Evaluation of capillary electrophoresis-mass spectrometry for the analysis of the conformational heterogeneity of intact proteins using beta$_2$-microglobulin as model compound

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Abstract

In this work we explored the feasibility of different CE-ESI-MS set-ups for the analysis of conformational states of an intact protein. By using the same background electrolyte at quasi physiological conditions (50 mM ammonium bicarbonate, pH 7.4) a sequential optimization was carried out, initially by evaluating a sheath-liquid interface with both a single quadrupole (SQ) and a time-of-flight (TOF) mass spectrometer; then a sheathless interface coupled with high-resolution QTOF MS was considered. Beta$_2$-microglobulin has been taken as a model, as it is an amyloidogenic protein and its conformational changes are strictly connected to the onset of a disease. The separation of two conformers at dynamic equilibrium is achieved all the way down to the MS detection. Notably, the equilibrium ratio of the protein conformers is maintained in the electrospray source after CE separation. Strengths and weaknesses of each optimized set-up are emphasized and their feasibility in unfolding studies is evaluated. In particular, ESI-TOF MS can assign protein forms that differ by 1 Da only and sheathless interfacing is best suited to preserve protein structure integrity. This demonstrates the CE-ESI-MS performance in terms of separation, detection and characterization of conformational species that co-populate a protein solution.

Keywords

Beta$_2$-microglobulin; protein conformation; capillary electrophoresis-mass spectrometry; folding; amyloidosis.

1. Introduction

A well-defined structure is considered an absolute requirement for a protein's biological function. The presence of non-native states or oligomers of an initially native protein plays an important role both in the quality control of biotechnological/biological medicinal products and in the study of conformational diseases. In fact, while conformation can be related to the desired biological activity of biopharmaceuticals [1], changes in conformation can be responsible for pathological conditions. Indeed, conformational heterogeneity is involved in the onset of a whole class of diseases, known as amyloidoses [2, 3]. In this respect, detection and separation of co-existing and transiently populated states in solution are not only a step forward in protein characterization but also the starting point to envisage new protein targets for drug molecules.

In this work we explore various CE-ESI-MS set-ups to separate and characterize, under non denaturing and denaturing conditions, the two conformers of beta$_2$-microglobulin (β_2-m), a protein which is often taken as a model of amyloidogenic globular proteins [4]. As previously demonstrated, these two conformers are at equilibrium in solution and the kinetics of their interconversion process is compatible with the time window of a CE or LC analysis [5-8]. Along with spectroscopic techniques, such as stopped flow fluorescence, circular dichroism, X-ray crystallography, NMR and mass spectrometry [9-12], CE-UV, LC-UV and LC-ESI-MS [5, 6, 8] have provided a complementary yet unique contribution to characterize β_2-m conformational states in solution. In this context, the relevant advantages of CE over LC were the high separation efficiency, the absence of a stationary phase (thus ruling out secondary equilibria), the possibility to work under mild and near-physiological conditions that preserve protein conformations, the high selectivity based on exposed charge differences, and the minimum sample and solvent volume consumption. The great potential of the CE-UV method mentioned above is witnessed by a number of applications, namely refolding kinetics studies [13, 14], analysis of mutated and truncated species of β_2-m [7, 8, 14, 15], accurate binding constant calculation by dynamic complexation CE [13, 14, 16, 17], and affinity screening to select new ligands of β_2-m [16, 18].

Since the introduction of “soft” ionization techniques, such as ESI, MS has evolved as a tool for studying the folding state of intact proteins and providing information on both the structure and the dynamics of proteins [19, 20]. The position and width of charge-state distributions (CSDs) in ESI mass spectra of proteins yield insight into the nature and population of conformational states at equilibrium [21]. Thus, coupled CE-MS can be a powerful analytical tool for detailed characterization of an intact protein in solution, as it combines the unique protein separation capacity of CE with the selectivity of MS. Further, MS can considerably enhance the utility of CE by providing information about the identity of the separated compounds [22-25]. Therefore, starting from the available CE-UV method for β_2-m, we set out to evaluate different CE-ESI-MS arrangements, either coupled by sheath-liquid or sheathless interfacing. The former interface is known to be robust, however, the high flow rate of the sheath liquid, as compared to that of CE, causes analyte dilution and, consequently lower sensitivity [26]. Moreover, the sheath liquid commonly comprises an organic solvent, which may affect protein conformation. Further, loss of separation efficiency can be ascribed to suction effects which are generated by both nebulizing gas and sheath liquid [27]. On the other hand, the main difficulty in the sheathless approach is to close the electrical circuit required for any CE separation, as the voltage is directly applied to the CE buffer. In this work we used a sheathless nanospray interface originally developed by Moini, which provides electric contact through the wall of the porous fused-silica capillary tip [28]. Compared to the sheath liquid or liquid-junction interfaces, no dilution or dead-volume effects occur and the flow rate is only due to the electrophoretic and electrospray processes. Furthermore, the analytes are directly electrosprayed from the used background electrolyte and will not be exposed to organic solvents. These features render the sheathless interface efficient and attractive for CE-ESI-MS of proteins and their potential conformers.

As witnessed by review articles [22, 23, 29], little effort has been put so far on the application of CZE-ESI-MS for the conformational analysis of intact proteins. Protein refolding/unfolding states have hardly been explored [30]. On this basis, a sequential optimization is here accomplished, and proof-of-concept experiments were carried out to separate and characterize β_2-m folding conformations in solution. The

sheath liquid interface is coupled either to single quadrupole (SQ) MS or TOF MS, then sheathless CE-nanoESI-QTOF MS is evaluated. Strengths and weaknesses of each arrangement are discussed.

2. Materials and methods

2.1 Samples and chemicals

Recombinant wild type β_2-m (M_r 11860 Da, pI 6.05) was expressed and purified as previously described [31]. Stock and diluted solutions of β_2-m were prepared in water and maintained at -20°C until use. To induce partial denaturation, different amounts of acetonitrile (30% and 50 % v/v) were added to the protein solutions and immediately injected without any further treatment. Acetonitrile and propan-2-ol of analytical reagent grade were from Panreac (Barcelona, Spain) and Acros Organics (Geel, Belgium), respectively. Ammonium bicarbonate and formic acid were purchased from Sigma-Aldrich (St.Louis, MO, USA). Ultrapure water was supplied by a Milli-Q purification unit from Millipore (Bedford, MA, USA).

