of standard capillaries and provides electrospray stability, making the performance virtually independent of the electroosmotic flow (EOF). However, due to the dilution of the analytes leaving the capillary by the sheath liquid, the detection sensitivity – which in ESI is predominantly concentration sensitive – is compromised. Moreover, as the sheath liquid composition usually is quite different from the BGE, the CE performance (peak widths, migration time order) may be affected [13–15].

In the second CE–ESI-MS approach, the terminating electrical contact for the CE process is made directly to the BGE just before or after it leaves the capillary. Such a so-called sheathless interface was actually used by Olivares et al. [1] to perform their first on-line CE–ESI-MS experiments. Usually, the capillary tip is tapered to reduce the capillary inner diameter providing good ESI conditions. As effluent volume flow rates are low, the initial droplets formed during the electrospray process are small [16], leading to more efficient ionization (i.e., nanospray). The sheathless approach also allows the ESI spray tip to be positioned closer to the MS inlet and, thereby, improving ion sampling efficiencies [16,17]. As a result, enhanced sensitivity and lower limits of detection (LODs) can be obtained.

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Over time, various sheathless CE–ESI-MS interface designs have been developed. The most widely used method for establishing the terminating electrical contact is coating the outer surface of the CE capillary tip with a conductive material [18–22]. Unfortunately, lifetimes of such coatings are generally limited as they suffer from deterioration under influence of the high voltages applied. Alternatively, a wire microelectrode can be inserted into the capillary channel [23,24], however, manipulation of such microelectrodes is not easy and they can cause turbulence and bubble formation in the separation capillary reducing the separation efficiency and electrospray stability. The CE effluent can also be brought in contact with an external electrode through a hole or a locally porous section in the capillary wall [25–27], but it is quite difficult to produce such microholes or sections in a reproducible and robust way. In order to circumvent the problem of closing the electrical circuit in sheathless CE–MS interfacing, some researchers have used separate ESI sprayer tips. The electrical contact for both the CE separation and ESI process is made at the "liquid junction" of the separation capillary and the spray tip, where the terminal electrode is placed into an electrolyte surrounding the junction [28–31]. However, the alignment of the tip is critical to maintain an efficient separation and stable electrospray. Moreover, the liquid junction may also lead to dilution of the CE effluent.

Recently, Moini has introduced a porous sheathless CE–MS interface that provides electrical contact without the need for microelectrodes or liquid junctions [32]. In this design (Fig. 1A), the last 3–4 cm of the bare fused-silica capillary are etched with hydrofluoric acid until the section becomes conductive, producing an ~5-μm thick porous wall, which is conductive when in contact with an electrolyte. The electrical contact for both the CE and ESI is achieved by letting the porous capillary outlet protrude from a stainless steel ESI needle filled with static conductive liquid allowing electrospray formation at the capillary tip. Any bubble formation at the electrode occurs outside the separation capillary, and no dilution of the CE effluent takes place. The capillary inner diameter remains unchanged (i.e. no tapering) so that chances of clogging are minimized. The potential usefulness of the porous tip sprayer was demonstrated by the analysis of amino acids, peptides, protein digests and protein–metal complexes [32,33].

Based on Moini's approach, a prototype high-sensitivity porous sprayer (HSPS) sheathless interface for CE–MS was recently developed in the laboratories of Beckman Coulter. We set out to test the performance of the HSPS for the CE–MS analysis of intact proteins. There is a definite requirement for increased sensitivities in CE–MS of intact proteins. Multiple charging of proteins occurring during ESI distributes the overall signal intensity over many charge states, thereby decreasing the achievable sensitivity for intact proteins [34]. Common LODs for CE–MS of intact proteins are in the low μM or high nM range, although it has been demonstrated that detection of protein concentrations below 200 nM is possible with CE–MS [27,35–37].

In this study, capillaries with HSPS tips were inserted in a stainless steel ESI needle filled with static conductive liquid. The inner wall of the capillaries was covalently coated with a positively charged polymer providing a significant EOF and avoiding protein adsorption when using low-pH BGEs. The performance of the sheathless interface was first tested using a conventional ESI source. With a mixture of model proteins, we investigated parameters such as migration time, reproducibility, detection linearity and limits of detection. Comparisons with sheath-liquid interfacing were made, paying attention to the role of organic solvent in the sheath liquid. Finally, the optimal system was tested in conjunction with a nanoESI source.

