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Liquid-phase and gas-phase investigation of biomolecules in a single experiment†

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Interrogating three-dimensional structures of biomolecules is instrumental to the fields of biochemistry and biophysics. Mass spectrometry is a technique that can provide important data on the molecular weight of protein molecules in the gas phase. However, the relationship between the structure of biomolecules in the native water-rich environment and the artificial gas-phase environment cannot always be established with high certainty. Here we communicate initial results obtained using a simple analytical approach which allows one to perform measurements of structural parameters of biomolecules in liquid phase and gas phase within a single experiment. Single-component samples are injected as plugs to a microscale capillary filled with aqueous medium and driven hydrodynamically. As they traverse the capillary, they undergo zone broadening. The extent of this broadening is measured at two points along the capillary by UV absorption detection, and used to compute hydrodynamic radii according to the Taylor dispersion analysis theory. Further downstream, the dispersed zones leave the capillary through the sharpened outlet aligned to the orifice of a mass spectrometer. This multiple detection strategy allows one to confront protein conformation in the liquid phase (deduced from the hydrodynamic size) and the gas phase (deduced from mass spectral patterns).

Introduction

Water is the most wide-spread solvent of biomolecules occurring in nature.[1] Through billions of years, biomolecules evolved their native functions being surrounded by the omnipresent molecules of water. However, water is not always the most desirable matrix for the studies of molecular structure and function. For example, analytical techniques such as crystallography[2] or mass spectrometry (MS)[3] require the samples to be delivered in solid or gaseous form, respectively. Due to their structural, energetic, and biocatalytic functions, proteins are essential to living organisms. MS is a powerful and versatile analytical tool with high specificity and sensitivity, and it has found applications in the study of protein structures.[3] For example, electrospray ionization (ESI)-MS,[4] nanoflow electrospray ionization (nanoESI)-MS,[5] and matrix-assisted laser desorption/ionization (MALDI)-MS[6] have been used to analyze proteins, and even study changes to their structures, or interactions with other biomolecules. Even though conformations of proteins can be affected by external factors such as solvent, temperature or pressure, mass spectra can provide a wealth of information on the structure of macromolecules. Results of such analyses are interpreted based on the assumption that the gas-phase conformations/functions of biomolecules reflect the native ones which exist in aqueous environments.[7,8] This assumption needs to be verified upon case-by-case basis limiting the usability of these powerful analytical platforms in bioscience.

Taylor dispersion analysis (TDA) is a simple method that enables measurements of diffusion coefficients and hydrodynamic radii of molecules driven by laminar flow.[9–14] TDA was already used with MS to measure diffusion coefficients of analytes.[15] However, TDA is often used in conjunction with UV absorption detection, in particular with multi-point detection systems, which are currently available commercially. Notably, excellent compatibility of TDA with UV imaging detection has been demonstrated.[16–18]

Here we introduce a simple approach for investigating conformations of biomolecules in liquid and gas phase almost simultaneously. The method is based on the hyphenation of capillary-flow TDA – using UV imaging detection at two detection windows on one capillary – with atmospheric pressure ionization mass spectrometry (Fig. 1). This simple setup enables measurement of hydrodynamic sizes of molecules by TDA performed right before the detection by mass spectrometry.

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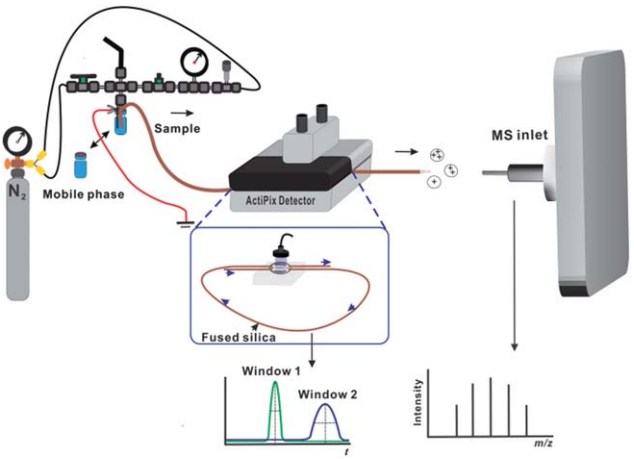

