Two decades of studying non-covalent biomolecular assemblies by means of electrospray ionization mass spectrometry

Gillian R. Hilton and Justin L. P. Benesch*

Department of Chemistry, Physical and Theoretical Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX3 1QZ, UK

Mass spectrometry (MS) is a recognized approach for characterizing proteins and the complexes they assemble into. This application of a long-established physico-chemical tool to the frontiers of structural biology has stemmed from experiments performed in the early 1990s. While initial studies focused on the elucidation of stoichiometry by means of simple mass determination, developments in MS technology and methodology now allow researchers to address questions of shape, inter-subunit connectivity and protein dynamics. Here, we chart the remarkable rise of MS and its application to biomolecular complexes over the last two decades.

Keywords: mass spectrometry; ion mobility; protein assembly; non-covalent complex; hybrid structural biology

1. INTRODUCTION

Since its invention at the beginning of the last century, mass spectrometry (MS) has been considered an essential tool for chemists and physicists alike, primarily being used to analyse small molecules and volatile compounds. At the end of the 1980s, its utility broadened dramatically, as its application to problems in biology began in earnest. The first reports on the MS of non-covalent complexes appeared in the literature in the early 1990s, with the initial studies focusing on protein–ligand complexes quickly joined by those in which protein–protein interactions were maintained [1]. Since those early days considerable improvements in instrument technology and experimental methodology have dramatically increased the range of protein assemblies amenable to MS analysis [2]. As a result, 20 years after the initial reports, assemblies as large as intact viruses; of as many components as ribosomes; as hydrophobic as membrane protein complexes; as heterogeneous as amyloidogenic oligomers; and as dynamic as molecular chaperones have all been successfully interrogated [3–7]. MS has therefore evolved to impact a wide range of applications in structural biology.

In this historical perspective, we chart some of the milestones in MS development as they pertain to the study of non-covalent complexes, and the novel applications they have enabled (figure 1). While multiple MS approaches can inform on such assemblies [4], we focus here on those in which they are examined intact in the gas phase. We also describe the current state of the art in MS instrumentation and sample preparation, and direct to the relevant literature. Having described the past and present of the MS of non-covalent complexes, we allow ourselves to indulge in speculation as to what the future of the field might hold. As such, our intention is that this review serves as a ‘primer’ for scientists new to the field, providing an entry point to the literature. This is inevitably a subjective undertaking, and though we have endeavoured to be as comprehensive as possible, we apologize in advance for any omissions and hope the interested reader will soon fill in the gaps.

2. THE DEVELOPMENT OF MS FOR STRUCTURAL BIOLOGY

2.1. The initial discoveries

In the early 1990s, a series of seminal studies demonstrated that bimolecular complexes held together by non-covalent interactions could be transferred into the vacuum of the mass spectrometer and analysed. While the preservation of non-covalent interactions, in the form of salt and solvent bound to proteins, had been observed a few years previously, this detection of
specific and biologically relevant complexes represented a major breakthrough [1,8,9].

The earliest of these reports appeared in the literature in 1991, concerning the receptor–ligand binding of FK binding protein and macrolides [10]; the enzyme–substrate pairing of lysozyme and a hexa-saccharide [11]; and the haem-binding of myoglobin [12]. These studies were soon followed by an array of other examples in which non-covalent interactions were measured by means of MS in 1991, and the following two decades have seen dramatic progress in both the technology and its application to problems in structural biology.

While these reports clearly showed the promise of MS for studying protein assemblies, much work remained in the development of both technology and methodology. Electrospray ionization (ESI) was an essential development in MS as it allowed the transfer of protein from solution molecules into gas-phase ions. The above studies all employed ESI, which in the late 1980s had been shown to enable the MS analysis of intact protein chains [14]. Such ‘soft’ or ‘gentle’ ionization was in stark contrast to previous approaches which caused extensive covalent bond fragmentation, and were effectively limited to molecular weights on the order of 10 kDa [15].

### 2.2. Optimizing transfer into the gas phase

#### 2.2.1. Electrospray mechanism

ESI is achieved by applying a potential difference between the inlet of the mass spectrometer and a conductive capillary containing the analyte solution [16]. This results in the production of charged droplets at the end of the capillary which evaporate solvent as they pass into the vacuum of the mass spectrometer. The droplets shrink until they reach the Rayleigh limit, the point at which the surface tension holding them together equals the Coulombic repulsion between the charges on their surface, and droplet fission occurs. Successive rounds of evaporation and fission occur until an analyte ion is formed via one of two different mechanisms. Analyte ions formed by the ‘ion evaporation model’ are expelled directly from the droplets [17], whereas those resulting from the ‘charged-residue model’ arise as the end product after droplet fission and solvent evaporation processes have reached exhaustion [18]. The current evidence suggests that folded protein ions are generated according to the latter [19].