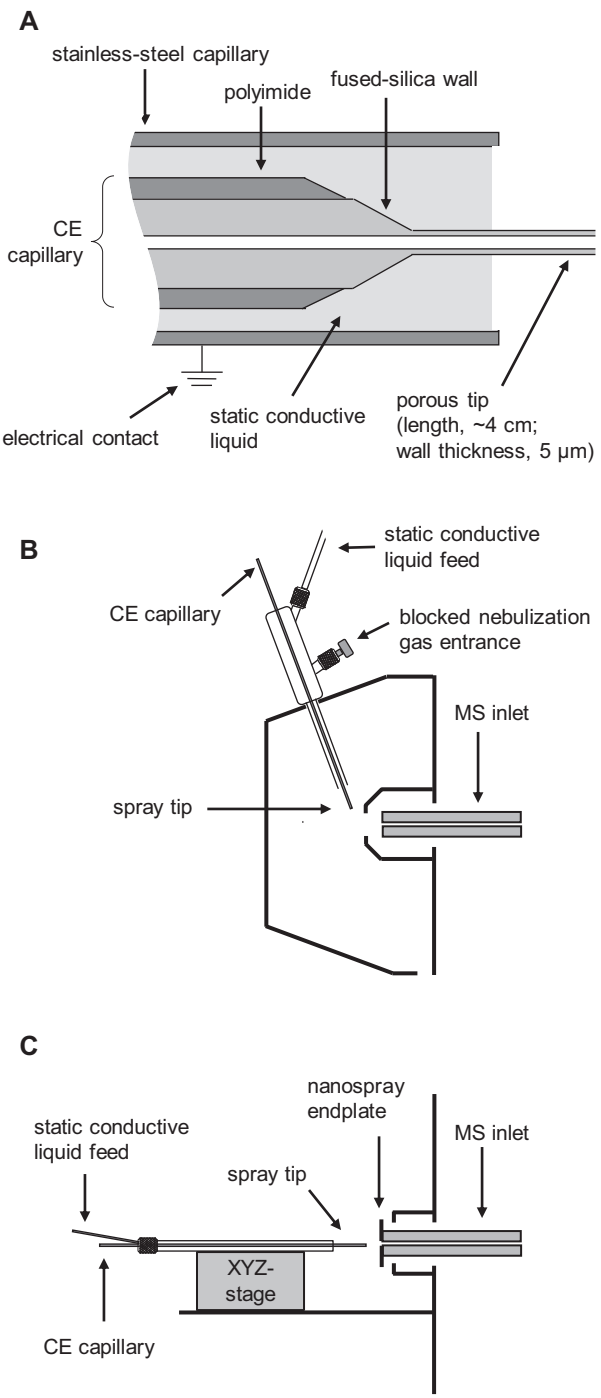

Fig. 1. (A) Detailed representation of the HSPS capillary positioned in the stainless steel capillary. Schematic representation of the sheathless CE–MS set-up using the HSPS tip (B) in the conventional ESI source and (C) in the nanospray source.

2. Materials and methods

2.1. Chemicals

Acetic acid (99.8%), ammonium hydroxide (25%) and isopropanol were obtained from Merck (Darmstadt, Germany). Anhydrous methanol, methionine enkephalin (M_w, 573.6; *pI*, 5.5), insulin (from bovine pancreas; M_w, 5733; *pI*, 5.6), carbonic anhydrase II (from bovine erythrocytes; M_w, 29,025 g/mol; *pI*, 5.9), ribonuclease A (from bovine pancreas; M_w, 13,680; *pI*, 9.7) and lysozyme (from chicken egg white; M_w, 14,304; *pI*, 11.0) were from

Sigma–Aldrich (Steinheim, Germany). Methionine enkephalin was prepared by diluting a stock solution (1 mg/mL) to a concentration of 25 μg/mL with BGE. Protein test mixtures were prepared by diluting protein stock solutions (1 mg/mL) to the appropriate concentration with deionized water. A BGE of 100 mM acetic acid (pH 3.1) was prepared by diluting 0.171 mL glacial acetic acid to 30 mL with deionized water and adjusting the pH with ammonium hydroxide.

