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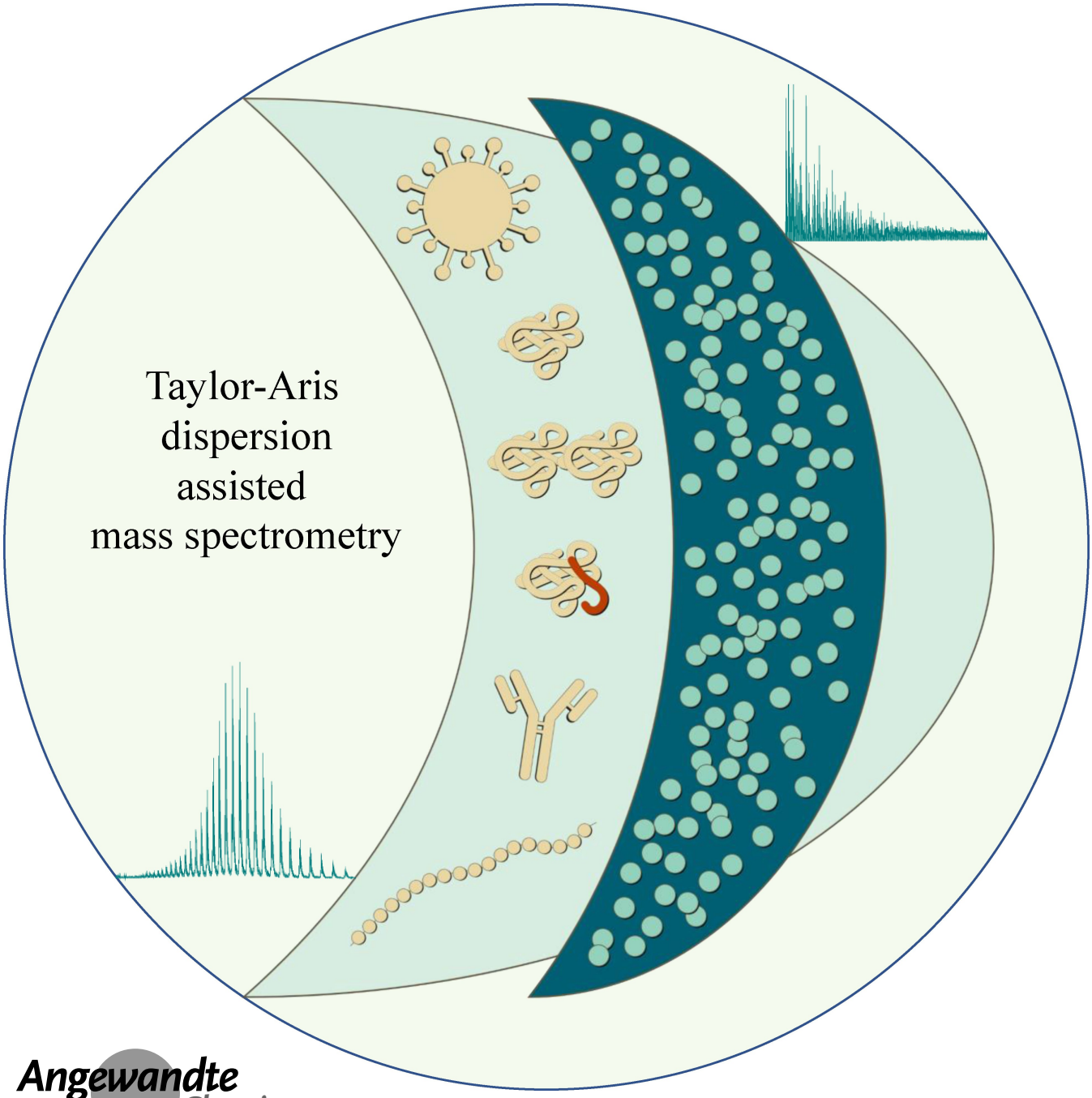
Analytical Chemistry

Direct Injection Electrospray Ionization Mass Spectrometry (ESI-MS) Analysis of Proteins with High Matrix Content:Utilizing Taylor–Aris Dispersion

*Ruben Szabo, Cynthia Nagy, and Attila Gaspar**



Abstract: This is the first work demonstrating the utility of the Taylor–Aris (TA) dispersion in avoiding serious interference issues commonly occurring in the electrospray ionization-mass spectrometric (ESI-MS) determination of therapeutic protein pharmaceuticals undergoing no pre-separation or sample purification. It was also pointed out that the TA dispersion conditions and its analytical utilization for proteomics can be easily accomplished in a commercial CE-MS instrument. In the proposed Taylor–Aris dispersion-assisted mass spectrometry (TADA-MS) analysis 0.5 μL sample is injected into a 65 cm long 50 μm i.d. capillary and pumped with 1 bar toward the MS. The procedure is efficient for the direct injection analysis of components having low diffusion coefficients (proteins) that are present in complex matrices of small organic and inorganic compounds.