#### 2.2.2. Nanoelectrospray and native MS

The use of ESI was not without its challenges for the analysis of biomolecules. Experiments required substantial sample volumes, and typically relied on a combination of organic solvents, acids and high temperatures to aid the desolvation and droplet fission processes, and thereby allow reliable ion production [20]. These conditions are generally not compatible with the preservation of biomolecular complexes in solution, and therefore limit the scope of conventional ESI in structural biology [2]. To overcome these difficulties miniaturized ESI [21–23] sources were designed which, by virtue of a smaller capillary diameter, lower the flow rate to nl min⁻¹ and therefore reduce sample consumption to only a few μl. The reduced flow rate has the added benefit of producing smaller initial droplet sizes [24], which both increases sensitivity and salt tolerance [25], and crucially negates the need for organic co-solvents and high interface temperatures. In this way, the examination of proteins in neutral aqueous buffers in which their structure is preserved has become possible, in a strategy often termed ‘Native MS’ [9,26] (figure 2a).

### 2.3. Transmitting and analysing large ions

The application of nano-ESI (nESI) not only enabled proteins to be analysed in their native form but also brought new challenges to MS technology. In order
that the large biomolecular complexes which could now be ionized (up to ≈100 MDa [28]) might be separated and measured by the mass spectrometer, significant technological improvements were required.

Experiments have shown that increasing the pressure in the early vacuum stages of the instrument dramatically improved the transmission of large ions [29–32]. This is owing to the increased number of collisions experienced by the analyte ions acting to focus them onto the appropriate trajectory in the instrument [33]. By dampening the trajectories of the ions, the literature of which has been discussed in detail [2,34], a dramatic reduction in the loss of high mass ions is observed. This discovery brought with it new and exciting opportunities, and before the turn of the century, species over 1 MDa were being successfully transmitted through the mass spectrometer [29,35–37].

2.3.1. Separation at high m/z

Many of the early studies of non-covalent complexes used triple-quadrupole mass spectrometers. These instruments have the advantage of allowing tandem-MS experiments (§2.4) but are typically limited to a maximum acquisition range of approximately 4000 m/z. To overcome this limitation, mass spectrometers were built incorporating a quadrupole operating at a lower radio-frequency, thereby allowing the separation of higher m/z species [38–40]. While this enabled the analysis of proteins in the 100 kDa range [41,42], the low resolving power at high m/z represented a considerable disadvantage.

In contrast, time-of-flight (ToF) mass analysers have a theoretically unlimited mass range and, when operated with a reflectron [43], can achieve high mass resolution and sensitivity on a very fast timescale. To take full advantage of the capabilities of ESI, novel instrumentation geometry was designed in which the continuous beam of ions was pulsed orthogonally into the ToF, allowing the identification of peaks well above 5000 m/z [44–46]. An early example demonstrating the utility of combining nESI with ToF was a study of the enzyme 4OT, where the sensitivity and resolution afforded by MS settled a conflict in the field as to the protein’s oligomeric state [47]. This heralded an important shift in the MS of protein assemblies from being a method which was considered a technical curiosity to one which could be used to provide novel structural biology insight.

Figure 2. Four example mass spectra of the same protein complex subjected to different experimental conditions. These spectra show the complex intact; solution adjusted conditions to reveal the presence of monomers and dimers; denaturing solution conditions (addition of organic solvent and acid); and finally MS activating conditions showing the gas phase fragmentation. The protein complex, a small Heat Shock Protein (sHSP) TaHSP16.9, is an oligomeric species comprising six dimeric building blocks to form a 12 mer (see inset 2(a)). (a) Mass spectrum of TaHSP16.9 under near ‘native’ conditions applying mild instrument conditions such as low collision voltages and ion guide pressures optimized to allow the transmission of the ions through the mass spectrometer. The spectrum shows a narrow charge series (30+ to 34+) corresponding to 202 237 Da, the mass of the intact 12 mer of HSP16.9 (see the inset). The multiple charge states are a direct result of the distribution of charges on the nESI droplet. (b) Example spectrum showing the effects of solution phase manipulation by the addition of isopropanol 10% (v/v), aqueous) reduces the 12 mer to monomeric units with a broad charge state distribution. The larger the surface area exposed, the more charges can be accommodated, and therefore a narrow distribution of low average charge suggests a folded protein state whereas an unfolded/disordered protein will have an extended highly charged distribution. (c) Spectrum under activating conditions in which monomers are ejected from the intact 12 mer to form an 11 mer and subsequently a 10 mer. Expelled monomers can be observed at low m/z. Unless otherwise stated, all spectra were obtained on a modified Q-ToF instrument (Waters, Manchester), as described previously [27], with a 10 μM monomer concentration of TaHSP16.9 in 200 mM ammonium acetate pH 6.9.
While other mass analysers have been used to examine intact protein complexes [48], the ‘hybrid’ Q-ToF has been the favoured instrument geometry for about a decade [32], capitalizing on the $m/z$-range benefits of ToF with the selection abilities of a quadrupole (Q) [49]. The decreased resolution of this first analyser does not impact on the final spectrum, as this is determined by the subsequent ToF stage. The great advantage of this instrument configuration is the ability to perform tandem-MS on high mass species to help elucidate their composition [32,34,50].

2.4. Gas phase manipulations

With the technology allowing the transmission of intact protein assemblies through the mass spectrometer and their mass measurement with unparalleled accuracy, attention shifted towards devising means for their gas-phase disassembly, such that their constituents might be probed. Multiple activation approaches have been developed to achieve this, including by impacting a surface [51,52], interactions with electrons [53,54] and absorption of infrared photons [55].

