MASS SPECTROMETRY OF MACROMOLECULAR ASSEMBLIES: PRESERVATION AND DISSOCIATION

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SUMMARY

Mass spectrometry not only plays a crucial role in the identification of proteins involved in the intricate interaction-networks of the cell, but is also increasingly involved in the characterization of the noncovalent complexes formed between interacting partners. Here we describe recent investigations in the study of the structure, dynamics and function of macromolecular assemblies by means of mass spectrometry. We detail developments that enable gas-phase dissociation to probe oligomeric organization and topology, and demonstrate how increased understanding of the electrospray process is leading to knowledge of the structure of protein assemblies both in solution and in the gas phase.

INTRODUCTION

With large-scale proteomics initiatives well underway, maps of human protein-protein interaction networks are beginning to emerge [1,2]. Mass spectrometry (MS) already plays a vital role in this endeavour, identifying the proteins involved, their expression levels, modifications, and contacts [3]. MS is also playing an increasingly prominent role in the study of the structure and behaviour of the macromolecular assemblies formed by the multitude of gene-products in cells. The vast majority of these MS investigations involve the preservation of noncovalent complexes intact in the gas-phase and employ electrospray ionization (ES), often in a miniaturized form such as nanoflow ES (nanoES). Accordingly, we will focus our review on this approach.

Since the last article on this topic in this journal [4], numerous reviews have been published [5-13]. These have focused on the application of MS to: the identification of interaction partners, and structural characterization [5,11]; the study of protein-ligand interactions, including small molecules, lipids, and nucleic acids [9]; the quantitative determination of non-covalent interaction strengths [13]; the analysis of intact viruses [10], and other homogenous and heterogeneous molecular systems [8]. Other reviews with a more extensive remit provide a detailed overview of this field [6,7,12]. A welcome addition to the literature is a comprehensive book which deals with not only the MS of macromolecular assemblies, but rather encompasses the whole field of MS applied to the study of conformation and dynamics of biomolecules [14]. Herein we describe recent advances in the ES-MS of macromolecular assemblies which illustrate the unique insight that can be gained into the structural organization of these assemblies. In the final section of this article we focus on how these species can be disassembled in the gas phase, and how this process can be used to garner additional information on the composition of heterogeneous macromolecular assemblies.

RECENT TECHNICAL DEVELOPMENTS: ‘The New Generation’

ES has been the ionization method of choice for the majority of macromolecular assemblies studied to date. Understanding and optimizing this process, in which highly charged droplets containing protein complexes are transformed into gas phase ions, is therefore central to the success of all investigations. In recent years several approaches have been proposed as means of reducing the extent of charging of the protein complex ions formed during electrospray with the overall goal of resembling more closely the charge on the complex in solution. Furthermore, such decharging also causes overlapping signal to be spread over a wider mass to charge (m/z) range, thereby facilitating the detection of individual components within a heterogeneous mixture. One such example dubbed ‘electrosonic spray ionization’ a novel variant of a low flow rate ES, uses a supersonic nebulizing gas [15]. Using this ionization method, narrower peaks in the mass spectrum were obtained than when using standard nanoES and charge states observed are generally lower suggesting more ‘native-like’ conformations are formed [15,16]. A similar study, which employed a different experimental set-up, showed comparable results, as well as an improved tolerance to preserving noncovalent interactions [17]. An alternative approach to charge-reduction involves the addition of solution additives with very high gas-phase basicity. In the presence of a proton sponge (1,5,7,-triazabicyclo(4.4.0)dec-5-ene) at pH7, the modal charge state of lysozyme dropped from 8+ to 3+ [18]. With a similar goal in mind the use of ionized air produced by a corona discharge [19], or an α -particle source such as ^{210}Po were shown to enable tunable charge reduction [20,21]. The usefulness of these approaches was demonstrated through their application to a fraction of whole cell lysate. An ES spectrum recorded without the aid of charge reduction was largely un-interpretable, but with charge reduction several different components could be identified [22]. These methods enable

disentanglement of overlapping peaks and may also prove invaluable for the study of heterogeneous macromolecular assemblies by MS.