2.2 Instrumentation

2.2.1 Sheath liquid CE-ESI-SQ MS

CE experiments were performed using a ProteomeLab™ PA 800 *plus* Protein Characterization System (Beckman Coulter, Brea, CA, USA) equipped with a temperature controlled autosampler. Analyses were carried out at +30 kV and capillary temperature was maintained at 15 °C. Uncoated capillaries (100 cm total length, 50 µm i.d., 365 µm o.d.) were from BGB Analytik AG (Böckten, Switzerland). New fused silica capillaries were conditioned by flushing sodium hydroxide, water and background electrolyte (BGE) for 100, 100 and 180 min, respectively. The BGE composition was 50 mM ammonium bicarbonate, adjusted to pH 7.4 with formic acid (buffer capacity of 9.8 mmol $L^{-1}pH^{-1}$). Prior to each sample injection, the capillary was rinsed at 2 bar with 0.01% (v/v) ammonium hydroxide and water (1.5 min each) and then with fresh BGE (3 min). Protein samples were hydrodynamically injected at 50 mbar for 30 s (equivalent to 1.0% of the capillary length).

CE was coupled to ESI-SQ (single quadrupole Agilent Series 1100 MSD) mass spectrometer via a sheath liquid electrospray interface equipped with a stainless steel needle (Agilent, Palo Alto, CA, USA). After careful evaluation, the coaxial sheath liquid composition was propan-2-ol-water-formic acid (49.5:49.5:1,

v/v/v) and the flow rate (delivered by a syringe pump) was set to 4 µL min^{-1}. The ESI voltage was set to +4500 V, the nebulizing gas pressure to 4 psi, the drying gas flow rate to 4 L min^{-1} and the drying gas temperature to 250°C. Fragmentor voltage was set at 250 V. Positive ionization was used and MS detection was carried out in single ion monitoring (SIM) mode for the following positive molecular ions: *z*=6, *m/z*= 1977; *z*=7, *m/z*= 1695; *z*=8, *m/z*= 1483; *z*=9, *m/z*= 1318.

2.2.2 Sheath liquid CE-ESI-TOF MS

CE experiments were performed using a G7100 CE system from Agilent Technologies (Waldbronn, Germany), equipped with an on-capillary diode array detector and an autosampler. Uncoated capillaries (50 µm i.d., 360 µm o.d.) with different total lengths (100 cm, 200 cm and 300 cm) were from BGB Analytik AG (Böckten, CH). Voltage, capillary conditioning protocol, capillary temperature and BGE composition were as in 2.2.1. Prior to each sample injection, the capillary was rinsed at 6 bar with 0.01% (v/v) ammonium hydroxide and water (5 min each) and then with fresh BGE (10 min). Protein samples were hydrodynamically injected at 50 mbar for 150 s (0.6% of the 300 cm capillary volume).

CE was coupled to an Agilent Technologies 6210 LC/MS TOF mass spectrometer via a commercial sheath liquid electrospray interface equipped with a stainless steel needle (Agilent). The coaxial sheath liquid composition was propan-2-ol-water-formic acid (49.5:49.5:1, v/v/v), and also other conditions were as in 2.2.1. Mass spectra were acquired in a positive ion mode and in the mass range *m/z* 800-2500 with an acquisition rate of 1 spectrum/s.

2.2.3 Sheathless CE-nanoESI-Q-TOF MS

Sheathless CE-MS was carried out on a CESI 8000 instrument (ABSciex, Brea, CA, USA). Uncoated capillaries (90 cm total length, 30 µm i.d., 150 µm o.d.) with a porous tip were used. The capillary temperature was 15°C. The BGE composition was 50 mM ammonium bicarbonate, adjusted to pH 7.4 with formic acid. Before each run, the capillary was flushed for 5 min at 75 psi with fresh BGE. Protein samples were injected at 5 psi for 20 s (13.4 nL, i.e. 2.1% of the capillary volume). The capillary was placed in a grounded stainless

steel needle that could be positioned by an XYZ-stage at the entrance of the Bruker maXis HD Ultra high resolution QTOF (Bruker Daltonics, Bremen, Germany). The stainless needle was filled with BGE (static electrolyte) for 2 min at 75 psi to establish the electrical contact. A specific end plate and gas diverter were installed in the ion source to allow nanoESI. The mass spectrometer was operated in positive mode using the following parameters: capillary voltage 1200–1400 V, drying gas temperature 180 °C, drying gas flow rate 2 L min^{-1}. Mass spectra were collected in profile between 500 and 3000 m/z. MS control and data acquisition were performed using QTOF control software (Bruker Daltonics). CE-MS data were analyzed using Bruker Daltonics data analysis software. Molecular mass determinations were performed using the 'Maximum Entropy' utility of the DataAnalysis software.

3. Results and Discussion

So far, only one CE-ESI-MS study on the separation and characterization of the conformational variants of β_2-m in solution has been reported [32]. Unfortunately this attempt failed, as the two conformers, which could be successfully separated and detected by CE-UV, were not distinguished by CE-ESI-MS. The electrophoretic selectivity that results in the separation of native β_2-m (higher apparent mobility) from the partially structured intermediate of its folding (lower apparent mobility) can be ascribed to differences in charge exposure and charge shielding, degree of hydration, gyration radius, and frictional coefficient associated to each state. As also reported in [32], besides the CE-UV separation of the conformers in solution, the further challenge of this particular case stands in demonstrating the maintenance of the equilibrium ratio between the two species, all the way through the ESI process and the MS detection. Moreover, using CE-UV we recently detected a third species in solutions of wild type β_2-m as well as of mutated forms [8]: thus a careful characterization of the separated peaks is needed, both in native (aqueous) and partially denatured samples.

Under the operative pH the protein migrates as anion, with migration times larger than EOF marker [8]. To be consistent with CE and LC methods previously developed [5, 6], acetonitrile was selected to partially denature the protein before injection and induce the appearance of the folding intermediate. The non-denaturing conditions, i.e. β_2-m solubilized in pure water, require the highest sensitivity, separation efficiency and selectivity, mainly for two reasons: (i) all the separated species, except for the native form, are scarcely populated; (ii) the partially folded intermediate structure is similar to that of the native β_2-m [33-35].