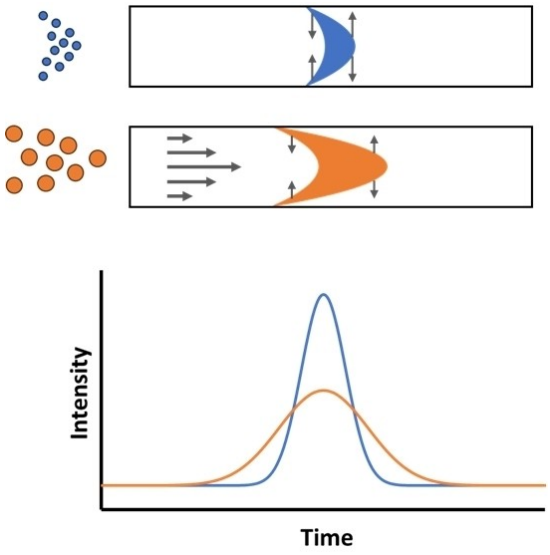


Figure 1. Schematic representation of the Taylor–Aris dispersion of molecules with various sizes.

Introduction

The components in a sample plug injected into a flowing fluid in a capillary that were initially near the center of the capillary are moving faster than the components being near the wall, therefore the components are dispersed due to the combination of the molecular diffusion and the different speeds in the cross-section of the capillary. Thus, the shape of the sample plug is determined by the combination of the parabolic velocity profile of pressure driven laminar flow (solvent, mobile phase) and the radial diffusion (convection) of the components. As a consequence of this Taylor–Aris (TA) dispersion, the molecules with low diffusion coefficients (proteins, monoclonal antibodies (mAbs)) move slowly across the parabolic velocity profile, resulting in a wider distribution of velocities, thus a larger band broadening. Since this broadening is around the population mean, the front of the band of a component with low diffusion constant is faster than the front of a component with higher diffusion constant (Figure 1). TA dispersion is significant if the residence time of the molecules in the capillary is large enough for them to cross the streamlines of different speeds along the cross section of the capillary, that is:

$$\frac{L}{u} \gg \frac{d^2}{D} \tag{1}$$

where L and d is the length and the inner diameter of the capillary, respectively; u is the average linear velocity and D is the molecular diffusion coefficient.[1,2] The TA dispersion is utilized mainly for the determination of diffusion coefficient values as Taylor dispersion analysis (TDA)[3–6] according to Eq. (2),

$$D = \frac{R^2\, t}{24\, \sigma^2} \tag{2}$$

when the axial diffusion is negligible and where R is the inner diameter of the capillary, t is the average elution time and σ is the peak variance of the component.[7,8] TA dispersion is also applicable for determining the hydrodynamic radii of molecules/particles,[9–13] separation of isotopes,[14] monitoring polymerization reactions,[15] studying ligand binding properties[16] or band broadening effects in chromatographic columns[2] and differentiating protein conformations.[17]

TA dispersion was utilized for the aforementioned goals in different flow systems: HPLC,[2,18] mass spectrometry (MS)[4,17] or capillary electrophoresis (CE)[3,5,6,9,10,12,13,15,16,19] instruments. The CE instrument seems especially advantageous for utilizing or studying TA dispersion since the movement of liquid in a long but small inner diameter capillary can be well regulated with low pressure (10–1000 mbar) and even nanoliter volume of samples can be injected.[7]

Since TA dispersion depends on the diffusion coefficients of the components, a partial separation between the components with largely different sizes (diffusion constants) can occur. In this work we demonstrated that transporting a small sample plug along a capillary, the large molecules/particles (having little D) like proteins, mAbs are partly separated from the small components in the sample and reach the MS detector in a pure, matrix-free form at the

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front of the sample plug dispersed by the TA effect. Since the MS analyses of proteins and mAbs in pharmaceutical products containing large amounts of matrix components are performed after SPE or HPLC purification/separation (otherwise the matrix components would strongly obscure the MS detection of proteins), the proposed direct injection ESI-MS analysis of proteins with high matrix content should have high importance for simple, fast and cheap MS analyses of intact proteins. In this work we propose for the first time that the utilization of TA dispersion using the flow system of a CE instrument can be an effective, routine tool for ESI-MS based intact protein analysis.