Charge reduction of multiply charged ions is also employed in a gas-phase electrophoretic mobility molecular analysis (GEMMA). In this case multiply charged ions produced by ES are transformed into singly charged or neutral particles that are subsequently separated according to their electrophoretic mobility as they pass through a differential mobility analyzer [23]. Using this approach the diameter of the intact 150S human rhinovirus particle has been measured in the gas phase as $29.8 \pm 0.3\text{nm}$ [23], and that of the *Methanosarcina thermophila* 20S proteasome as 15.1nm [24]. Both of these dimensions are in good agreement with those obtained by electron microscopy or X-ray crystallography, suggesting their gas-phase structures are similar to those in solution. On a smaller scale a hybrid mass spectrometer which allows novel ion mobility separation in a drift tube with concurrent ES MS was employed to investigate non covalent protein complexes [25]. Gas-phase conformations of the 11-membered ring formed by *trp* RNA-binding attenuation protein (TRAP) were deduced from collision cross sections measured using this experimental setup. It was found that higher charge states in the ES spectrum corresponded to somewhat collapsed structures, whereas the lower charge states matched very well with the ‘native’ topology predicted by the X-ray structure [26]. Moreover in the presence of a specific RNA molecule that binds to the perimeter of the protein complex, collapse of the ring structure was prevented. This provides conclusive evidence that protein quaternary structure can be preserved *in vacuo*, and opens a new door in the study of the structure of heterogeneous macromolecular assemblies.

Advances have also been made in understanding the relationship between the structure of proteins and their charge states observed in electrospray mass spectra. The square-root relationship between

the charge state and mass of the protein [27] has been shown to extend to noncovalent complexes up to and larger than 1MDa [12], and two recent studies have correlated the charge state with the surface area of the proteins [28,29], but reach differing conclusions. The first proposed a linear relationship [28], and the second suggests the properties are related through a power function [29]. This difference appears to arise from the methods used in the estimation of surface area (Fig. 1). The values calculated directly from the relevant X-ray structures provide a relationship which may allow the estimation of the *solution-phase* surface area. The other study extrapolated the surface area from the mass, assuming a constant protein density and smooth spherical shape [28]. The linear relationship between this ‘spherical’ surface area and charge appears to hold true even for noncovalent complexes (Fig. 1), implying that they have a similar density to the individual proteins, and therefore perhaps suggesting that they are, on average, somewhat collapsed in the gas-phase. While more investigations are needed to define fully the relationship between structure and charge, greater insight may lead to be a simple and rapid assessment of the gas and solution phase structures of macromolecular assemblies.

RECENT MS STUDIES: ‘Sticking Together’

It is established that MS is an accurate way of determining the stoichiometries and interactions of macromolecular assemblies. Two major advantages of this approach over traditional biophysical techniques are the ability to determine mass directly, and the facility to detect different species within the same solution, even when their masses are very similar. For example, MS showed that a small heat shock protein (Acr1/HSP16.3 from *M. tuberculosis*) forms dodecamers, despite the fact that it had previously been reported to be nonameric. This observation catalyzed the determination of new electron microscopy reconstruction with tetrahedral symmetry [30]. TRAP, previously thought to be exclusively undecameric, was shown to exist concurrently in a dodecameric form, and in the presence of the amino acid tryptophan, as a double-ringed 24mer [31]. Similarly the established pentameric and decameric forms of serum amyloid P component were shown to assemble into 20mers and 30mers in the presence of dAMP [32]. Moreover, a dynamic interaction with the related C-reactive protein to form mixed decamers was observed [32]. This latter study highlights one of the main strengths of this approach, namely the ability to follow the formation of mixed complexes, directly, in solution.

A recent study which exploited this advantage investigated the dynamics of the tetrameric protein transthyretin by monitoring the subunit exchange of the wild-type and an aggressively amyloidogenic variant. Detailed kinetic modeling allowed the authors to show that the exchange process was not only faster in the case of the variant, but also proceeded via a different exchange pathway [33]. Transient species were also probed in two studies into the interactions within haemoglobin. An investigation of the pH-induced denaturation of the protein under equilibrium conditions demonstrated the differential haem-binding behaviour of the different globin chains [34].

Subsequently, the kinetics of haemoglobin denaturation were monitored using electrospray and a rapid online mixing device [35]. A simple capillary mixer [36] was used, allowing measurement as early as 9ms after initiating unfolding. Together these studies demonstrate not only how changes in stoichiometry of heterogeneous systems can be followed, but also how the kinetics of their solution-phase disassembly and assembly can be monitored.

Of particular interest in the study of transient species is the use of a ES probes modified to allow the study of reversible temperature-induced changes in macromolecular organization [37,38] In the case of two different small heat shock proteins [37,39] dodecamers were found to dissociate into dimers and monomers, thereby suggesting a mode of action for the chaperone-like function of these proteins [37,39]. The best-characterized of the molecular chaperones, GroEL, was the focus of an elegant study which investigated the assisted refolding of the bacteriophage capsid protein pg23 [40]. The authors showed that the chaperonin complex of the tetradecameric GroEL with its heptameric co-chaperone gp31 could be detected in the gas phase. Moreover, the stoichiometry of substrate binding, the refolding reaction, and the co-chaperone requirements were monitored using this nanoES approach (Fig. 2) [40].