Fig. 1 Schematic diagram of the system for TDA combined with quadrupole time-of-flight (Q-TOF) mass spectrometry. The sample was injected hydrodynamically by pressurizing gas in the headspace of the sample vial, while the applied pressure was controlled with a digital manometer (left). Following the injection, the system was vented, and the vial with a mobile phase was mounted in the inlet. A UV imaging detector was used to record the separated zones traversing two detection windows along the fused-silica capillary column. The outlet of the capillary was set in front of the orifice of the mass spectrometer in order to enable transfer of molecules into gas phase and their ionization in a manner similar to nanoESI.

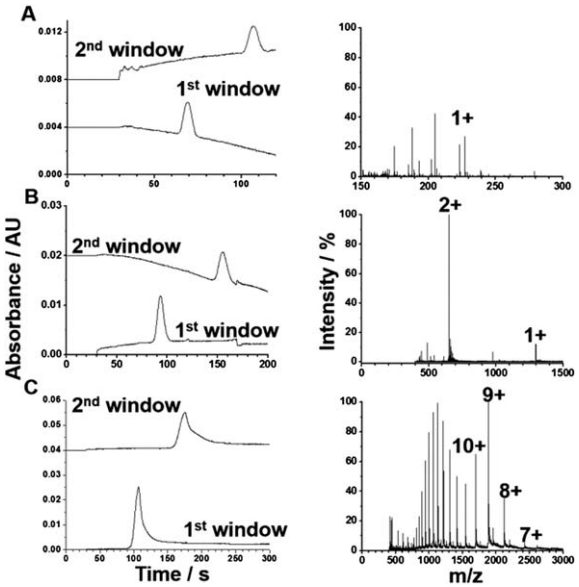

Fig. 2 TDA result plots and mass spectra obtained using the system shown in Fig. 1 after the optimization of relevant parameters: (A) tryptophan, (B) angiotensin II, and (C) myoglobin. See the ESI† file for experimental details. Running buffer (20 mM ammonium acetate at pH 7.4) was pumped by applying a pressure of ~100 kPa (slight differences in migration times are due to the inability to control the pressure precisely).

Results and discussion

In the proposed TDA-MS setup, setting up the ion emitter was straightforward since – unlike in conventional nanoESI – there was no need for establishing a direct electric contact with ground or potential bias.[19,20] Table S1† summarizes the main experimental parameters used in this study. As the nL volume sample plug traverses the capillary, it is recorded three times: first, in two windows of a UV imaging detector, and, second, using a quadrupole time-of-flight (Q-TOF) mass spectrometer with an ion source operating at atmospheric pressure (Fig. 1). Please note that this setup and the data treatment method described below are different from the ones implemented in a previous study,[15] in which electrospray ionization mass spectrometry was used to monitor Taylor dispersion of an analyte. In the current study, UV absorption signals – representing analyte peaks at two detection windows – are used to compute the hydrodynamic sizes (and/or diffusion coefficients), while the MS signal is used to measure molecular weight and draw conclusions on the conformations of biomolecules in the gas phase.

Combined TDA and MS measurements

Performance of the proposed TDA-MS system was first examined using test samples containing analytes with different molecular weights, including an amino acid (tryptophan, 10^{-5} M), a peptide (angiotensin II, 10^{-5} M), and a protein (myoglobin, 10^{-4} M). Fig. 2 shows the TDA results (left) as well as mass spectra (right) of tryptophan, angiotensin II, and myoglobin, respectively. The data describing the peaks of the analyte zones traversing the two UV absorbance detection windows (t_1 and t_2, σ_1 and σ_2) were used in the determination of hydrodynamic radii and diffusion coefficients. The parameters of the peaks recorded in the first and the second detection window of the UV imaging detector were obtained by fitting curves (*e.g.* Haarhoff–Van der Linde) to the raw data, and inputted to the following equation:[21]

$$R_h = \frac{4k_B \times T(\sigma_2^2 - \sigma_1^2)}{\pi\eta r^2(t_2 - t_1)} \quad (1)$$