Results and Discussion

TA Dispersion of Different Components in a CE-MS Instrument

There are two conditions to that need to be satisfied to ensure the domination of the Taylor dispersion over the longitudinal dispersion: (1) the elution/residence time should be longer than the characteristic time of diffusion of the analyte across the capillary radius and (2) the peak variance caused by the parabolic velocity profile should be much larger than the dispersion caused by the longitudinal diffusion.[1] In order to ensure these conditions (Eq. 1), earlier TDA was mainly employed in long open tubular columns (around 30 m[20,21] or even 92 m[22]) or very small linear flow rate was applied (around or less than 1 mm/s[5,9]). Recently, several works appeared suggesting the use of the commercial capillary electrophoresis instruments for TDA.[3,5,6,9,16] In their study on the determination of D values, Cottet et al.[7] concluded that the optimal operating conditions are the 60 cm×50 μm i.d. capillary with 40–100 mbar pumping pressure for components of 1–100 nm size. While these conditions for the TA dispersion are easily realizable in a CE instrument, they are hard to achieve in commercial LC systems (e.g.[18,20,21,23]). The application of TDA or the study of TA dispersion using CE instruments have been carried out only with UV detection[3,5,6,7,10,11,13,15,16,19,24,25] or recently with ICP-MS,[12] but not with ESI-MS detection. However, the CE-MS hyphenation is already a stable, efficient and commercialized accomplishment and the ESI-MS is the most effective detector for investigating large biomolecules (proteins, mAbs) since the elucidation of structural information or determination of molecular mass is possible. The application of CE in a CE-MS system can conveniently provide not only the necessary conditions for the TA dispersion but the triaxial interface with the sheath liquid (G1607B, Agilent commonly used in such combined systems) ensures the required liquid flow for the optimal ESI operation, as well. It should be highlighted that the volumetric flow optimal for TA dispersion (which is typically below 1 μl/min) should be completed to around 5–10 μl/min. The sheath liquid flow with the triaxial interface can be efficiently and flexibly selected and applied for sensitive MS detection.

In our experiments we used similar parameters as were suggested by Cottet[7] but considerably higher liquid flow rate (1 bar pumping pressure instead of 0.1 bar) was applied in order to increase the detection sensitivity, shorten the analysis time and eliminate the need for sheath liquid, resulting in an even simpler system. (It should be noted that based on our experiments and the conclusions made by Cottet,[7] similar TA dispersion can be obtained as long as the operating conditions (length and i.d. of the capillary and pumping pressure) correspond to the validity of Eq. 2.) The conditions that we propose for the CE-MS utilizing TA dispersion partly satisfy Eq. 1 since L/u is only slightly (and not at least 10×) larger than d^2/D, but due to the high time-resolution of the MS detection the degree of the observed TA dispersion was adequate for our analytical purposes.

In our experiments the analyzed sample contained a protein (β-casein, D: $6.05\cdot10^{-11}$ m^2/s,[26] MW: 24 kDa) and small inorganic (KSCN, D: $1.743\cdot10^{-9}$ m^2/s)[27] and organic (histidine, D: $7.328\cdot10^{-10}$ m^2/s)[28] components. Both with UV and MS detection the same degree of TA dispersion was obtained (Figure 2A–B and Figure S1), that is the protein peak was much wider than those of the smaller components and the front and the rear parts of the protein plug included exclusively the protein, while the middle part of the sample plug included the smaller components and the rest of the protein together. In the case of UV detection the measured absorbance is the sum of the absorbances of all components present in the sample plug, therefore we measured each component separately and the signals obtained were superimposed. (This was applicable due to the good reproducibility of the precisely controllable pressure systems applied in the Agilent CE instruments (Figure S2 and Table S3)) These UV measurements have the advantage of showing KSCN directly and eliminating special effects arising during ESI process (e.g., ion suppression, different adduct ion formation). In the case of MS detection each component can be separately detected from a mixture using selective m/z values (m/z 1411.65 for β-casein $[M+17H]^{17+}$, m/z 156.08 for histidine $[M+H]^{+}$) while KSCN was detected through the formation of potassium adduct ions (i.e., at m/z 1416.23 as β-casein $[M+15H+2K]^{17+}$). Since the ESI-MS detection of proteins is largely influenced and generally suppressed by other components present in the sample, the detection sensitivity is much higher at the front and the rear part of the sample plug.