2.2. CE system

Experiments were carried out on a P/ACE MDQ™ capillary electrophoresis instrument (Beckman Coulter, Brea, CA, USA). The separation voltage was −30 kV and the capillary temperature was 20 °C. Fused-silica capillaries (total length, 100 cm; inner diameter, 30 μm, outer diameter, 150 μm) equipped with a porous tip (length, 3–4 cm) were supplied by Beckman Coulter. The HSPS tip is under development by Beckman Coulter and is not available for commercial use. The capillaries were coated with polyethylenimine (PEI); the positively charged PEI coating is described in US Patent 6,923,895 B2. During storage (overnight or longer), the capillaries were filled with methanol and the tips of the capillary were immersed in methanol. At the beginning of each day before analyses were performed, the coated capillary was conditioned by flushing the capillary at 50 psi with air (10 min), methanol (20 min), deionized water (5 min) and BGE (10 min). Before each run, the capillary was flushed for 3 min at 50 psi with fresh BGE. The sample was injected for 10 s at 5 psi (equal to 1% of the capillary volume).

2.3. CE–MS

MS detection was performed using a micrOTOF orthogonal-accelerated time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany). Transfer parameters were optimized by direct infusion of an ESI tuning mix (Agilent Technologies, Waldbronn, Germany).

2.3.1. Sheathless CE–MS

Two different set-ups were used to perform sheathless CE–MS interfacing. In the first approach, the capillary with the HSPS tip was placed in a grounded coaxial CE–MS sprayer (Agilent Technologies, Waldbronn, Germany) as schematically shown in Fig. 1B. The coaxial sprayer was positioned in a conventional ESI source comprising the standard ESI endplate and capillary cap. The stainless steel sprayer was filled with BGE via the sheath liquid entrance to establish electrical contact with the porous tip. Neither sheath flow (static conductive liquid) nor nebulization gas was applied. The protruding distance of the porous tip was optimized by electrokinetically infusing methionine enkephalin (25 μg/mL) through the PEI coated capillary at a potential of −30 kV. Optimal dry gas flow rates (see below) were lower than the minimal flow rate (∼4 L/min) recommended by Bruker Daltonics for the micrOTOF instrument. However, flow rates above the optimal flow rate resulted in distortion of the electrospray during CE–MS, and no or low protein signals were obtained. As low dry gas flow rates could result in contamination of the mass spectrometer over prolonged periods of time, the dry gas flow was increased to 5 L/min when not performing CE–MS experiments. The optimized spray conditions were as follows; dry gas temperature, 180 °C; dry gas nitrogen flow, 1.0 L/min; nebulizer pressure, 0.0 bar. Electrospray in positive ionization mode was achieved using an ESI voltage of −2.3 kV.

In a later stage of the research, the porous tip capillary was placed in a grounded stainless steel needle that could be positioned by an XYZ-stage (Beckman Coulter) fitting the Bruker micrOTOF instrument (Fig. 1C). A nanospray end plate and gas diverter were installed to allow nanoESI. The porous tip protruded the grounded needle approximately 0.5 cm and the needle was filled with BGE to establish the electrical contact. After optimization, the spray conditions were the same as with the former set-up, except for the dry gas nitrogen flow (3.0 L/min) and the ESI voltage (−2.1 kV).

2.3.2. Sheath-liquid CE–MS

For sheath-liquid interfacing the capillary with HSPS tip was withdrawn into the coaxial sprayer until the tip still protruded approximately 1 mm. A flow of 2 μL/min of isopropanol–water–acetic acid (75:25:0.1, v/v/v) was applied as sheath liquid using a 1.0 mL gas-tight syringe (Hamilton, Reno, NV, USA) on a syringe pump of Cole-Parmer (Vernon Hill, IL, USA). The optimized conditions for sheath-liquid interfacing were: dry gas temperature, 180 °C; dry gas nitrogen flow, 4 L/min; nebulizer pressure, 0.4 bar; ESI voltage, −4.0 kV.

2.3.3. Data analysis

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

3. Results and discussion

3.1. Sheathless CE–MS using HSPS capillaries

The capillary with HSPS tip was put into a coaxial CE–MS sprayer installed on a conventional ESI source (Fig. 1B). The electrical contact was established by filling the stainless steel needle with BGE, which remained static as no pressure was applied and no nebulization gas was used. The contact liquid did not leave the needle (despite its tilted position) and the same liquid could be used for an entire day of measurements. As the sprayer served as the common ground for the CE and ion source, voltages for both the separation and ESI process could be independently altered. When performing sheathless CE–ESI-MS, a significant and stable EOF is of great importance to obtain good analyte signal intensities [22,38–40]. Furthermore, a typical problem in CE of proteins is that they tend to adsorb onto the fused-silica capillary wall causing band broadening and changes in EOF [41,42]. Therefore, in order to achieve stable CE–MS of intact proteins, the HSPS capillaries were coated with positively charged PEI, which induces an anodic EOF when an acidic BGE of 100 mM acetic acid (pH 3.1) is used. Under these conditions peptides and proteins exhibit a net positive charge and adsorption to the capillary wall will be avoided due to electrostatic repulsion.