This way, the hydrodynamic radii of tryptophan, angiotensin II, and myoglobin were estimated to be ~0.40, ~0.48 and ~2.1 nm, respectively. In eqn (1), the parameter k_B is the Boltzmann constant (1.38×10^{-23} Pa m^3 K^{-1}), T is the absolute temperature (~293 K), η is the viscosity of medium (in this case, water; 0.0010 Pa s) while r is the radius of the capillary used (25 μm). It was pleasing to note that the measured hydrodynamic radii of the test analytes are similar to the values reported previously (tryptophan: 0.32 nm,[22] angiotensin II: <1 nm,[23] myoglobin: ~2.1 nm.[24] However, it should be noted that the peak of myoglobin exhibited strong tailing. Therefore, deconvolution had to be conducted by manual fitting. Incorrect fitting led to erratic R_h values (Fig. S1†). The obtained diffusion coefficients of tryptophan, angiotensin II, and myoglobin were 5.4×10^{-10} m^2 s^{-1}, 4.5×10^{-10} m^2 s^{-1}, and 1.0×10^{-10} m^2 s^{-1}, respectively. As expected, the diffusion coefficient of myoglobin is in the order of the values reported for proteins.[25]

After passing through the two UV detection windows of the TDA system, the eluting analytes could also be detected using the Q-TOF mass spectrometer. One should note that the mass spectra obtained with this setup (Fig. 2) look similar to conventional ESI mass spectra (*cf.* ref. 4) because multiply charged ions derived from peptides and proteins can be

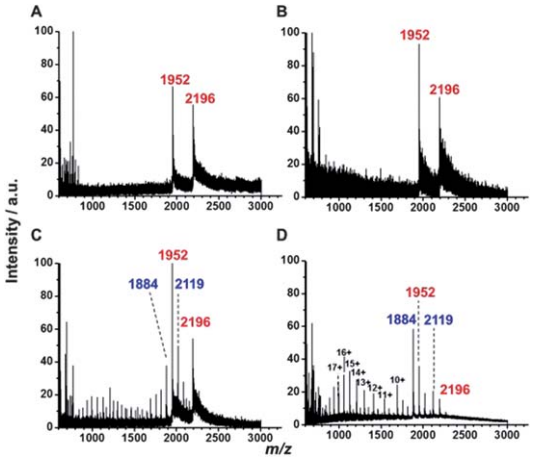

Fig. 3 TDA-mass spectra of myoglobin (10^{-4} M) obtained at different dry gas temperatures: (A) 160 °C, (B) 180 °C, (C) 220 °C, and (D) 260 °C. See the ESI† file for experimental details. The running buffer (20 mM ammonium acetate at pH 7.4) was pumped by applying a pressure of ~100 kPa. The peak labels highlighted with blue colour font correspond to unfolded myoglobin, while the peak labels highlighted with red colour font correspond to folded myoglobin.

observed. Singly charged ions were recorded for tryptophan ($[M + H]^+$ at the m/z 205; Fig. 2A) while multiply charged ions were observed when larger molecules, such as peptides (Fig. 2B) and proteins (Fig. 2C), were used as test analytes. Thus, the molecular weights of the analytes traversing the TDA column could be confirmed using MS. These results demonstrate that the on-line coupling of TDA and MS is useful for obtaining the information of hydrodynamic radii and molecular weights of analytes within a single run.