All of these approaches have their advantages, but collision-induced dissociation (CID) remains the most commonly applied approach for activation. CID was developed in the 1960s and is based on the analyte ions colliding with inert gas, resulting in activation as their translational energy is converted into internal modes by many consecutive collision events [56].

2.4.1. Effects of collisional activation

CID is typically performed in two regions of the mass spectrometer where the pressure is relatively high: in the source region, or in a specifically designed collision cell. Irrespective of the location within the instrument, this thermal heating incurs the same consequences on biomolecular assemblies: cleaning, restructuring, unfolding, dissociating and fragmenting [57].

Under non-denaturing conditions the measured mass of a large protein complex is higher than that calculated from the sequence alone, owing to the addition of salt and solvent during the ESI process [58]. The process of activation results in ‘cleaning’ of the protein by removal of these bound species, and thereby provides an increase in effective mass accuracy and resolution in the spectra [59]. Further increases in internal energy can lead to structural distortions of the protein assembly, such as the collapse of cavities within the structure [60].

At elevated activation conditions the individual protein chains begin to unfold, a process which continues until a threshold is reached and a subunit is ejected from the complex [61]. Dissociation occurring via unfolding leads to the expelled subunit typically carrying a proportion of the charge disproportionately high relative to its mass [41,62–64]. This process can occur repeatedly, with multiple subunits being removed sequentially from the assembly [65]. At the highest energies the unfolded monomers can undergo covalent fragmentation after their expulsion from the complex [66]. There has been considerable interest in recent years to manipulate the pathway of dissociation in order to obtain more information, including the adjustment of charge states [67,68], or effecting ion activation through collision with a surface [52,69]. The latter approach, surface-induced dissociation, shows particular promise for the analysis of protein assemblies in potentially allowing the determination of the building blocks of the oligomers [70].

2.4.2. Deconvoluting heterogeneity with tandem-MS

The ability of gas-phase activation to afford information on the components within a protein assembly is particularly powerful when employed in the form of tandem-MS (also referred to as MS/MS). In this approach ions can be selectively subjected to CID, and the resulting dissociation products measured in a second analysis stage [71–73]. This approach became established in the study of protein assemblies after the development of Q-ToF instruments with a high-$m/z$ quadrupole [32,34,50].

The high resolution of MS can be exploited to allow the different components within a mixture to be individually interrogated. Furthermore, in cases where the MS spectrum cannot be unambiguously assigned, knowledge that dissociation products must be complementary leads to tandem-MS aiding the assignment [32]. Another advantage of the nature of gas-phase dissociation is that the removal of highly charged monomers results in an effective charge reduction of the parent oligomers [65]. This has been exploited to quantify the relative distribution of the species comprising polydisperse ensembles which cannot be deconvoluted by MS alone [74] (figure 3).

2.4.3. Examining membrane proteins

Membrane-associated proteins are among the most challenging of protein systems for structural biology owing to their solubility requirements. While a vacuum can be regarded as hydrophobic and therefore a suitable environment in which to study such proteins [75], transferring them intact into the mass spectrometer has been a challenge. The first approach which brought success was to prepare a protein in a concentration of a detergent sufficient to solubilize the exposed hydrophobic surfaces, but not so high as to obscure the signal corresponding to protein [76].

The observation that detergent micelles could apparently be maintained in the gas phase [77,78] led to the development of an alternative strategy. In this approach, the protein assembly is encapsulated within a micelle to enable its transfer into the gas phase, whereupon the detergents are subsequently removed by collisional activation [79,80]. This process has the potential to be applicable to various membrane protein systems [81], and recent evidence suggests that it might even be possible to remove the detergent without excessive structural rearrangement of the protein [82].

Considering the importance of membrane proteins as drug targets, perhaps the most exciting aspect of this application is the ability to detect the presence of small molecules, and their influence on the structure and stability of the complex [83].
2.5. Solution phase manipulations

In the early 1990s, it was noted that solution conditions could affect the ESI mass spectra of proteins. Reduction of disulphide bonds [84], manipulation of pH [85], ionic strength [86], temperature [87] or addition of organic co-solvent [88] causes a change in the folding state of the protein chain which is reflected in the distribution of charge states. Typically a native globular protein will populate a narrow distribution of low average charge, whereas its denatured counterpart will feature a broad and highly charged distribution because of the additional sites available for protonation [89] (figure 2). This behaviour can be exploited to monitor the unfolding pathway of proteins [90–92].

Such solution-phase destabilization has been extended to provide a means for studying the composition of protein assemblies [93]. With careful adjustment, solution conditions can be found which effect disassembly of the complex yet stop short of denaturing the constituent protein chains (figure 2). This allows sub-complexes, that is oligomeric species smaller than the original assembly, to be generated in solution and measured in the mass spectrometer. Such equilibrium experiments can therefore be used to reveal the building blocks of assembly [94] and the thermodynamics of the subunit interfaces [95,96]. Furthermore, in the case of heteromeric proteins when multiple sub-complexes can be observed [97], the overlap can be used to generate an interaction map of the protein complex [98,99].

2.6. Determining protein quaternary structure and dynamics

The ability of MS to inform on the oligomeric and disassembled states of proteins renders it very attractive for structural biology. The issue of whether such gas-phase measurements can be directly related to the native form has however been a controversial topic [75,100].