3.1 Sheath liquid CE-ESI-SQ MS experiments

Approaching native conditions while attaining MS compatibility, a BGE of 50 mM ammonium bicarbonate (pH 7.4) was selected. The sheath liquid composition (propan-2-ol-water-formic acid (49.5:49.5:1, v/v/v)) and flow rate (4 µL min^{-1}) were accurately selected to achieve efficient ionization of β_2-m. The four main

charge states obtained for β_2-m with z=6-9 were monitored by SIM. This set-up afforded to obtain the analysis of β_2-m under quasi native conditions by using a separation technique coupled to MS [6]: from the extracted-ion trace (Fig. 1A) and by comparison with the CE-UV electropherograms under native conditions [5], it is conceivable that the small, later migrating peak (2) can be attributed to the partially unfolded conformer of the native main peak (1). Because equilibrium studies by adding denaturant are accessible [5, 8, 36], this was confirmed by the addition of 30% (v/v) acetonitrile to the protein sample (Fig. 1B). While the quantitative equilibrium between the two species does not exactly correspond to that observed in CE-UV using a non-volatile BGE [5, 8], it is evident that upon partial denaturation the second conformer increases at the expense of the native protein peak.

The CE separation of a third, later migrating and unidentified species was also observed in wild type as well as in mutated and truncated species of β_2-m, mostly after adding denaturants to the sample and in a few cases under native conditions [8].

Notwithstanding the step forward achieved, some drawbacks characterize this CE-MS configuration. First, although detection of peak 2 is also reached under non-denaturing conditions (Fig.1A), insufficient sensitivity hinders its identification by MS. Further, the same difficulty is still encountered under denaturing conditions (Fig. 1B), as the limited performance of the SQ does not allow to obtain good-quality ESI mass spectra for the individual species (peaks 1, 2 and 3).

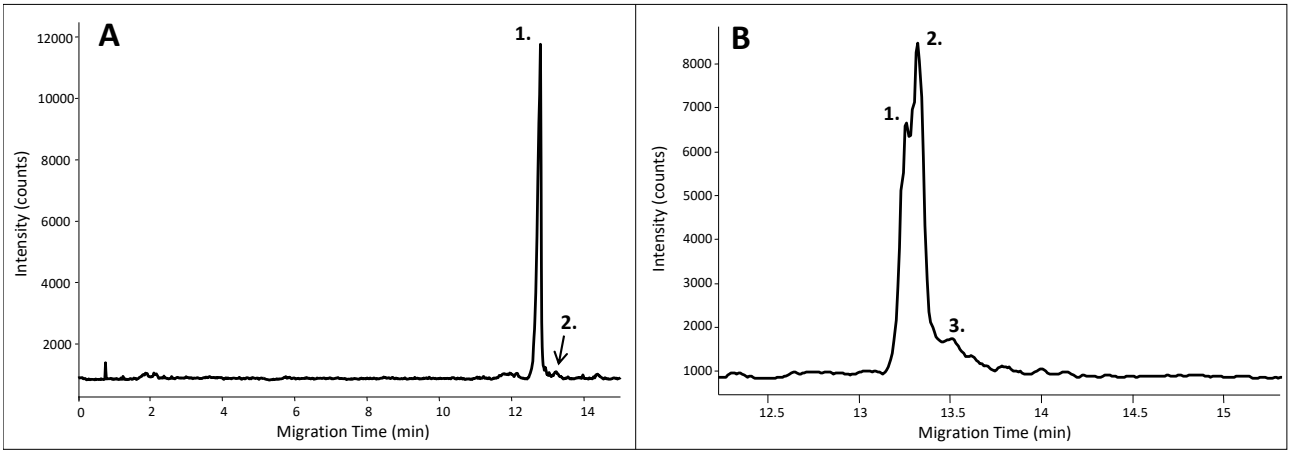

Fig. 1: Sheath liquid CE-ESI-SQ MS. Extracted ion electropherograms of 60 µM β_2-m in water (A) and 30% v/v acetonitrile (B).

3.2 Sheath-liquid CE-ESI-TOF MS experiments

Time-of-flight (TOF) technology presents advantages such as high mass accuracy and resolution, sensitivity, large mass range, and high data acquisition rate that, overall, meet the requirements for CE detection [37]. This was found to be the case also for the analysis of β_2-m conformers here presented. Employing the same BGE made of 50 mM ammonium bicarbonate (pH 7.4), CE was hyphenated with ESI-TOF MS using the sheath-liquid interface and all settings (i.e. sheath liquid composition, ESI source parameters, protein concentration, injection volume, electric field and capillary total length) were accurately tuned leading to similar conditions as applied with SQ MS.

To attain detection of all protein species, wild-type β_2-m concentration was increased up to 210 µM in the presence of 50% (v/v) acetonitrile in the sample, when the population of the partially structured conformer is maximized upon partial unfolding. Using a capillary length of 100 cm (Fig. 2A), a similar separation as observed with CE-ESI-SQ MS was obtained. However, when increasing the capillary length to 200 and 300 cm (Fig. 2B-C), the peak resolution was enhanced. Deconvolution of the mass spectra obtained for peaks 1 and 2 yielded exactly the same protein mass, confirming the conclusion that peaks 1 and 2 originate from conformers of the same protein (Fig. S1-S3 Supplementary material). Interestingly, the ratio of peaks 1 and 2 appeared to depend on the capillary length.