To optimize the sheathless interfacing conditions, a solution of the peptide methionine enkephalin (25 μg/mL) was infused electrokinetically through the PEI-coated capillary by applying a CE voltage of −30 kV. Stable electrospray formation and an analyte signal could be established, indicating that the porous tip provided adequate electrical contact. By varying the tip protruding distance, the most optimal tip position could be determined, while simultaneously assessing the electrical contact and the stability of the electrospray process. A stable electrospray was obtained when the tip protruded the sprayer approximately 5 mm and had a distance to the MS inlet of about 10 mm, with typical electrospray currents of 130–140 nA. Subsequently, the basic performance of the PEI coating was investigated by repetitive CE–MS analysis of methionine enkephalin (25 μg/mL). In a series of 15 runs, the RSD of the EOF

velocity and peptide migration time was less than 1.1%, and the RSD of the peptide peak area was less than 7%. Clearly, the PEI coating generated an EOF that provided stable separation and electrospray conditions for the 30-μm ID HSPS tip.

To study sheathless CE–HSPS ESI-MS of proteins, a mixture of insulin, carbonic anhydrase II, ribonuclease A and lysozyme was analyzed. Using a BGE of 100 mM acetic acid (pH 3.1), the four proteins were baseline separated within 10 min (Fig. 2A). Good quality mass spectra were obtained (Fig. 2B) allowing the assignment of the molecular masses of the respective proteins. The relatively low signal for carbonic anhydrase II in the base-peak electropherogram (BPE) can be explained by the fact that the protein signal is distributed over a considerable number of charge states. Deconvolution of the mass spectra yielded masses of 5733.6 Da (insulin), 29,024.6 Da (carbonic anhydrase II), 13,681.8 Da (ribonuclease A) and 14,304.5 Da (lysozyme), respectively, which agreed well with their expected molecular masses. The efficient CE–MS analysis also revealed the presence of two impurities (indicated by asterisks in Fig. 2A). Considering the recorded mass spectrum (Fig. 2B; deconvoluted mass, 8564.6 Da), the impurity migrating between insulin and carbonic anhydrase II most probably is ubiquitin. The compound migrating between ribonuclease A and lysozyme had a mass spectrum (Fig. 2B; deconvoluted mass, 14,304.3 Da) very similar to lysozyme indicating a highly related impurity of lysozyme. Interestingly the shorter migration time with respect to lysozyme suggests the impurity to be a modification involving a loss of postive charge. A possible explanation could be the substitution of a lysine for glutamine in the lysozyme molecule leading to a less basic protein and a mass difference of only 0.04 Da [43].

The overall performance of the sheathless CE–HSPS ESI-MS system was further evaluated by assessing the repeatability, detection linearity and limits of detection (LODs) for the test proteins. Migration time RSDs for all four proteins were less than 1%, whereas peak area RSDs were within 9% (Table 1). Plate numbers ranged from 0.5×10^5 (insulin) up to 1.5×10^5 (ribonuclease A). Clearly, the sheathless CE–MS system allows repeatable and efficient analyses of intact proteins. To check for linearity of signal, protein mixtures with concentrations between 0.1 and 100 μg/mL of each protein were prepared and every solution was analyzed in triplicate. Extracted ion electropherograms (EIEs) were constructed for each protein using the m/z-values of their most abundant signals (see Section 2) and peak areas were determined. For all proteins, good linear relationships (Table 2; $R^2 > 0.974$) between injected concentration and obtained peak areas were obtained. Table 2 also lists the LODs ($S/N = 3$) achieved with sheathless CE–HSPS ESI-MS. Low-nanomolar concentrations could still be detected indicating very favorable LODs for CE–MS of intact proteins. This good sensitivity is primarily due to enhanced protein responses and reduced noise, as will be outlined in the next section.

Table 1
Repeatability of migration time and peak area ($n = 15$) of the four model proteins (each 50 μg/mL) analyzed with sheathless CE–MS using capillaries with HSPS tips.