2.6.1. Specificity of protein complexes in the gas phase

While it had clearly been demonstrated that specific protein oligomers could be maintained intact within the mass spectrometer, early reports raised the possibility of observing false positives in mass spectra [101,102]. Such non-specific oligomers arise from those electrospray droplets containing more than one analyte molecule, and their artefactual association during droplet fission and evaporation [16]. This effect is concentration-dependent, and therefore the improved sensitivity of modern mass spectrometers as well as the smaller initial droplet sizes resulting from nESI have largely removed the appearance of these unwanted artefacts when determining protein oligomeric state [2]. For cases where experiments necessitate high protein concentrations, methods have been developed to deconvolute the contributions of specific and non-specific protein oligomers [103–105].

Similarly, false positives can be observed in spectra of oligonucleotides [106], and in ligand-binding studies in which the ligand is typically in considerable excess in solution [107,108]. In both cases a large contributor to this effect is the fact that the strength of molecular interactions change upon transfer into the gas phase. Those based on electrostatics, dipoles and polarizability are strengthened owing to the removal of 'competition' from water, and conversely hydrophobic associations are weakened [109]. As such the risk of 'false negatives', in which contacts present in solution are not represented in the ESI spectra, needs to be considered [110]. Experiments have, however, shown that van der Waals interactions remaining after dehydration can effectively act to retain contacts driven by water [111]. As such the extent to, and timescale on, which hydrophobic associations can be maintained in the gas phase remains an active area of study [112]. Typically large protein assemblies are held together by a large number of individual contacts, and therefore even those dominated by hydrophobic effects such as membrane protein oligomers [113] or molecular chaperone : target complexes [95] can successfully be interrogated in the gas phase.

2.6.2. Preservation of structure in the gas phase

While protein stoichiometry can be faithfully preserved in the gas phase, the question arises as to whether solution-phase structure is similarly maintained. Various strands of evidence combine to indicate that this is possible, at least on the timescale of typical MS measurements [114]. Protein complexes transmitted through the mass spectrometer and examined ex situ by electron microscopy retain their global topology [115], and in the case of viruses and enzymes can retain infectivity [116] and activity [117], respectively. Similarly, infrared [118] and fluorescence [119] measurements of proteins trapped in vacuum have demonstrated the retention of aspects of solution structure, evidence backed up by molecular dynamics studies [120].

Perhaps the most compelling evidence comes from ion-mobility (IM) spectrometry measurements, which enable the direct determination of molecular size in terms of a rotationally averaged collisional cross section (CCS), in the gas phase. Evidence suggests the experimental CCS of proteins to be similar to those estimated from atomic constraints [121,122] (figure 4), and that different conformations do not exchange on the timescale of milliseconds [124,125]. Moreover, even the size of fragile protein complexes has been observed to match what is expected from their structure [126]. These observations combine to demonstrate that, on the timescale of typical MS measurements, tertiary and quaternary structures of the protein can be preserved in the gas phase [60].

2.6.3. Structural restraints

The observations outlined above motivate the use of MS for determining structural restraints on protein complexes [4]. Stoichiometry and size information can be obtained from ‘top-down’-type experiments, in which the intact assembly is measured in the gas phase by means of IM – MS. These experiments coupled with the use of gas-phase dissociation can also generate composition and connectivity information.

J. R. Soc. Interface (2012)
Alternatively, information can be obtained using a ‘bottom-up’ approach, through the interrogation of sub-complexes [127] and folded subunits [128] released from the assemblies in solution under destabilizing conditions. This can provide data on the monomeric, protomeric and oligomeric levels [129], providing valuable information to enable the modelling of protein complex architecture [130]. Furthermore, by combining IM–MS with the additional separation afforded by tandem-MS, candidate structures of polydisperse protein assemblies can be filtered according to their correspondence with measurement [123].

Spatial restraints obtained in this way can be augmented by those obtained from other MS-based approaches [4]. For example, hydrogen/deuterium exchange [131] and oxidative footprinting [132] experiments can be used to reveal secondary structure via solvent accessibility. Limited-proteolysis [133] experiments can provide information as to the domains of the proteins, and cross-linking experiments can reveal protein fold and inter-subunit connectivity [134]. Ultimately, MS-derived structural information can be integrated with restraints obtained from different sources, enabling the modelling of ‘hybrid’ structures which best fit all the available data [135].

**2.6.4. Monitoring dynamics**

The function of protein complexes hinges not only on their structure, but also on the dynamic processes they undergo, both before and at equilibrium. The speed of analysis and separation afforded by MS renders it well suited to analysing such fluctuations in real time [136,137]. In fact one of the earliest studies to show the preservation of non-covalent interactions in the gas phase monitored in real time the turnover of substrate by the enzyme lysozyme [11]. Furthermore, MS has been used to monitor various other dynamic aspects of proteins, including the folding and conformational fluctuations of protein chains [138].