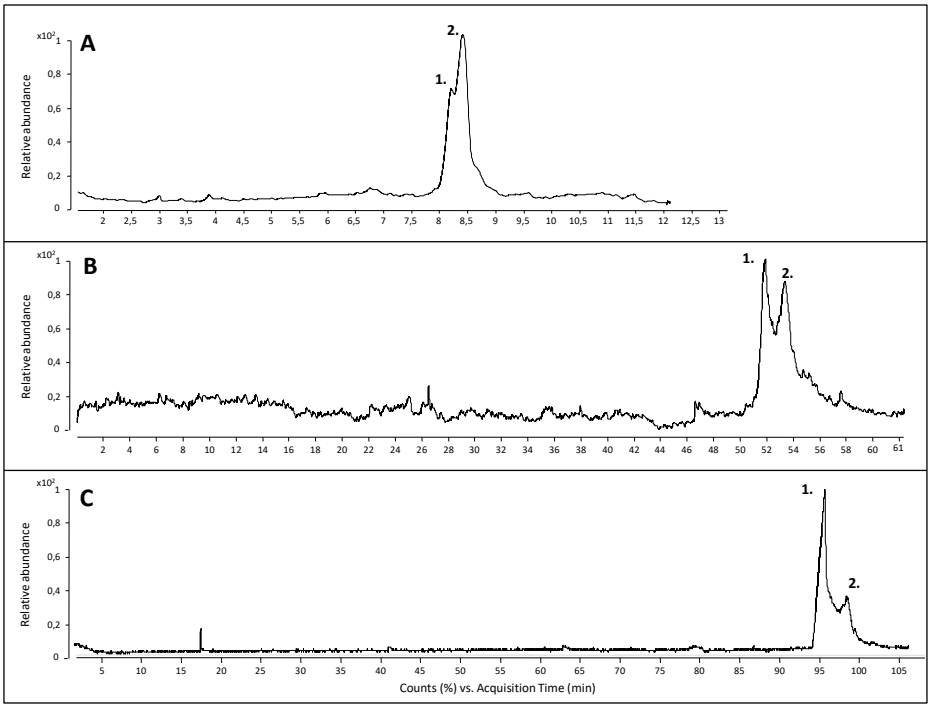

Fig. 2: Sheath liquid CE-ESI-TOF MS. Total ion electropherogram of 210 µM β_2-m in 50% v/v acetonitrile using a capillary length of 100 cm (A), 200 cm (B) and 300 cm (C).

Despite the separation of peaks 1 and 2, they were not baseline resolved. The elevated baseline is to be ascribed to the isomerization of a fraction of molecules in either state and it is expected for two conformers at equilibrium during the separation. This phenomenon has been already observed for the same peaks separated by CE-UV [5, 8, 38] and LC-MS [6] and it is consistent with the hypothesized kinetics of interconversion between the two isoforms [35]. The changing peak ratio is due to protein refolding and it is consistent with the increasing residence time of β_2-m in a non-denaturing BGE [5, 7, 32]

The TOF MS detection provides high-resolution mass spectra of the analyzed protein species giving access to the charge state distribution (CSD) of the β_2-m conformers. Being partly folded, the intermediate state (peak 2) could give rise to a shift of the CSD to more charged ions (lower m/z) with respect to native β_2-m (peak 1). However, the recorded CSDs of peaks 1 and 2 were clearly superimposable (Fig. S1-S3 Supplementary material). The mixing of the nanoflow of the CE separation capillary to the microflow of the propan-2-ol-containing sheath liquid could induce a normalization of the mass spectra in terms of number

and relative abundance of charges. The composition of the sheath liquid may favor protein unfolding. Surprisingly, as shown in Fig. 3A-C, the CSD of the protein species shifted to higher charges (lower m/z) when the capillary length increased. As recently commented [8, 39], the conformational integrity of the protein may be also influenced by temperature gradients, non zero shear components, interaction with the hydrophilic surface of the fused-silica over the long migration in the capillary.

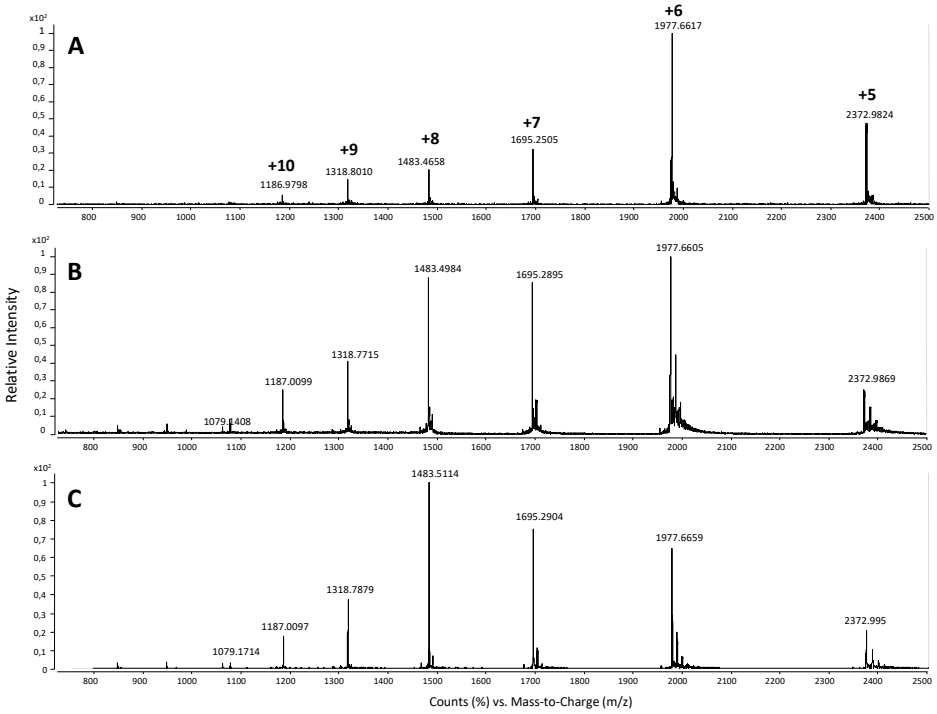

Fig. 3: Sheath-liquid CE-ESI-TOF MS. Mass spectra (full scan mode, 800-2500 m/z range) of peak 1 obtained during analysis of β_2-m in 50% v/v acetonitrile using a capillary length of 100 cm (A), 200 cm (B) and 300 cm (C). The corresponding electropherograms are shown in Fig. 2.

CE-TOF-MS was also used to analyze β_2-m under native conditions. Notably, the obtained electropherogram (Fig. 4A) shows the presence of a main peak, and a second small well-separated peak characterized by a significantly lower mobility. The TOF mass spectra obtained are depicted in Fig. 4B-C allowing calculation of the accurate protein's molecular masses by spectral deconvolution. Interestingly, the molecular mass of the minor species showed a mass difference of +1 Da with respect to β_2-m. Considering the observed mobility difference as well, we hypothesize the compound to be a deamidated form of β_2-m. Deamidation involves

the transition of an asparagine residue into aspartic acid, leading to an extra negative charge (i.e. lower mobility) at pH 7.4 and a gain in molecular mass of 1 Da.