Protein	Migration time RSD (%)	Peak area RSD (%)
Insulin	0.63	8.5
Carbonic anhydrase II	0.61	6.3
Ribonuclease A	0.68	8.4
Lysozyme	0.74	7.0

3.2. Comparison with sheath-liquid CE–MS

In order to assess the actual gain in signal provided by the sheathless CE–MS set-up with the HSPS, a comparison with sheath-liquid CE–MS was made. The analyses were performed on the same

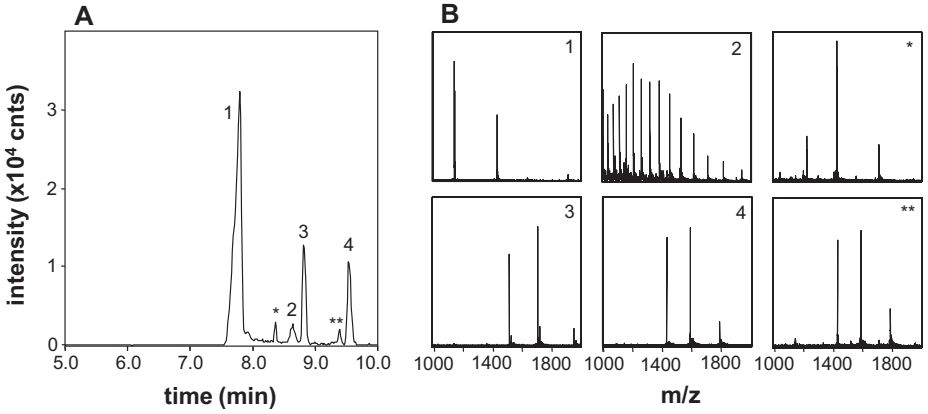

Fig. 2. (A) BPE obtained with sheathless CE–MS of a mixture of insulin (1), carbonic anhydrase II (2), ribonuclease A (3) and lysozyme (4) (each 50 μg/mL) using the conventional ESI source. (B) Mass spectra obtained at the apices of peaks 1–4 and of peaks denoted with the asterisks. Further conditions, see Section 2.

Table 2
Linearity (R^2)[a] and LODs (nM)[b] for the four model proteins obtained with CE–MS with HSPS sheathless and sheath-liquid interfacing with a conventional ESI source, and HSPS sheathless interfacing with a nanoESI source.

Protein	Sheathless – conventional ESI source[c]		Sheath liquid[c]		Sheathless – nanoESI source[d]	
	R^2	LOD	R^2	LOD	R^2	LOD
Insulin	0.998	16	0.992	106	0.999	1.28
Carbonic anhydrase II	0.974	4.4	0.981	79	0.989	0.58
Ribonuclease A	0.983	2.3	0.989	33	0.992	0.62
Lysozyme	0.997	2.1	0.990	41	0.997	0.50

[a] Concentration range, 0.1–100 μg/mL (sheathless conventional ESI source), 1–100 μg/mL (sheath liquid) and 0.05–25 μg/mL (sheathless nanoESI source).
[b] Concentration to yield S/N ratio of 3 as calculated by extrapolation from 1-μg/mL injection. For carbonic anhydrase analyzed with sheath-liquid interfacing, a 5-μg/mL injection was used.
[c] BGE, 100 mM ammonium acetate (pH 3.1).
[d] BGE, 100 mM ammonium acetate (pH 3.1) containing 5% (v/v) isopropanol.