The purity of the protein at the edges of the TA dispersed peak can be illustrated by plotting the UV signal ratio of β-casein and histidine against the elution time (Figure 2C). The few second interval covering the pure and relatively high concentration of protein in the sample plug (the top third of the sharp peaks) is a proper duration for MS detection (not too short for providing enough data points for averaging mass spectra to obtain more accurate mass, and not too long that would cause long analysis time and lower sensitivity). Mass spectra from different time windows can be seen in Figure S3.

Changing the operational conditions by increasing the inner diameter of the capillary with a factor of 2 and decreasing the pumping pressure with a factor of 10, TA dispersion seemed even larger, but the analysis time was doubled and the detection sensitivity was not improved at

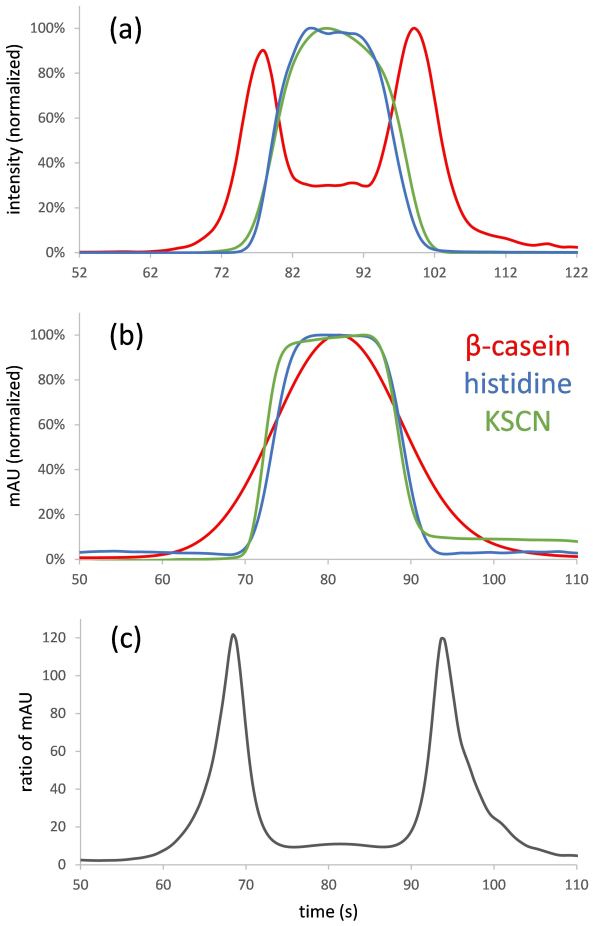

Figure 2. (a) MS and (b) UV signals for a protein and two small components and (c) UV signal ratio of a protein and a small component. The signals are normalized for their highest intensities. (a) MS signals for β-casein $[M+17H]^{17+}$ (BPC at m/z 1411.65) in red, histidine $[M+H]^{+}$ (BPC at m/z 156.08) in blue, and β-casein $[M+15H+2K]^{17+}$ (BPC at m/z 1416.23) in green. (b) UV signals recorded at 200 nm for the three components measured separately. Conditions: capillary: fused silica 50 µm i.d., 65 cm total length; injection: 1 bar×18 s; elution: 0.1% formic acid, 1 bar. Sample: 1 mg/mL β-casein, 0.1 mg/mL histidine, 0.1 mg/mL KSCN, all containing 0.1% formic acid.

the front/rear part of the sample plug (in UV detection the increased diameter results in a better sensitivity, but not in MS detection) (Figure 3). The effects of mobilizing pressure and sample injection on the dispersion of different molecules were also studied (Figure S4). Naturally, decreasing the mobilizing pressure from 100 mbar to 25 mbar resulted in the 4-fold increase in peak width if the injected sample volumes were the same. The lower flow rate of the liquid increased not only the Taylor–Aris dispersion, but the laminarity of the flow, as well. The larger laminarity (lower Reynolds number) resulted in a relatively smaller flow dispersion of the sample (even a short plato of the signal was observed for 25 mbar). Based on these conclusions and other measurements, the operational parameters used in Figure 2 are proposed.