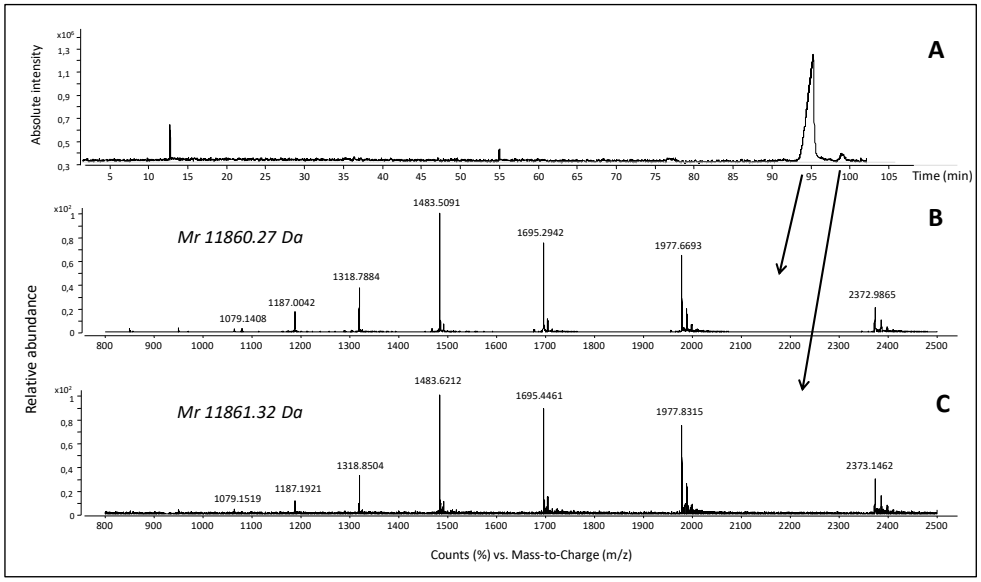

Fig. 4: Sheath-liquid CE-ESI-TOF MS. Electropherogram of β_2-m in water using a capillary of 300 cm (A); MS spectra of the main (B) and minor (C) peak, respectively. M_r states the relative molecular masses resulting from deconvolution of the mass spectra.

3.3 Sheathless CE-nanoESI-Q-TOF MS experiments

$Beta_2$-m was also analyzed by CE-MS using a porous-tip sheathless interface and QTOF mass spectrometer. This interface, which was first developed by Moini *et al.* [28], has shown particularly useful for the analysis of intact proteins [40-42], offering high sensitivity [43]. Moreover, it prevents the analytes coming into contact with organic solvent or vapor during the entire CE-MS process. Fig. 5 shows the sheathless CE-MS analysis of β_2-m in water (28 µM; 4.5 ng injected) and after incubation in 50% v/v acetonitrile (42 µM; 6.7 ng injected). CE-MS revealed the presence of at least three species (peaks 1-3). For the native β_2-m sample (Fig. 5A), the most abundant peak 1 is followed by a poorly resolved second peak (2). For the partially denatured β_2-m sample (Fig 5B), the intensity of peak 2 is clearly increased at the expense of peak 1. For repeated analysis (n=3) of the β_2-m conformers RSD% for migration time and peak area were lower than

1.7% and 2.5%, respectively. The mass spectra of peaks 1 and 2 (Fig.6A) show a very simple CSD with only two major ions at m/z 1695.26 (7+) and m/z 1977.64 (6+). Compared to the mass spectra obtained with sheath-liquid interfacing, there are a smaller number of charge states and a minor shift towards fewer charges. This is most probably caused by the fact that with sheathless interfacing the protein species are not exposed to organic solvent. Deconvolution of the mass spectra of peaks 1 and 2 yielded exactely the same molecular mass. However, for peak 1 the 6+ charge state showed the highest relative intensity, whereas for peak 2 the 7+ charge state is the most abundant. This observation supports the conclusion that peaks 1 and 2, respectively, are the native (fully folded) and partly folded species of β_2-m.

The mass spectra recorded for peak 3 in both β_2-m samples, show a strong resemblance with the mass spectra obtained for peaks 1 and 2. However close inspection of the acquired isotope pattern and molecular mass resulting from spectral deconvolution, revealed a small but significant difference of +1 Da with respect to the earlier migrating β_2-m species (Fig.6B and Fig.S4). From this we conclude that peak 3 is the deamidated form of β_2-m which was also observed with sheath-liquid interfacing.

Digestion of recombinant β_2-m by Endoproteinase Asp-N and analysis by MALDI-TOF MS support this conclusion (Fig.S5)

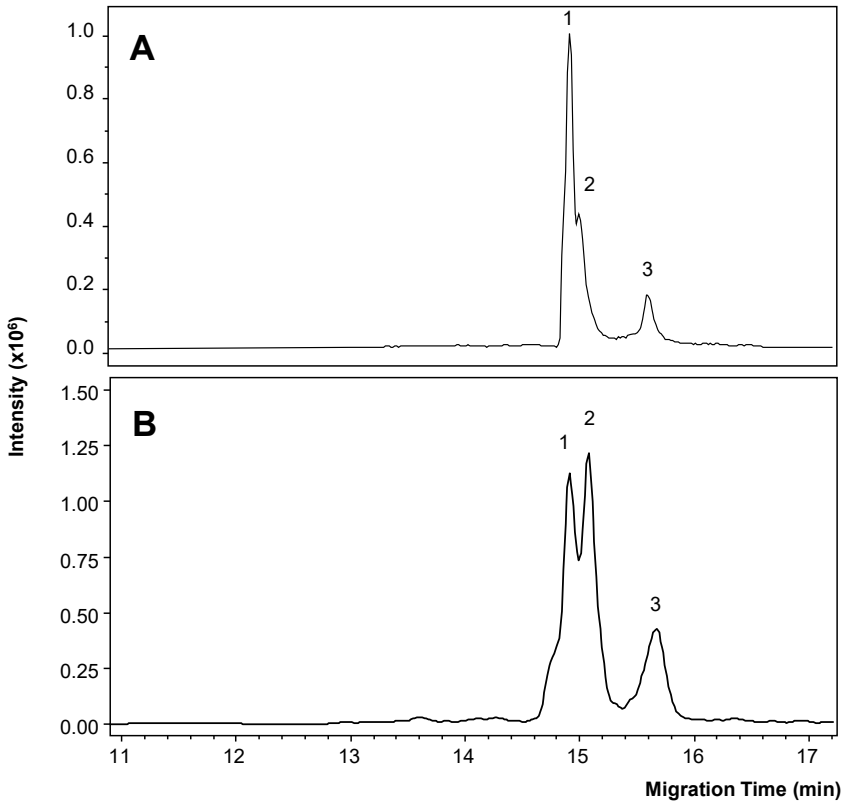

Fig. 5: Sheathless CE-nanoESI-Q-TOF MS. Cumulative extracted-ion electropherograms of m/z 1695-1696 (6+) and 1977-1978 (7+) obtained during analysis of 28 µM β_2-m in water (A) and 42 µM β_2-m in 50% v/v acetonitrile (B).