Influence of system parameters on the mass spectra

In the following experiments we tested the influence of various parameters on the assayed form of the analyzed protein in the gas phase. We hypothesized that the hydrodynamic pressure applied in TDA may affect the determination of hydrodynamic radii of the target analytes. Thus, TDA was further performed using tryptophan, platinum nanocluster, myoglobin, and ovalbumin as test samples and with different hydrodynamic pressures applied to the inlet vial (~7–70 kPa). As the hydrodynamic pressure increased, the apparent hydrodynamic radii of proteins (myoglobin and ovalbumin) became smaller (Fig. S2†). On the other hand, the hydrodynamic radii of tryptophan and platinum nanoclusters (1 mg mL^{-1}) were unaffected by the change of hydrodynamic pressure. It is known that hydrodynamic pressure may have a significant impact on protein structures.[26–30] Protein unfolding (denatured) occurs when exposed to high pressure. Various analytical methods have been used to study the structure of proteins when exposed to high hydrodynamic pressures. These methods include dynamic light scattering (DLS),[31] infrared spectroscopy (IR),[32] rapid mixing methods,[33] Taylor dispersion analysis (TDA),[12] and high-pressure NMR spectroscopy.[34] While the influence of pressure on protein structure was apparent at higher pressures (~700 kPa), here we observe a change of R_h when applying relatively low pressures (~7 kPa). We deduce the cause of the variation of apparent hydrodynamic radii of proteins in the current study is different from the ones discussed in the previous studies which tested the influence of very high pressures (≥200 MPa).[35,36] These reasons for elevated hydrodynamic radius at low pressures (Fig. S2†) can be shear stress induced by the laminar flow in the capillary column, adsorption on the capillary wall, and possible curve fitting bias. Further discussion on the influence of hydrodynamic pressure and capillary geometry on the folding state is presented in the ESI† file.

It is generally known that the temperature of dry gas (N_2) released through the MS inlet may also affect protein conformation in the course of desolvation when using conventional ESI sources. Since the ionization method used in this study is not exactly the same as conventional ESI or nanoESI, but more similar to contactless atmospheric pressure ionization (C-API),[20] it was appealing to examine the effects of dry gas temperature. Myoglobin was used as the test sample in this experiment since the molecular weights of the folded form (M_w = 17 566 Da) and the unfolded form (M_w = 16 949 Da) of myoglobin are different: this is because heme molecules are only retained in folded myoglobin.[37] Thus, according to the m/z distribution of peaks corresponding to the multiply charged ions of myoglobin in mass spectra, the information about the folding state of myoglobin molecules can be obtained. Fig. 3 shows the mass spectra of myoglobin obtained at different dry gas temperatures (160, 180, 220, and 260 °C). The peaks at m/z 1952 (M^{9+}) and 2196 (M^{8+}) – observed at lower temperatures – correspond to multiply charged ions of folded myoglobin (Fig. 3A and B). However, at higher temperatures of dry gas, the spectra reveal the peaks at m/z 1884 (M^{9+}) and 2119 (M^{8+}), which correspond to the multiply charged ions of unfolded myoglobin (Fig. 3C and D). Thus, the structure of protein in the gas phase is influenced by the temperature of the dry gas. If the information about the folding state of proteins is expected to be obtained, the use of a lower temperature of the dry gas (*e.g.* 180 °C) is recommended.

The above results suggest that the current approach can be used to study protein conformation by obtaining hydrodynamic sizes and mass spectral profiles in one run. They also show that the aspect ratio of capillary channels used for transferring protein samples, and as ion source emitters, as well as dry gas temperature, may influence the mass spectral patterns, and even give rise to system-related artefacts.

Conclusions

In summary, we have proposed a fast, easy, and inexpensive approach to study biomolecules in the liquid phase and in the gas phase. Both datasets provide orthogonal and complementary information for the studies in protein confirmations. This approach provides various benefits: two pieces of information (hydrodynamic size and molecular weight) are obtained in one run of one analytical instrument, saving time and workload. The time gap between the measurement of hydrodynamic size and the measurement of molecular weight is decreased down to seconds, which may be important when studying some of the dynamic biomolecular systems. The total sample volume used

in analysis is very small (nanoliters). Coupling TDA to MS is straightforward, and it does not require utilization of additional accessories. Since this simple hyphenated system provides the values of hydrodynamic radii along with mass spectra, it may further be used to study the stability of folded structures of native and modified proteins. Furthermore, this approach can potentially be used while investigating non-covalent interactions between small molecules and their target proteins in liquid and gas phase, which would be useful in the study of therapeutic proteins with potential applications in biomedicine. To this end, there is a need for further validation studies involving the described hyphenation of TDA with MS. Testing many other kinds of biomolecules (amino acids and peptides) using the developed TDA-MS platform will certainly extend the practicality of this approach. It would also be appealing to couple the TDA-MS system with upstream separation techniques (*e.g.* nanoflow liquid chromatography and capillary electrophoresis) to enable direct analysis of mixtures.

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