MS is particularly useful in the study of protein dynamics on the quaternary level, such as monitoring protein complex assembly [139], disassembly [140] and subunit exchange [141]. By virtue of intrinsic mass separation, different oligomeric states can be monitored individually; whereas the quaternary dynamics of...
advice reader consult recently published protocols for detailed analysis of protein assemblies, but also suggest that the brief description the current state of the art of nESI MS technology have developed rapidly [144]. In this section, we complexes in the gas phase, instrumentation and methodology have. Since the early experiments in examining non-covalent dynamics as well as details of their architecture to be ascertained [143].

3. TECHNOLOGY AND METHODOLOGY: THE STATE OF THE ART

Since the early experiments in examining non-covalent complexes in the gas phase, instrumentation and methodology have developed rapidly [144]. In this section, we briefly describe the current state of the art of nESI MS analysis of protein assemblies, but also suggest that the reader consult recently published protocols for detailed advice [93,145–149].

3.1. Protein preparation

Aqueous solutions of protein complexes are typically prepared at concentrations of 1–10 μM (oligomer), in a volatile buffer. The low concentration guarantees minimal non-specific association during nESI, while the buffer ensures electrochemical effects in the capillary do not affect solution pH. The most commonly used buffered standard is ammonium acetate which, unadjusted, gives a neutral solution even up to high ionic strengths [150], and readily evaporates during ion desolvation. When necessary for the stabilization of the protein assembly, low concentrations of involatile salts or other kosmotropes can be added and still result in tolerable mass spectra [151]. Membrane protein assemblies have specific solubilization requirements, either through stabilization with the minimum amount of a specific detergent [76], or by their release from intact micelles into the gas phase [81].

Spectra of the denatured proteins allow the determination of the masses of the individual subunits, information often essential for establishing oligomeric stoichiometry. These are typically achieved by the addition of organic solvents and acid to the protein solution to degrade the quaternary and tertiary structures. Similarly, identifying the protein chains themselves and the location of post-translational modification through typical proteomic means, either from fragmentation within the mass spectrometer or proteolysis in solution, can provide valuable additional information in the case of purified rather than recombinant sample. Such MS-based proteomics is well established [152], and an important complement to the interrogation of intact protein assemblies described here.

3.2. Nanoelectrospray ionization

As described in §2.2, MS analyses of protein assemblies are generally performed using nESI owing to the low sample volumes required, and its tolerance of mild interface conditions. nESI is typically performed using borosilicate glass capillaries that have been pulled to form micro-pipettes. The ends can then be manually clipped under a stereo-microscope to provide an orifice size on the order of 1–5 μm in diameter. Electrospray is initiated by applying a potential difference between the capillary and the inlet to the mass spectrometer, and current is delivered to the solution by either making the capillary conductive via gold coating, or the introduction of a platinum wire. Alternatively nESI can be performed using a chip-based robotic infusion system [153].

3.3. Transmission and analysis

As discussed in §2.3, the transmission of large protein assemblies is aided by collisional focusing in the early vacuum stages of the mass spectrometer. Typically this is achieved by reducing the pumping efficiency at the front end of the instrument. Alternative methods exist, and all similarly rely on increasing the number of collisions with background gas experienced by the analyte [32,34]. Additional stabilization of non-covalent complexes can also be achieved in this region by using a curtain gas such as sulphur hexafluoride [154].

These considerations are sufficient for analysing protein complexes on a simple ToF mass spectrometer. The majority of such experiments are performed on
Q-ToF instruments, incorporating a modified quadrupole which allows the selection of high \( m/z \) ions \([32,34,50]\). Optimum instrument parameters, i.e. operating pressures and voltages, are somewhat sample-dependent, however conditions are typically adjusted to achieve maximum removal of adducts while still maintaining the protein complexes intact.

### 3.4. Ion mobility MS

The current state-of-the-art mass spectrometers for the analysis of protein assemblies incorporate an IM stage, thereby providing two dimensions of separation: effectively mass and size \([155]\). A number of different means exist to effect IM–MS separation \([156]\) and several have applied to the interrogation of protein multimers, including drift-tube IM \([157,158]\), differential-mobility analysis \([159]\) and energy-loss experiments \([160]\). The majority of studies on macromolecular assemblies have however employed travelling-wave IM \([161]\), a high-transmission approach which is available on commercial platforms \([162,163]\).

In all cases, the IM measurement can be related to a rotationally averaged CCS of the ion. In the case of travelling-wave experiments this conversion is enabled by calibration using protein standards of known CCS. To this end, a number of protocols \([164–166]\) and CCS databases \([167–169]\) have been published, and it is advisable to use standards of similar mobility to the unknown when performing a calibration \([167,170]\). It is important to note that even mildly activating conditions within the mass spectrometer, typically used to obtain good quality mass spectra, can cause unwanted structural changes in the protein complexes \([57,164]\). It is therefore of paramount importance to employ low acceleration voltages prior to IM separation. Additional stabilization can be afforded by charge-reduction \([68]\) or by the addition of kosmotropes \([151]\).

The experimental CCS can be compared with those calculated from atomic structures in silico. A number of algorithms exist to achieve this, with the simplest employing a ‘projection approximation’ (PA) \([165,171–175]\). More sophisticated approaches, including the exact hard-sphere scattering \([176]\) and trajectory methods \([177]\) can also be used. These latter methods, though providing CCS estimates matching experimental values more closely than PA approaches, are more computationally expensive, particularly in the case of the trajectory method \([178]\).