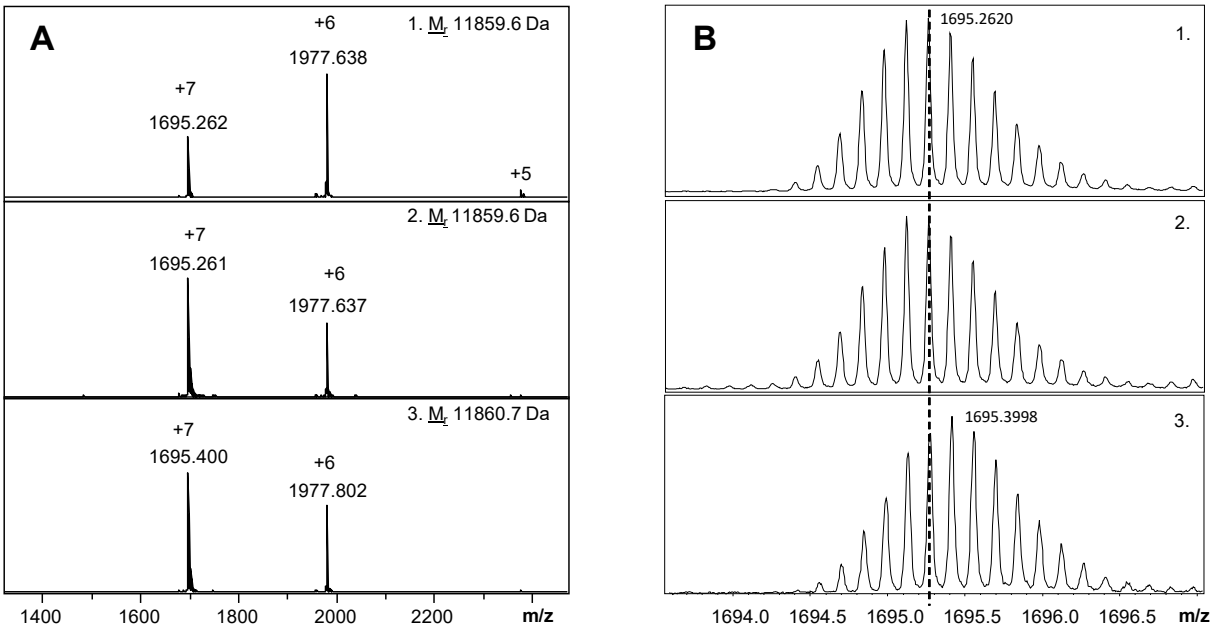

Fig. 6: Sheathless CE-nanoESI-Q-TOF MS. MS spectra (A) and isotopic pattern (B) of charge state 7+ of peaks 1-3 detected during analysis of β_2-m (Fig. 5B). M_r states the relative molecular masses resulting from deconvolution of the mass spectra.

The deamidated form of β_2-m is quite abundant under both native and denaturing conditions. While spontaneous deamidation may well depend on sample preparation and/or method conditions, this chemical modification is nevertheless not unexpected in β_2-m. The presence of deamidation products at Asn17 or Asn42 in ex-vivo β_2-m fibrils has been previously described [44, 45], amyloid fibril formation from the deamidated variant Asp17 has been studied, and the structures of wild type β_2-m and this variant have been demonstrated to be very similar [46]. Deamidation causes a distinct change in protein charge-to-size

ratio, facilitating CE separation [47]. A recent work reported that β_2-m spontaneously deamidates under defined conditions and over long incubation times [48]. Notwithstanding these studies, an estimation of the susceptibility of this protein to deamidate is still challenging and the involvement of deamidation in β_2-m amyloid formation remains so far unclear.

4. Conclusions

In this work we presented CE-ESI-MS for the separation and characterization of the intact protein folding conformers [22, 23, 29] of β_2-m [32]. While quantitative analysis was beyond the scope of this work, the maintenance of protein conformers under CE and ESI conditions was demonstrated. In particular, sheathless interfacing appears attractive for preserving the protein structure integrity, while ESI-TOF-MS can discriminate conformers and reliably assign protein forms that differ by 1 Da only. To achieve protein resolution the CE method neither needs BGE additives nor capillary coating procedures.

Given the clinical importance of β_2-m conformational heterogeneity in the context of amyloidosis, the separated species are potential drug targets and this confers an added value to the obtained results. In principle, mobility shift ACE with small drug-like molecules to investigate binding is made possible by CE-ESI-MS [47].

Acknowledgments

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References

[1] F.D.A. USA, I.C.o. Harmonisation, Quality of biotechnological products stability testing of biotechnological/biological products, U.S. Dept. of Health and Human Services, Food Drug Administration, Rockville, MD, 1996.
[2] M.B. Pepys, Amyloidosis, Annu Rev Med, 57 (2006) 223-241.
[3] F. Chiti, C.M. Dobson, Protein misfolding, functional amyloid, and human disease, Annu Rev Biochem, 75 (2006) 333-366.
[4] T. Eichner, S.E. Radford, A generic mechanism of beta2-microglobulin amyloid assembly at neutral pH involving a specific proline switch, J Mol Biol, 386 (2009) 1312-1326.
[5] F. Chiti, E. De Lorenzi, S. Grossi, P. Mangione, S. Giorgetti, G. Caccialanza, C.M. Dobson, G. Merlini, G. Ramponi, V. Bellotti, A partially structured species of beta 2-microglobulin is significantly populated under physiological conditions and involved in fibrillogenesis, J Biol Chem, 276 (2001) 46714-46721.
[6] L. Bertoletti, L. Regazzoni, G. Aldini, R. Colombo, F. Abballe, G. Caccialanza, E. De Lorenzi, Separation and characterisation of beta2-microglobulin folding conformers by ion-exchange liquid chromatography and ion-exchange liquid chromatography-mass spectrometry, Anal Chim Acta, 771 (2013) 108-114.
[7] E. De Lorenzi, S. Grossi, G. Massolini, S. Giorgetti, P. Mangione, A. Andreola, F. Chiti, V. Bellotti, G. Caccialanza, Capillary electrophoresis investigation of a partially unfolded conformation of beta(2)-microglobulin, Electrophoresis, 23 (2002) 918-925.
[8] L. Bertoletti, F. Bisceglia, R. Colombo, S. Giorgetti, S. Raimondi, P.P. Mangione, E. De Lorenzi, Capillary electrophoresis analysis of different variants of the amyloidogenic protein β2 -microglobulin as a simple tool for misfolding and stability studies, Electrophoresis, 36 (2015) 2465-2472.
[9] F. Chiti, P. Mangione, A. Andreola, S. Giorgetti, M. Stefani, C.M. Dobson, V. Bellotti, N. Taddei, Detection of two partially structured species in the folding process of the amyloidogenic protein beta 2-microglobulin, J Mol Biol, 307 (2001) 379-391.
[10] G. Verdone, A. Corazza, P. Viglino, F. Pettirossi, S. Giorgetti, P. Mangione, A. Andreola, M. Stoppini, V. Bellotti, G. Esposito, The solution structure of human beta2-microglobulin reveals the prodromes of its amyloid transition, Protein Sci, 11 (2002) 487-499.
[11] A. Corazza, E. Rennella, P. Schanda, M.C. Mimmi, T. Cutuil, S. Raimondi, S. Giorgetti, F. Fogolari, P. Viglino, L. Frydman, M. Gal, V. Bellotti, B. Brutscher, G. Esposito, Native-unlike long-lived intermediates along the folding pathway of the amyloidogenic protein beta2-microglobulin revealed by real-time two-dimensional NMR, J Biol Chem, 285 (2010) 5827-5835.