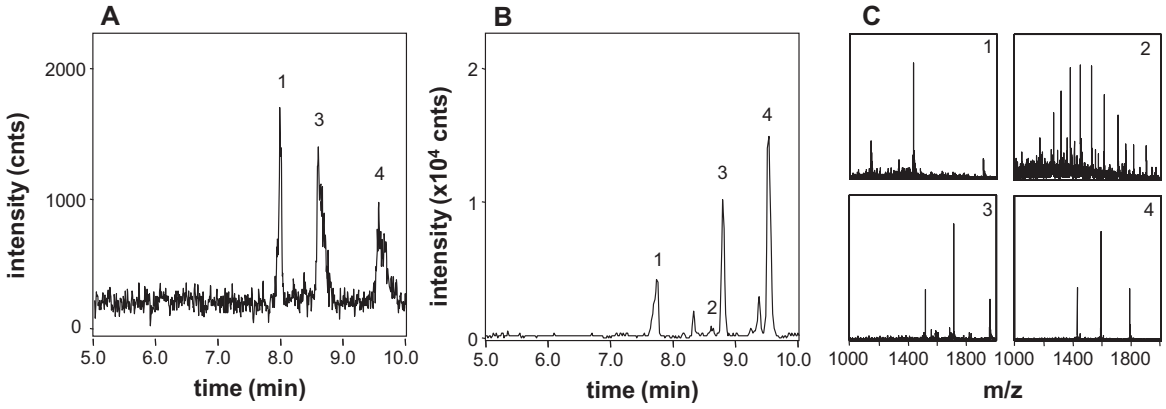

Fig. 3. BPE obtained with sheath-liquid CE–MS of a mixture of insulin (1), carbonic anhydrase II (2), ribonuclease A (3) and lysozyme (4) (each 50 μg/mL) using the conventional ESI source and a sheath liquid of (A) 100 mM acetic acid (pH 3.1) and (B) isopropanol–water–acetic acid (75/25/0.1, v/v/v). (C) Mass spectra as obtained in the apices of peaks 1–4 in panel B. Further conditions, see Section 2.

days, in order to prevent day-to-day variability from influencing the results. The same capillary (that is, with the HSPS tip) was used to carry out sheath-liquid interfacing using the same BGE and injection volume. As a coaxial sprayer has been used to support the HSPS capillary, sheath-liquid interfacing conditions could be achieved simply by withdrawing the porous tip into the stainless steel needle (leaving a 1-mm protrusion) and connecting the sheath liquid supply and nebulization gas. To study the effect of the sheath flow, BGE was first used as sheath liquid at a flow rate of 2 μL/min. CE–MS analysis of the protein test mixture under these conditions showed a dramatic decrease of analyte response as compared to sheathless CE–MS with the HSPS (Fig. 3A). These lower protein signals can probably be explained by the dilution effect of the sheath liquid causing reduced concentrations of analyte in the solution that is electrosprayed. Moreover, the larger volume flow will induce formation of larger electrospray droplets, which may lead to less efficient protein ionization.

It is well known that addition of organic solvents, such as acetonitrile, methanol, and isopropanol, to the sheath liquid can significantly enhance analyte ionization [44]. In a former sheath-liquid CE–MS study of intact proteins [45], we have found a sheath liquid of isopropanol (IPA)–water–acetic acid (75/25/0.1, v/v/v) very suitable to achieve good protein signals. Applying this sheath liquid at a flow rate of 2 μL/min resulted in much improved signal intensities for the test proteins with the CE–MS system (Fig. 3B). Furthermore, good protein separation and linear protein signals (Table 2) were obtained, indicating the proper functioning of the sheath-liquid interfacing. The increased ionization efficiency with respect to the aqueous sheath liquid was a direct result from the addition of IPA. Compared to water, IPA has a lower surface tension and evaporates faster. Consequently, smaller initial electrospray droplets are formed and a more efficient disintegration of the droplets occurs, resulting in more gas phase analyte ions and a higher MS signal [44]. Additionally, compared to sheathless interfacing, the introduction of the sheath liquid induced an alteration of the protein mass spectra (Fig. 3C). A shift towards lower charge states (higher m/z) is observed, which is most likely caused by the higher gas-phase basicity of IPA compared to water [46]. As expected, the deconvoluted masses of the proteins did not differ from those obtained with sheathless interfacing.

The addition of IPA to the sheath liquid resulted in a significant gain in protein signal (Fig. 3B) bringing the absolute protein signals to a level that was similar (ribonuclease A, lysozyme) or about five times lower (insulin, carbonic anhydrase II) than obtained with sheathless CE–HSPS ESI-MS. (*cf.* Fig. 3A). However, the LODs for sheath-liquid CE–MS as obtained from the EIEs of the proteins (33–106 nM) are considerably higher than for sheathless CE–HSPS ESI-MS (Table 2). This can be explained by the fact that the baseline noise in sheath-liquid CE–MS is much higher, as is illustrated in Fig. 4. This leads to lower S/N ratios and, thus, impaired LODs. Clearly, the use of sheath liquid causes a significant chemical noise, which is nicely avoided with sheathless CE–MS.