Currently the most convenient strategy for structural biology applications is to employ a scaled PA estimate, as it affords equivalent accuracy and also allows the assessment of coarse-grained molecular models \([4]\).

### 4. THE NEXT TWO DECADES: MS IN STRUCTURAL AND DYNAMICAL BIOLOGY

With the dramatic advances since the first measurements of intact non-covalent complexes, and the excellent instrumentation and protocols now available, MS appears to have a large role to play in the evolution of structural and dynamical biology. While anticipating future advances is naturally more difficult than describing past developments, there are several research areas we feel are likely to go beyond just incremental advances to see exciting progress over the coming years.

#### 4.1. Standardized and quantitative MS analyses

With the proliferation of structural information stemming from MS experiments, there is an emerging need for the development of experimental standards, and independent criteria for evaluating data quality. Such principles of best practice are either already established or are undergoing development for other structural biology techniques and MS-based proteomics. What form these will take is still unclear, but ultimately we can expect the annotation of protein databases with information from MS-based ‘structural proteomics’ experiments, with the associated requirement for data integrity and deposition.

Equally important to ensuring robust structural information on proteins is the accurate determination of the associated thermodynamic and kinetic parameters that describe their stability and dynamics. As we have described here, MS is well placed to bridge this gap between structural biology and biophysics. Crucial to these efforts is the necessity for MS to accurately reflect the distribution of all molecules in solution \([179]\). This has been shown to be the case for similar protein species in solution \([4]\); however, care needs to be taken to overcome the \( m/z \) dependence of both current mass analysers \([180]\) and detectors \([181]\). It is probable that future improvements in MS instrumentation will act to overcome these difficulties, ultimately leading to absolute quantification of varied species in solution based on signal intensity alone. This will enable MS to provide not only structural information but also reliably the strength and dynamics of interfaces within diverse macromolecular assemblies.

#### 4.2. Structural proteomics through automated multiplexed MS

In order to appropriately characterize the stoichiometry of an unknown protein assembly three crucial elements of information are required: the mass of the intact complex, and the identity and masses of the constituent subunits. As discussed in this article, obtaining the former is now well-established and, building on previous studies \([182,183]\), will probably allow for the automated screening of simple unknowns. The latter two are generally trivial to obtain in the case of recombinantly expressed assemblies, but not in the case of heteromeric complexes isolated from cells, where subunit masses are often considerably different to that expected from genomic databases \([184]\). It is necessary to perform experiments which separate the protein subunits and in parallel allow their mass measurement and the determination of sequence information \([185,186]\). We envisage the development in the coming years of MS platforms in which all these levels of information can be obtained in a single multiplexed experiment, thereby providing an automated accurate and reliable means for characterizing protein stoichiometry.
Furthermore, while in this review we have focused on the direct analysis of protein assemblies, there are a plethora of MS-based technologies which can inform on a wide range of structural aspects and timescales [4]. In fact, the vast majority of MS experiments performed on proteins rely on examining the array of peptides produced by enzymolysis of cell extracts or purified components [152,187]. These experiments can be highly automated both in terms of software and hardware [144], and thereby provide a vast amount of data on the sequence level of proteins [188]. In this way, structural probes which have been introduced through, for example, chemical cross-linking, hydrogen/deuterium exchange or oxidative-foot-printing experiments, can be localized. A major goal for MS is to combine these approaches into an integrated structural proteomics platform, enabling the determination of spatial and dynamical restraints spanning the residue to oligomer levels.

4.3. Visualizing gas phase ions

In addition to combining these existing MS technologies into a synergistic whole, there are a number of exciting frontiers in the gas-phase visualization of protein assemblies. The opportunities afforded by the possibility of interrogating mass-selected ions, in the absence of solvent background, are significant, and gas-phase spectroscopy of isolated proteins [118,119] and complexes [189] promises to provide considerable insight into their conformation. IM–MS is likely to evolve considerably too, through not only incremental improvements in resolution, but also potentially by dipole alignment in the gas phase [189], or through the use of specific dopants in the IM gas [190].

The use of MS as a high-resolution purification method is likely to prove very useful, allowing ex situ analysis of selectively deposited material by electron microscopy [115]. This will allow the construction of initial models to guide downstream single-particle electron microscopy analysis. Higher resolution structural information on isolated biomolecules is promised by the advent of free-electron laser single-molecule X-ray diffraction [191]. Combining this ability of determining atomic structures with the separation and manipulation afforded by MS represents an exciting frontier for the characterization of heterogeneous macromolecules.

4.4. From structural to cell biology

As we have discussed, MS can already contribute significantly towards structural biology, both in isolation and in combination with other techniques. With the continual development of computational structural biology, it is anticipated that ever fewer spatial restraints will be required to produce high-fidelity structures. This is likely to lead to an increasing role for MS, as its generality, speed, and sensitivity will outweigh the fact that it provides fewer restraints than some other structural biology techniques.

Crucial over the coming years are efforts to bridge the gap between structural biology in vitro, and the situation in vivo [192]. MS has the potential to play an important role in this regard. Already MS-based approaches dominate the field of proteomics (and are likely to play a similar role in metabolomics, lipidomics and glycomics [193]), informing as to the identity, modification and abundance of different proteins in the cell [188]. Furthermore, the high sensitivity of MS allows the interrogation of protein complexes affinity-purified directly from cells [98]. Indeed, when the protein complexes are in high abundance, they can be measured intact directly from diluted crude cell extracts [194], and potentially even from individual cells [195].