The examples outlined above highlight many of the methodologies taken in solution to bring about change, subsequently monitored by ES MS. Since solutions of protein complexes are examined from near-native conditions to determine stoichiometries of binding and interaction, or under the influence of disruptive factors such as temperature, e.g. [37,39]; pH changes, e.g. [34,35]; organic solvents, e.g. [41]; or concentration changes, e.g. [39]. These approaches are therefore capable of detecting stable and transient species, under both equilibrium and kinetic conditions.

RECENT CID-MS STUDIES: ‘*Drifting Apart*’

While the majority of MS investigations have concentrated on maintaining interactions there is a rapidly growing area of research focused on the structural information obtainable through the dissociation of large macromolecular complexes in the gas phase. The majority of these investigations involve collision-induced dissociation (CID), where ions are accelerated into inert collision gas, and the dissociation products analyzed. These experiments are typically conducted in the front stages of the mass spectrometer (known as ‘in source CID’) or in a specifically-designed collision cell. In the latter approach, ions can, if desired, be pre-selected by m/z for dissociation, thereby enabling the fragmentation of specific components of a mixture (known as ‘tandem MS’ or ‘MS/MS’).

An example of CID applied to a typical large macromolecular complex is shown in figure 3. *MjHSP16.5* is a protein which exists exclusively as a 24mer of 395kDa. The isolation of one charge state of the 24mer and its CID is shown in figure 3A. Collisional activation of this complex results in the removal of up to three monomers from the 24mers, in a sequential manner. The pathway of this dissociation process appears to consist of the ejection of unfolded monomers [42,43], which carry away a large amount of charge. The consequence of this removal of charge results in the formation of low charge-density ‘stripped oligomers’ (Fig. 3B), and as such they appear at higher m/z values than the parent oligomers. The removal of each successive monomer results in the signal being distributed over a wider m/z range, and as a consequence, results in a greater separation between adjacent charge states (Fig. 3C). CID can therefore function as another method for achieving charge reduction analogous to the electrospray approaches detailed above.

This strategy has been used to disentangle the complicated overlap of peaks observed in the mass spectra of the polydisperse α -crystallins. By removal of two monomers, peaks become sufficiently separated such that the relative populations of the different components which make up the polydisperse assembly can be quantified [44]. Further tandem MS studies of the α -crystallins has revealed how the quaternary assembly of these polydisperse proteins is affected by phosphorylation [45] and truncation [46]. Phosphorylation, specifically at *Ser*-45, was found to cause α B-crystallin to lose its preference for adopting oligomers comprised of an even number of subunits, through a disruption of its dimeric interfaces [45]. Truncation of five C-terminal residues of α A-crystallin was found to have the opposite effect. Furthermore, by coupling this CID approach with a heating device [37] and an online reaction monitoring technique [47], a reduction in the subunit exchange rate was observed and could be attributed specifically to this truncation [46].

Membrane proteins are notoriously challenging targets for structural biologists. MS is no exception since a prerequisite for the ES approach is solubility of the protein complex in a compatible solvent. The transfer of *E. coli* multidrug resistance protein E (EmrE) with the drug tetraphenyl phosphonium bound between protein molecules, and solubilized in detergent, into the gas phase as a protein-drug-micelle ternary complex was demonstrated. The heterogeneity of binding of small molecules could be ameliorated using a tandem MS approach which involves the transfer of protein-containing micelles into the gas phase and their subsequent dissociation [48]. The study showed release of the drug implying that the functional dimeric species of EmrE is preserved in gas-phase micelles. Alternatively by using the minimum amount of detergent required to solubilize membrane-bound microsomal glutathione S-transferase 1, peaks corresponding to intact trimeric form of this protein could be observed despite a preponderance of distracting detergent signal [49]. Performing in-source CID, enabled the authors to observe binding of one glutathione ligand molecule to the noncovalent

complex [49]. While the MS of membrane proteins undoubtedly remains a significant challenge these studies show much promise.

At over 2MDa, and composed of more than 50 different proteins, the prokaryotic ribosome is at the upper end of the mass and complexity range of species currently amenable to study by MS. The mass determination of the whole ribosome from *T. thermophilus* was recently reported as 2.33MDa [50]. Moreover, through using tandem MS the authors were able to investigate the elusive ‘stalk’ region of the ribosome. They found that this stalk complex, thought to be universally pentameric, is heptameric for ribosomes from the thermophiles investigated. Furthermore, a likely phosphorylation of the stalk protein L12, and the possible interaction of the ribosome with enolase, a component of the degradosome, were also revealed by tandem MS [50].