Recently, one of the major challenges in analytical chemistry is the analysis of proteins in their native state.[29–32] The most suitable sample matrix of these studies includes phosphate buffer in NaCl/KCl at pH 7 (PBS) and no organic solvent is allowed during the analysis in order to mimic physiological conditions. These are particularly harsh requirements imposed on ESI-MS systems in general for several reasons: (i) phosphate is a non-volatile buffer component, rendering it incompatible with MS, (ii) the large concentration of NaCl and KCl cause intensive adduct formation ultimately leading to considerable signal spreading[33] and (iii) the lack of organic solvent (MeOH, ACN, IPA, etc.) leads to poor ESI efficiency. The application of truly native conditions may cause a dramatic decrease in MS detection sensitivity, often to a point where no meaningful information can be extracted from the spectra. TADA-MS, however, has the potential to offer the direct MS analysis (no sample clean-up prior to analysis) of proteins dissolved in non-MS-compatible solutions that preserve the native state of the proteins. In effect, the technique can be considered a means of on-line buffer exchange, since a part of the protein present in the original native conditions (PBS buffer) can enter an MS-compatible, "close-native" matrix (volatile NH_4Ac, pH = 7, no organic solvent), thus largely increasing sensitivity. In Figure 4 the TADA-MS analysis of β-casein in native conditions is shown. For the native MS of large proteins and protein complexes, averaging a large number of spectra is often necessary, which requires relatively long acquisition times (even minutes). Depending on the applied mobilization pressure (25–100 mbar or 1 bar is possible in Agilent CE instruments) the speed of analysis and the length of acquisition time for the target protein can be tuned (1 bar mobilization pressure provides a fast measurement (less than 2 min) and ~10 s long segment of clean MS spectrum for the protein, while a lower mobilization pressure of 25 mbar allows 1 min length of such spectrum but with 8 min analysis time). The native MS signal (Figure 4A) showed a similar pattern to the signal acquired in denaturing mode (Figure 2). The mass spectra obtained from the front and the rear parts of the TA dispersed sample plug (Figure 4B and 4D) demonstrate native characteristics, i.e., high intensity MS signals in the larger m/z range (1900–2500), where the dominant charge numbers are 10–12. However, the middle part of the sample can provide only low intensity and highly adducted signals due to the high interferences of the matrix (Figure 4C). Therefore, useful spectra can only be gathered from the TA-dispersed regions of the sample plug (Figure 4E and 4G). While the TA-dispersed front part of the sample is free from Na or K adducts, in the rear part there is more substantial adduct formation as can be seen in Figure 4E and Figure 4G, respectively (the assignments of the adducts for Figure 4E are detailed in Figure S4). Native MS measurements of protein complexes were also studied. Hemoglobin consists of two peptide chains (α and β) that can exist in apo and holo form, the latter containing the heme prosthetic group. In the native spectrum both apo and holo forms of α and β chains and the tetrameric protein complex ($\alpha_2\beta_2$) can be seen, however in denatured condition only the apo form could be well observed (Figure S6).