The advent of desorption ESI [196], which has been shown to allow the transfer of even protein complexes into the gas phase [197] raises the possibility of probing macromolecular assemblies directly from cell or tissue surfaces. Combining this with the gas-phase separation of different classes of biomolecules in IM-MS spectra [198], leads to the prospect of not only extracting good quality mass spectra of protein complexes despite a high solute background [164], but also the tantalizing prospect of interrogating protein assemblies within the context of their cellular milieu.

5. CONCLUSIONS

Over the past two decades native MS has evolved to become a structural biology approach of remarkably general utility, providing insights into the composition, architecture, and dynamics of protein complexes. With the realization that the study of the most challenging systems is likely to require a combination of approaches [199,200] and an appreciation of the cellular environment [192], MS will have a crucial role in characterizing the molecular structure, dynamics, and interactions of molecules in the cell.

We thank Helena Hernández for the review of the manuscript: Carol Robinson (University of Oxford), Joe Loo (University of California, Los Angeles), Matthias Mann (Max Planck Institute for Biochemistry), James Scrivens (University of Warwick) and Mark McDowall (Waters UK Ltd) for stimulating discussions; and the Wellcome Trust, and Royal Society for funding.

REFERENCES

1 Loo, J. A. 1997 Studying noncovalent protein complexes by electrospray ionization mass spectrometry. Mass Spectrom. Rev. 16, 1–23. (doi:10.1002/(SICI)1098-2787(1997)16:1<1::AID-MAS1>3.0.CO;2-L)
2 Benesch, J. L. P., Ruotolo, B. T., Simmons, D. A. & Robinson, C. V. 2007 Protein complexes in the gas phase: technology for structural genomics and proteomics. Chem. Rev. 107, 3544–3567. (doi:10.1021/cr068289b)
3 Ashcroft, A. E. 2005 Recent developments in electrospray ionisation mass spectrometry: noncovalently bound protein complexes. Nat. Prod. Rep. 22, 452–464. (doi:10.1039/b41772j)
4 Benesch, J. L. P. & Ruotolo, B. T. 2011 Mass Spectrometry: an approach come-of-age for structural and dynamical biology. Curr. Opin. Struct. Biol. 21, 641–649. (doi:10.1016/j.sbi.2011.08.002)
5 Heck, A. J. 2008 Native mass spectrometry: a bridge between interactomics and structural biology. Nat. Methods 5, 927–933. (doi:10.1038/nmeth.1265)
6 Sharon, M. & Robinson, C. V. 2007 The role of mass spectrometry in structure elucidation of dynamic protein complexes. *Annu. Rev. Biophys. Bioeng.*, 36, 167–193. (doi:10.1146/annurev.bioeng.36.061005.090816)

7 Wytenbach, T. & Bowers, M. T. 2007 Intermolecular interactions in biomolecular systems examined by mass spectrometry. *Annu. Rev. Phys. Chem.*, 58, 511–533. (doi:10.1146/annurev.physchem.58.032806.104515)

8 Loo, J. A. 2000 Electrospray ionization mass spectrometry: a technology for studying noncovalent macromolecular complexes. *Int. J. Mass Spectrom.* 200, 175–186. (doi:10.1016/S1387-3806(00)00298-0)

9 Winston, R. L. & Fitzgerald, M. C. 1997 Mass spectrometry as a readout of protein structure and function. *Mass Spectrom. Rev.* 16, 165–179. (doi:10.1002/(SICI)1099-2787(199716)16:5<165::AID-MAS1>3.0.CO;2-F)

10 Ganem, B., Li, Y. T. & Henion, J. D. 1991 Detection of noncovalent receptor ligand complexes by mass spectrometry. *J. Am. Chem. Soc.*, 113, 6294–6296. (doi:10.1021/ja00016a069)

11 Ganem, B., Li, Y. T. & Henion, J. D. 1991 Observation of noncovalent enzyme-substrate and enzyme-product complexes by ion-spray mass-spectrometry. *J. Am. Chem. Soc.*, 113, 7818–7819. (doi:10.1021/ja00020a085)

12 Katta, V. & Chait, B. T. 1991 Observation of the heme-globin complex in native myoglobin by electrospray ionization-mass spectrometry. *J. Am. Chem. Soc.*, 113, 8534–8535. (doi:10.1021/ja0022a058)

13 Baca, M. & Kent, S. B. H. 1992 Direct observation of a ternary complex between the dimeric enzyme HIV-1 protease and a substrate-based inhibitor. *J. Am. Chem. Soc.*, 114, 3992–3993. (doi:10.1021/ja00036a006)

14 Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F. & Whitehouse, C. M. 1989 Electrospray ionization for analysis of large biomolecules. *Science*, 245, 68–71. (doi:10.1126/science.2675315)

15 Smith, R. D., Loo, J. A., Edmonds, C. G., Barinaga, C. J. & Udseth, H. R. 1990 New developments in biochemical mass spectrometry: electrospray ionization mass-spectrometry. *Anal. Chem.*, 62, 882–899. (doi:10.1021/ac00208a002)