CONCLUSIONS

In this short review we have demonstrated the exciting potential of MS in the study of macromolecular assemblies, both through maintaining their contacts in the gas-phase, and through their subsequent selective disruption. This combination of ‘preservation’ and ‘dissociation’ can provide detailed structural information both globally, as exemplified by the polydisperse α -crystallin assemblies, and locally by deducing the heptameric ‘stalk’ complex within the context of intact ribosomes. Moreover, monitoring the dynamics and interactions of these complexes can provide the elusive details of their exchange reactions in solution.

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[49] The first direct observation of a membrane-protein complex in the gas-phase, achieved through solubilization with a minimum amount of detergent.

[50] Mass measurement of intact prokaryotic ribosome, and disassembly by means of tandem MS to reveal post-translational modification and potentially novel interactions.

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[14] Comprehensive book covering many aspects of MS applied to biomolecules. Provides an overview of the field of molecular biophysics and the role MS does and can play in this field, with particular attention to protein folding and higher-order structure.

[26] Ion mobility and MS study of the gas-phase structure of TRAP. The first study that shows conclusively that quaternary structure can be preserved in the gas phase. Paves the way for macromolecular structure information to be obtained through gas-phase measurements.

[33] Detailed study into the mechanism and kinetics of subunit exchange of the amyloidogenic protein transthyretin and its aggressive L55P mutant. Shows how detailed kinetic elucidations can be made as to transient reaction intermediates.

[35] A study of the acid-induced denaturation of haemoglobin involving the use of rapid online mixing. Demonstrates how the kinetics of disassembly of a heterogenous system can be monitored by ESI-MS.

[40] Investigation of the assisted refolding of gp23 by the GroEL/gp31 chaperonin complex. Shows how stages of the mechanism of action of an important cellular machine can be monitored by MS.

[46] Comparative study of full length α -crystallin and a truncated form. Tandem MS allows the determine the relative populations of the different oligomers that compose the polydisperse assembly, show how this is affected by truncation, and how truncation compromises the subunit exchange dynamics of this protein.

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FIGURE LEGENDS

Figure 1: *Relationship between surface area and charge state.*

There is a power relationship between charge state and the surface area of the protein/assembly in solution, as determined from its crystal structure (green line, ‘crystallographic’) [29]. Re-plotting this charge-state data against an a surface area extrapolated from the measured mass (by assuming a constant protein density of $0.83\text{Da}/\text{\AA}^3$ and smooth-sphere shape, essentially as in [28]) results in a linear relationship (red line, ‘spherical’). This may suggest that noncovalent complexes appear to be, on average, somewhat collapsed in the gas-phase, such that their density is closely similar to that of folded monomeric proteins.

Figure 2: *Refolding of gp23 by the GroEL:gp31 chaperone complex.*

A GroEL (blue), gp31 (green), and gp23 (black) in the presence of Mg^{2+} and ADP results in the formation of a 944kDa species, corresponding to GroEL:gp31 binding one gp23 substrate molecule. Replacement of ADP with ATP, **B**, leads to the formation of refolded gp23 hexamer (yellow), GroEL:gp31 chaperone complex, and GroEL tetradecamer (peaks labelled by green and blue circles respectively). Inset is a spectrum of gp23 hexamer as purified from *E. coli*. Replacement of gp31 with GroES (red), **C**, results in the observation of the GroEL:GroES complex binding one gp23, but no refolding is observed Adapted, with permission from [40], copyright 2005 Nature Publishing Group.

Figure 3: *Collision-induced dissociation of protein oligomers.*

A The 46+ charge state of the 24mers formed by *MjHSP16.5* is isolated and subjected to collisions with argon atoms. At the lowest accelerating voltages only these isolated 24mers are observed. Once the accelerating voltage is raised, the amount of 24mers observed decreases, and concomitantly monomers and 23mers appear. As the accelerating voltage is increased further still, the 23mers start to decay and 22mers are observed. At the highest voltages 21mers are also detected, demonstrating that, in this case, as many as three monomers can be stripped from the *MjHSP16.5* 24mer. The dissociation process occurs via unfolding of one monomer from the oligomer, **B**. With sufficient activation, the highly charged parent oligomer undergoes three successive dissociation steps, each reducing its charge density (charge density, relative to surface area, decreasing from red to blue). This effective charge-reduction leads to vastly improved peak separation in the mass spectra, **C**. Peaks corresponding to the parent 24mers are observed only in the narrow range of ~ 8000 to 9500 m/z (red), whereas after three dissociation steps (blue) the signal is spread over a much wider range. Consequently peaks are significantly more separated.

Figure 1

Figure 3