[12] E. Rennella, A. Corazza, F. Fogolari, P. Viglino, S. Giorgetti, M. Stoppini, V. Bellotti, G. Esposito, Equilibrium unfolding thermodynamics of beta2-microglobulin analyzed through native-state H/D exchange, Biophys J, 96 (2009) 169-179.
[13] C. Carazzone, R. Colombo, M. Quaglia, P. Mangione, S. Raimondi, S. Giorgetti, G. Caccialanza, V. Bellotti, E. De Lorenzi, Sulfonated molecules that bind a partially structured species of beta2-microglobulin also influence refolding and fibrillogenesis, Electrophoresis, 29 (2008) 1502-1510.
[14] N.H. Heegaard, E. De Lorenzi, Interactions of charged ligands with beta(2)-microglobulin conformers in affinity capillary electrophoresis, Biochim Biophys Acta, 1753 (2005) 131-140.
[15] M.C. Mimmi, T.J. Jørgensen, F. Pettirossi, A. Corazza, P. Viglino, G. Esposito, E. De Lorenzi, S. Giorgetti, M. Pries, D.B. Corlin, M.H. Nissen, N.H. Heegaard, Variants of beta-microglobulin cleaved at lysine-58 retain the main conformational features of the native protein but are more conformationally heterogeneous and unstable at physiological temperature, FEBS J, 273 (2006) 2461-2474.
[16] M. Quaglia, C. Carazzone, S. Sabella, R. Colombo, S. Giorgetti, V. Bellotti, E. De Lorenzi, Search of ligands for the amyloidogenic protein beta2-microglobulin by capillary electrophoresis and other techniques, Electrophoresis, 26 (2005) 4055-4063.
[17] L. Regazzoni, R. Colombo, L. Bertoletti, G. Vistoli, G. Aldini, M. Serra, M. Carini, R.M. Facino, S. Giorgetti, M. Stoppini, G. Caccialanza, E. De Lorenzi, Screening of fibrillogenesis inhibitors of β2-microglobulin: integrated strategies by mass spectrometry capillary electrophoresis and in silico simulations, Anal Chim Acta, 685 (2011) 153-161.
[18] M. Quaglia, E. De Lorenzi, Capillary electrophoresis in drug discovery, Methods Mol Biol, 572 (2009) 189-202.
[19] M. Landreh, J. Astorga-Wells, J. Johansson, T. Bergman, H. Jörnvall, New developments in protein structure-function analysis by MS and use of hydrogen-deuterium exchange microfluidics, FEBS J, 278 (2011) 3815-3821.
[20] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Electrospray ionization for mass spectrometry of large biomolecules.
[21] A. Dobo, I.A. Kaltashov, Detection of multiple protein conformational ensembles in solution via deconvolution of charge-state distributions in ESI MS, Anal Chem, 73 (2001) 4763-4773.
[22] R. Haselberg, G.J. de Jong, G.W. Somsen, Capillary electrophoresis-mass spectrometry for the analysis of intact proteins 2007-2010, Electrophoresis, 32 (2011) 66-82.
[23] R. Haselberg, G.J. de Jong, G.W. Somsen, CE-MS for the analysis of intact proteins 2010-2012, Electrophoresis, 34 (2013) 99-112.
[24] J.A. Olivares, N.T. Nguyen, C.R. Yonker, R.D. Smith, On-line mass spectrometric detection for capillary zone electrophoresis, Analytical Chemistry, 59 (1987) 1230-1232.
[25] K. Klepárník, Recent advances in combination of capillary electrophoresis with mass spectrometry: Methodology and theory.
[26] M. Mokaddem, P. Gareil, J.E. Belgaied, A. Varenne, New insight into suction and dilution effects in CE coupled to MS via an ESI interface. II - Dilution effect, Electrophoresis, 30 (2009) 1692-1697.
[27] G. Bonvin, J. Schappler, S. Rudaz, Capillary electrophoresis-electrospray ionization-mass spectrometry interfaces: Fundamental concepts and technical developments, J Chromatogr A, 1267 (2012) 17-31.
[28] M. Moini, Simplifying CE-MS operation. 2. Interfacing low-flow separation techniques to mass spectrometry using a porous tip, Anal Chem, 79 (2007) 4241-4246.
[29] R. Haselberg, G.J. de Jong, G.W. Somsen, Capillary electrophoresis-mass spectrometry for the analysis of intact proteins, J Chromatogr A, 1159 (2007) 81-109.
[30] P.K. Jensen, A.K. Harrata, C.S. Lee, Monitoring protein refolding induced by disulfide formation using capillary isoelectric focusing-electrospray ionization mass spectrometry, Anal Chem, 70 (1998) 2044-2049.
[31] G. Esposito, R. Michelutti, G. Verdone, P. Viglino, H. Hernández, C.V. Robinson, A. Amoresano, F. Dal Piaz, M. Monti, P. Pucci, P. Mangione, M. Stoppini, G. Merlini, G. Ferri, V. Bellotti, Removal of the N-terminal hexapeptide from human beta2-microglobulin facilitates protein aggregation and fibril formation, Protein Sci, 9 (2000) 831-845.