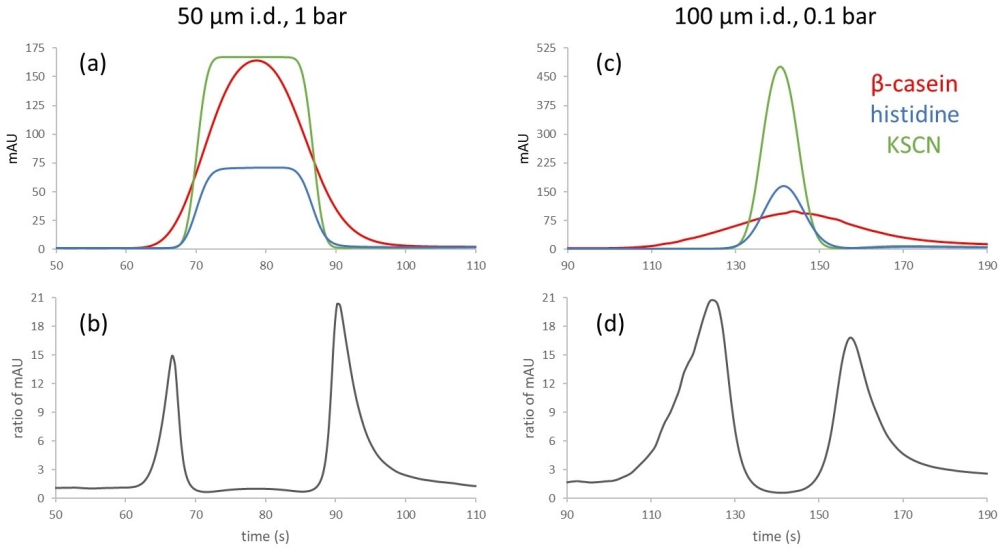

Figure 3. UV signals of a protein and 2 small components using a) 50 µm with 1 bar and c) 100 µm with 0.1 bar as capillary i.d. and pumping pressure, respectively. UV signal ratio of a protein and a small component (KSCN) using b) 50 µm with 1 bar and d) 100 µm with 0.1 bar as capillary i.d. and pumping pressure, respectively. Conditions: UV signals separately recorded at 200 nm for the β-casein, histidine and KSCN; capillary: fused silica 65 cm total length; injection: 1 bar×18 s for 50 µm capillary (0.5 µL, 26 cm sample plug) and 0.1 bar×12 s for 100 µm capillary (0.5 µL, 7 cm sample plug). Samples: 1 mg/mL β-casein (in red), 1 mg/mL histidine (in blue), 1 mg/mL KSCN (in green).

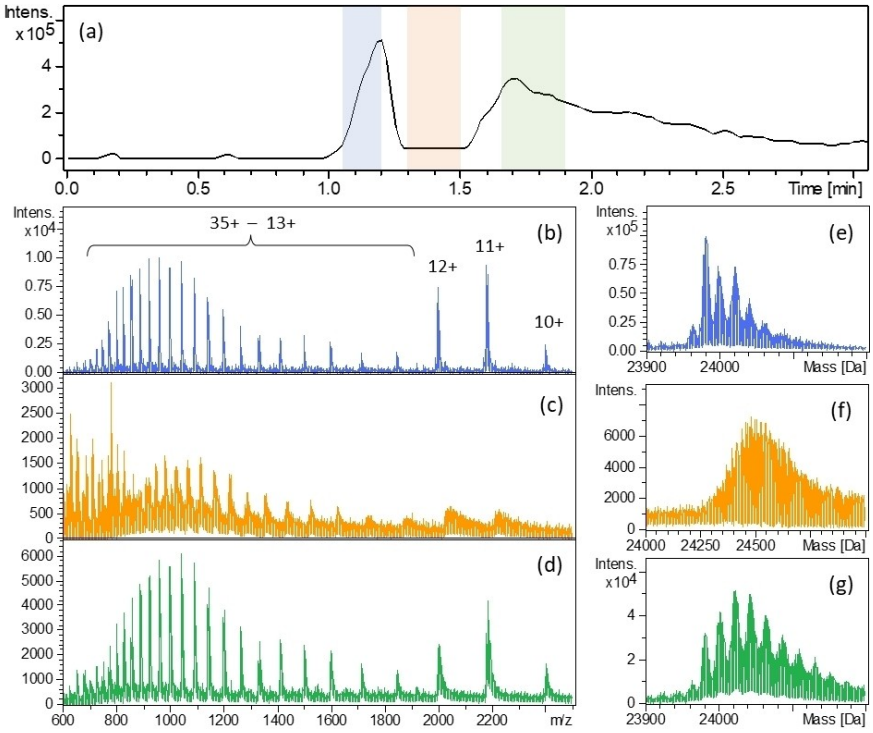

Figure 4. TADA-MS analysis of β-casein in native conditions. a) MS signal for the +11 charge of β-casein (EIC at m/z 2180–2190). Mass spectra from different time intervals: b) 1.05–1.2 min, c) 1.3–1.5 min, d) 1.65–1.9 min, and the corresponding deconvoluted spectra (e, f, g). Sample: 5 mg/mL β-casein in PBS (10× diluted). Conditions: capillary: fused silica 50 µm i.d., 65 cm total length; injection: 1 bar×18 s; elution: 40 mM ammonium acetate, 1 bar.

Analysis of mAb in a High Matrix Content Pharmaceutical Product

The therapeutic protein (often mAbs) pharmaceuticals contain large amount of matrix material (e.g., buffers (10–50 mM citrate, histidine, acetate, phosphate, etc.), salts (50–200 mM NaCl or KCl), detergents (0.0003–0.3 % polysorbates), polyols, sugars, amino acids, or amines) in a relatively high concentrations in order to stabilize and keep proteins in their active form.[34] The proteins and mAbs should not be isolated from these components before their analysis to avoid deformation, aggregation, precipitation. Therefore, these components are (at least partly) separated only minutes before their detection in a pure form. This means that the intact mass determination of mAbs under denaturing conditions typically requires an on-line chromatographic (HPLC) separation before MS detection. The importance of the proposed TADA-MS method lies in its ability to largely simplify and accelerate the standard protocol for such determinations.