Considering the positive effect of IPA in sheath-liquid CE–MS, we checked whether addition of IPA to the BGE could be used to enhance protein MS signals in sheathless CE–MS. The effect of IPA on protein ionization was determined by infusion of protein solutions in BGE containing different concentrations of IPA. The solutions were pushed through the capillary with HSPS tip at a

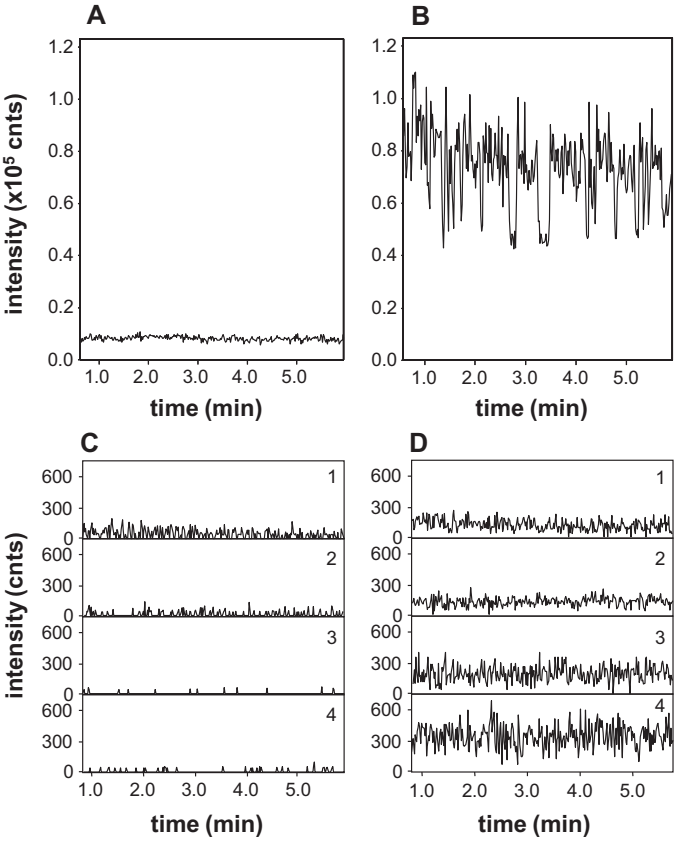

Fig. 4. Baseline signal and noise in the 0.9–5.9 min interval during (A, C) sheathless CE–MS and (B, D) sheath-liquid CE–MS of the protein test mixture using the conventional ESI source. (A, B) Total-ion trace; (C, D) Extracted-ion traces for insulin (1; m/z 1434.1), carbonic anhydrase II (2; m/z 1262.9), ribonuclease A (3; m/z 1711.1) and lysozyme (4; m/z1590.3). Further conditions, see Section 2.

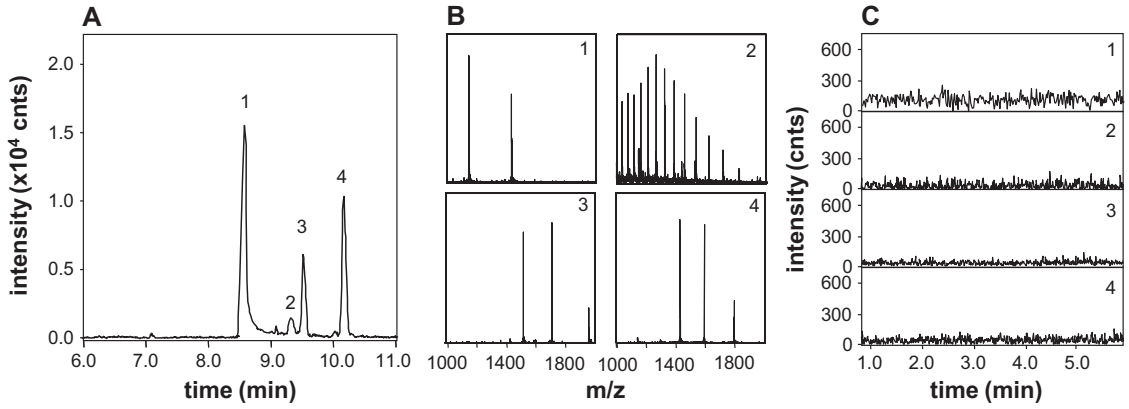

Fig. 5. (A) BPE obtained with sheathless CE–MS of a mixture of insulin (1), carbonic anhydrase II (2), ribonuclease A (3) and lysozyme (4) (each 5 μg/mL) using the nanoESI source. (B) Mass spectra obtained in the apices of peak 1–4. (C) Baseline signal and noise of the extracted-ion traces of the test proteins (*m*/*z*-values used, see Fig. 4). BGE, 100 mM acetic acid (pH 3.1) containing 5% (v/v) isopropanol. Further conditions, see Section 2.