[32] N.H. Heegaard, L. Rovatti, M.H. Nissen, M. Hamdan, Structural and conformational variants of human beta2-microglobulin characterized by capillary electrophoresis and complementary separation methods, J Chromatogr A, 1004 (2003) 51-59.
[33] T.R. Jahn, M.J. Parker, S.W. Homans, S.E. Radford, Amyloid formation under physiological conditions proceeds via a native-like folding intermediate, Nat Struct Mol Biol, 13 (2006) 195-201.
[34] M. Sakata, E. Chatani, A. Kameda, K. Sakurai, H. Naiki, Y. Goto, Kinetic coupling of folding and prolyl isomerization of beta2-microglobulin studied by mutational analysis, J Mol Biol, 382 (2008) 1242-1255.
[35] A. Mukaiyama, T. Nakamura, K. Makabe, K. Maki, Y. Goto, K. Kuwajima, Native-State Heterogeneity of β(2)-Microglobulin as Revealed by Kinetic Folding and Real-Time NMR Experiments, J Mol Biol, 425 (2012) 257.
[36] N.H. Heegaard, J.W. Sen, N.C. Kaarsholm, M.H. Nissen, Conformational intermediate of the amyloidogenic protein beta 2-microglobulin at neutral pH, J Biol Chem, 276 (2001) 32657-32662.
[37] A. Staub, J. Schappler, S. Rudaz, J.L. Veuthey, CE-TOF/MS: fundamental concepts, instrumental considerations and applications, Electrophoresis, 30 (2009) 1610-1623.
[38] V. Bellotti, G. Caccialanza, F. Chiti, E. De Lorenzi, C.M. Dobson, S. Giorgetti, S. Grossi, P. Mangione, G. Merlini, G. Ramponi, Separation and quantification of two beta2-microglobulin isoforms, University of Pavia, WO 2003/021270 A1, 2003.
[39] G. Brancolini, A. Corazza, M. Vuano, F. Fogolari, M.C. Mimmi, V. Bellotti, M. Stoppini, S. Corni, G. Esposito, Probing the Influence of Citrate-Capped Gold Nanoparticles on an Amyloidogenic Protein, ACS Nano, (2015).
[40] R. Haselberg, C.K. Ratnayake, G.J. de Jong, G.W. Somsen, Performance of a sheathless porous tip sprayer for capillary electrophoresis-electrospray ionization-mass spectrometry of intact proteins, J Chromatogr A, 1217 (2010) 7605-7611.
[41] R. Haselberg, G.J. de Jong, G.W. Somsen, Low-flow sheathless capillary electrophoresis-mass spectrometry for sensitive glycoform profiling of intact pharmaceutical proteins, Anal Chem, 85 (2013) 2289-2296.
[42] R. Haselberg, S. Harmsen, M.E. Dolman, G.J. de Jong, R.J. Kok, G.W. Somsen, Characterization of drug-lysozyme conjugates by sheathless capillary electrophoresis-time-of-flight mass spectrometry, Anal Chim Acta, 698 (2011) 77-83.
[43] G. Bonvin, J.L. Veuthey, S. Rudaz, J. Schappler, Evaluation of a sheathless nanospray interface based on a porous tip sprayer for CE-ESI-MS coupling, Electrophoresis, 33 (2012) 552-562.
[44] H. Odani, R. Oyama, K. Titani, H. Ogawa, A. Saito, Purification and complete amino acid sequence of novel beta 2-microglobulin, Biochem Biophys Res Commun, United States, 1990, pp. 1223-1229.
[45] M. Stoppini, P. Mangione, M. Monti, S. Giorgetti, L. Marchese, P. Arcidiaco, L. Verga, S. Segagni, P. Pucci, G. Merlini, V. Bellotti, Proteomics of beta2-microglobulin amyloid fibrils, Biochim Biophys Acta, 1753 (2005) 23-33.
[46] N.M. Kad, N.H. Thomson, D.P. Smith, D.A. Smith, S.E. Radford, Beta(2)-microglobulin and its deamidated variant, N17D form amyloid fibrils with a range of morphologies in vitro, J Mol Biol, 313 (2001) 559-571.
[47] E. Domínguez-Vega, R. Haselberg, G.W. Somsen, G.J. de Jong, Simultaneous assessment of protein heterogeneity and affinity by capillary electrophoresis-mass spectrometry, Anal Chem, 87 (2015) 8781-8788.
[48] M. Fukuda, T. Takao, Quantitative analysis of deamidation and isomerization in β2-microglobulin by 18O labeling, Anal Chem, 84 (2012) 10388-10394.

FIGURE CAPTIONS

Figure 1: **Sheath liquid CE-ESI-SQ MS.** Extracted ion electropherograms of 60 µM β_2-m in water (A) and 30% v/v acetonitrile (B); mass spectrum of peak 1 showing indicated charge states at m/z 1318, 1483, 1695, and 1977 (C).

Figure 2: Sheath liquid CE-ESI-TOF MS. Total ion electropherogram of 210 µM β_2-m in 50% v/v acetonitrile using a capillary length of 100 cm (A), 200 cm (B) and 300 cm (C).

Figure 3: Sheath-liquid CE-ESI-TOF MS. Mass spectra (full scan mode, 800-2500 m/z range) of peak 1 obtained during analysis of β_2-m in 50% v/v acetonitrile using a capillary length of 100 cm (A), 200 cm (B) and 300 cm (C). The corresponding electropherograms are shown in Fig. 2.

Figure 4: Sheath-liquid CE-ESI-TOF MS. Electropherogram of β_2-m in water using a capillary of 300 cm (A); MS spectra of the main (B) and minor (C) peak, respectively. Mr states the relative molecular masses resulting from deconvolution of the mass spectra.

Figure 5: Sheathless CE-nanoESI-Q-TOF MS. Cumulative extracted-ion electropherograms of m/z 1695-1696 (6+) and 1977-1978 (7+) obtained during analysis of 28 µM β_2-m in water (A) and 42 µM β_2-m in 50% v/v acetonitrile (B).

Fig. 6: Sheathless CE-nanoESI-Q-TOF MS. MS spectra (A) and isotopic pattern (B) of charge state 7+ of peaks 1-3 detected during analysis of β_2-m (Fig. 5B). M_r states the relative molecular masses resulting from deconvolution of the mass spectra.