In our experiments the partial separation of the proteins from the matrix was performed without a separation technique (chromatography or electrophoresis) but with the utilization of TA dispersion. Since there are very large differences between mAbs and the relatively small matrix ingredients of the pharmaceutical product samples regarding their size (and thus D values), considerable TA dispersion could be expected using the proposed operational conditions in the CE-MS system. Furthermore, the adverse interferences of matrix compounds during ESI are much larger for high molecular mass components, therefore the proposed method can be especially beneficial in the case of large proteins such as mAbs. We used 10 mg/mL rituximab for our study simply because that is its concentration in the pharmaceutical product, which also meant there was no dilution of the matrix components either, prior to the analysis. (Recent developments and optimization in upstream processing provide mAb concentration in 1–20 mg/mL range.[35]) This high concentration of rituximab resulted in proper, clear MS spectra even with the accompanying high matrix content. In order to achieve better signal-to-noise ratio, the acquisition time should be extended. By applying a smaller mobilization pressure, the frontal part of the TA-dispersed zone can be stretched (clean mass spectrum). This can be required for mAbs or native protein measurements where a high detection sensitivity is desired.

The intensity profile in time (Figure 5A) of the small sample plug of MabThera® containing rituximab (composition details in Table S1) was similar as it was obtained for the mixture of protein and two small additives shown in Figure 2A. The ESI-MS signal was much higher in the front part of the sample plug than in its middle section (the sensitivity difference is hard to express with a factor value because peaks corresponding to the mAb were not observed at all in the center of the sample plug). The mass spectra obtained in the front part of the sample provide clear and well-evaluable spectra for the mAb suggesting that the mAb is present in a pure form in that part of the sample plug (Figure 5B). However, in the middle part of the sample plug strong interferences (ion suppression, formation of many different adduct ions) of the sample matrix (salts, buffer compounds, detergents) occurs (Figure 5C). This intensity