flow rate of 140 nL/min, similar to the velocity of the EOF in our system, and sheathless ESI was carried out. With respect to the BGE without IPA, a gain in signal was observed with increasing percentage of IPA. Most pronounced signal increase occurred between 0% and 25% IPA, with the protein signals reaching a maximum plateau between 50% and 75% IPA. Maximum gain factors reached from 2.2 (carbonic anhydrase II) to 4.6 (ribonuclease A). Apparently, IPA also enhances the signal in sheathless CE–MS although gains are lower than in sheath-liquid CE–MS. Unfortunately, addition of IPA to the BGE at concentrations of 10% and higher caused serious broadening of the test protein peaks and loss of resolution [47,48]. A BGE with 5% IPA still gave good separation while providing a modest gain in protein signal intensities (see also below).

3.3. Sheathless CE–HSPS ESI-MS with a nanoESI source

In the final stage of this research project we came into possession of the nano-electrospray ion source for our TOF-MS instrument. This source is especially designed to handle very low flow rates typical for CE. We carried out some preliminary experiments to find out whether further gain in sensitivity could be achieved using the sheathless HSPS capillary in combination with the nanoESI source. The porous tip capillary was placed in a grounded needle that was positioned on an XYZ-stage (Fig. 1C). The distance of the spray tip with respect to the MS inlet was briefly optimized by electrokinetic infusion of a methionine enkephalin solution. A distance of 3 mm provided highest peptide signals. Subsequently, the protein test mixture was analyzed at different concentrations using a BGE of 100 mM acetic acid (pH 3.1) with 5% IPA (Fig. 5A). Despite the addition of isopropanol, the protein separation was nicely maintained and the mass spectra were not affected by the IPA present in the BGE (Fig. 5B). More importantly, for all proteins a significant increase in sensitivity was observed when compared to sheathless CE–MS using the conventional ESI source. Overall, this led to LODs which were factors of 4–13 lower than obtained with the standard ESI source, and even 50–140 times lower than for sheath-liquid CE–MS (Table 2). With the sheathless CE–HSPS ESI-MS using the nanoESI source, sub-nM levels could even be detected for carbonic anhydrase II, ribonuclease A and lysozyme. These are highly favorable sensitivities which, to our knowledge, have not been achieved before with CE–MS for intact proteins. The sheathless CE–MS system with nanoESI source also showed good linearity (Table 2). A preliminary repeatability study indicated that RSDs ($n = 5$) for protein migration time and peak area were below 0.8% and 8%, respectively. It should be noted that the baseline noise levels in the extracted-ion traces of the respective proteins were a factor 2–3 higher using nanoESI source in comparison to sheathless CE–MS with the conventional ESI source (Fig. 5C). So, there is still room for further improvement of the system and protein detection limits in the mid-pM range might be well achievable.

4. Concluding remarks

The performance of sheathless CE–MS using a prototype HSPS for the analysis of intact proteins was studied. Stable CE and ESI conditions were established by placing the HSPS capillaries in a stainless steel needle filled with static conductive liquid. A positively charged capillary coating in combination with a low-pH BGE provided the conditions for efficient protein separation and the EOF for effective ESI. Low-nanomolar LODs were obtained with the HSPS sheathless CE–MS system using a conventional ion source, which is a considerable improvement over sheath-liquid CE–MS employing the same capillary. This gain in performance is the result of reduced noise levels and increased analyte responses as obtained with sheathless CE–MS. The sheathless CE–HSPS ESI-MS sensitivity could be further improved by use of a nanoESI source and the addition of IPA to the BGE, achieving sub-nM LODs for three test proteins. These very favorable LODs indicate that the sheathless CE–HSPS ESI-MS can be highly useful for intact protein analysis. Considering the significant role of (reduced) noise in achieving enhanced detection limits, it might be interesting to also evaluate and compare the *S*/*N* ratios of proteins in sheathless and sheath liquid CE–MS under MS/MS conditions. The porous tip is fragile, but once the sprayer is installed in the stainless steel needle, the HSPS capillaries are easy to handle and a single capillary could be used for more than 100 runs over prolonged periods of time without any loss of performance. Beckman Coulter has developed an improved prototype containing a retractable cover on the HSPS housing to protect the porous tip when not in use. Currently, we are studying the potential of the CE–HSPS ESI-MS system for the characterization of drug–protein conjugates and the profiling of biopharmaceutical impurities, degradation products and glycoforms.

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