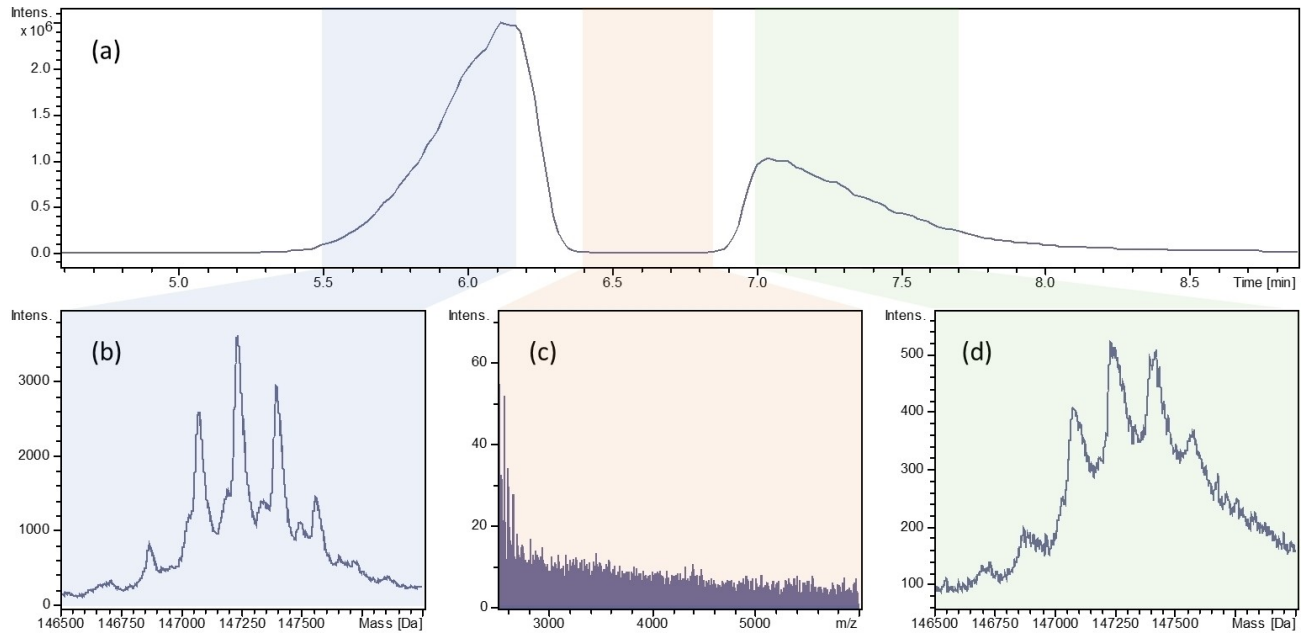

Figure 5. ESI-MS determination of direct injected pharmaceutical product containing monoclonal antibody: a) extracted ion chromatogram (EIC) recorded in 3000–5000 m/z range, b) deconvoluted spectrum from 5.5–6.15 min time range, c) average mass spectrum from 6.4–6.85 min time range, d) deconvoluted spectrum from 7–7.7 min time range. Conditions: sample: MabThera® (containing Rituximab); capillary: fused silica 50 µm i.d., 65 cm length; injection: 100 mbar×25 s; elution: 0.1 M formic acid, 100 mbar, sheath liquid: IPA:water = 1 : 1 containing 0.1 % acetic acid, 7 µl/min.

profile clearly suggests that there is no need for a larger sample volume for the analysis, because the increase of the sample volume would increase only the width of the middle part of the peak having minimal signal intensity thus it is useless for the analysis. Since the volume of the sample is minimal (only around 70 nl) the non-MS compatible content of the matrix does not cause considerable contamination for the subsequent MS measurements. In the rear part of the sample plug the mAb can be sensitively detected as well, but the signal intensity is smaller due to the presumably smaller purity level of the mAb in the rear part of the sample plug (compared to that of the front part, potentially caused by the tailing of certain matrix compounds (e.g. Tween 80)) (Figure 5D).

The analysis of mAbs under denaturing conditions provided sensitive determination of the molecular mass of intact mAbs with discernible glycosylation pattern. The MS spectrum obtained from the front (Figure 5B) or the rear part (Figure 5B) of the sample is able to provide useful information about the mAb target molecule such as intact molecular mass, glycosylation pattern, and drug-to-antibody ratio in the case of antibody-drug conjugates (the three main peaks in Figure 5B can be observed at 147076.7 Da, 147238.2 Da and 147399.8 Da corresponding to the G0F/G0F, G0F/G1F, G1F/G1F or G0F/GF2 glycoforms, respectively).

Conclusion

In this work, we demonstrated for the first time that the TA dispersion can be utilized for avoiding the serious interference issues commonly occurring during the ESI-MS determination of therapeutic protein pharmaceuticals (i.e. samples that include proteins or mAbs together with a large amount of matrix materials) without resorting to any preseparation or sample purification techniques. The proposed method is only applicable for the analysis of large components (especially for very large mAbs) since their diffusion constants are very small compared to those of the other (small) components in the sample.

We also firstly pointed out that the TA dispersion conditions and its analytical utilization can be easily accomplished in a commercial CE-MS instrumentation. Although the application of high voltage (key element in the CE systems) is disenabled in the TA dispersion-assisted MS system, there are several advantageous facilities of the CE instrument constructions: (1) reproducible injection of submicroliter volume of samples into a small inner diameter fused silica capillary, (2) well-controlled, stable pressure system to transport the liquid/sample either with low (10–1000 mbar) or higher (1–10 bar) pressure, (3) totally automable injection for series of samples and mobilizing liquids from vials and (4) the sheath liquid of the triaxial interface that ensures the required liquid flow for optimal ESI operation.

Instead of the commonly applied HPLC-MS instrumentation, we suggest the use of the much simpler and cheaper TADA-MS analysis, which is a kind of direct injection determination method for proteins which are present in complex matrices. TADA-MS can be considered as a form of direct injection, however, it only serves as a means of separating the proteins of interest from interfering matrix components and is not suitable for the separation of different proteins in a complex protein mixture. Nevertheless, the approach is applicable for the determination of a single protein (or large component) present in a complex, ESI-MS incompatible matrix including even a large number of lower MW components. This is typically the case for pharmaceutical products (mAbs, therapeutic proteins). The TADA-MS approach could also be essential for native protein analysis since the proteins can be transported to native conditions just a few seconds before their MS detection so problems regarding their aggregation/solubilization can be avoided (liquid providing native conditions is transported before and after the sample plug).

Supporting Information

Supporting Information for this article is given via the following link.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Taylor–Aris dispersion · proteomics · monoclonal antibody · mass spectrometry · capillary electrophoresis

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