16 Kebarle, P. & Verkerk, U. H. 2009 Electrospray: from ions in solution to ions in the gas phase, what we know now. *Mass Spectrom. Rev.* 28, 898–917. (doi:10.1002/mas.20247)

17 Iribarne, J. V. & Thomson, B. A. 1976 On the evaporation of small ions from charged droplets. *J. Chem. Phys.*, 64, 2287–2294. (doi:10.1063/1.432536)

18 Dodge, M., Luce, L. A. & Hines, R. L. 1968 Molecular beams of macromolecules. *J. Chem. Phys.*, 49, 2240–2249. (doi:10.1063/1.1670391)

19 de la Mora, J. F. 2000 Electrospray ionization of large multiply charged species proceeds via Dolé’s charged residue mechanism. *Anal. Chim. Acta.*, 406, 93–104. (doi:10.1016/S0003-2670(99)00601-7)

20 Karas, M., Bahr, U. & Duclaux, T. 1999 Nano-electrospray ionisation mass spectrometry: addressing analytical problems beyond routine. * Fresenius’ J. Anal. Chem.*, 366, 669–676. (doi:10.1007/s002160051561)

21 Emmett, M. R. & Caprioli, R. M. 1994 Micro-electrospray mass-spectrometry—ultra-high-sensitivity analysis of peptides and proteins. *J. Am. Soc. Mass Spectrom.*, 5, 605–613. (doi:10.1016/1044-0395(94)85001-1)

22 Gale, D. C. & Smith, R. D. 1993 Small-volume and low flow-rate electrospray-ionization mass-spectrometry of aqueous samples. *Rapid Commun. Mass Spectrom.*, 7, 1017–1021. (doi:10.1002/rcm.1290071111)

23 Wilm, M. S. & Mann, M. 1994 Electrospray and Taylor-cone theory, Dolé’s beam of macromolecules at last?
implications of high mass-to-charge ratio ions from electrospray mass spectrometry. *J. Am. Soc. Mass Spectrom.* **4**, 536–545. (doi:10.1016/S1044-0395(93)85015-P)

40 Collings, B. A. & Douglas, D. J. 1997 An extended mass range quadrupole for electrospray mass spectrometry. *Int. J. Mass Spectrom. Ion Process.* **162**, 121–127. (doi:10.1016/S0168-1176(96)04178-3)

41 Light-Wahl, K. J., Schwartz, B. L. & Smith, R. D. 1994 Observation of the noncovalent quaternary associations of proteins be electrospray ionization mass spectrometry. *J. Am. Chem. Soc.* **116**, 5271–5278. (doi:10.1021/ja00091a035)

42 Light-Wahl, K. J., Winger, B. E. & Smith, R. D. 1993 Observation of the multimeric forms of concanaavalin-a by electrospray-ionization mass-spectrometry. *J. Am. Chem. Soc.* **115**, 5869–5870. (doi:10.1021/ja00660a083)

43 Mamyrin, B. A., Karataev, V. I., Shmikk, D. V. & Zagulina, V. A. 1973 Mass-reflection a new nonmagnetic time-of-flight high-resolution mass-spectrometer. *Z. Eksperimentalnoi I Teoreticheskoi Fiziki* **64**, 82–89.

44 Coles, J. & Guilhaus, M. 1993 Orthogonal acceleration— a new direction for time-of-flight mass spectrometry: fast, sensitive mass analysis for continuous ion sources. *Trends Anal. Chem.* **12**, 203–213. (doi:10.1016/S0168-1176(96)00478-3)

45 Verentchikov, A. N., Ens, W. & Standing, K. G. 1994 Reflecting time-of-flight mass spectrometer with an electrospray ion source and orthogonal extraction. *Anal. Chem.* **66**, 126–133. (doi:10.1021/ac90073a022)

46 Dawson, J. H. J. & Guilliaux, M. 1989 Orthogonal- acceleration time-of-flight mass spectrometer. *Rapid Commun. Mass Spectrom.* **3**, 155–159. (doi:10.1002/rcm.1290030511)

47 Fitzgerald, M. C., Chernushevich, I., Standing, K. G., Whitman, C. P. & Kent, S. B. 1996 Probing the oligomeric structure of an enzyme by electrospray ionization time-of-flight mass spectrometry. *Proc. Natl Acad. Sci. USA* **93**, 6851–6856. (doi:10.1073/pnas.93.14.6851)

48 Heck, A. J. R. & van den Heuvel, R. H. 2004 Investigation of intact protein complexes by mass spectrometry. *Mass Spectrom. Rev.* **23**, 368–389. (doi:10.1002/mas.10081)

49 Morris, H. R., Paxton, T., Del, A., Langhorne, J., Berg, M., Bordoli, R. S., Hoyes, J. & Bateman, R. H. 1996 High sensitivity collisionally-activated decomposition tandem mass spectrometry on a novel quadrupole/orthogonal-acceleration time-of-flight mass spectrometer. *Rapid Commun. Mass Spectrom.* **10**, 889–896. (doi:10.1002/(SICI)1097-0231(19960610)10:8<889::AID-RCM615>3.0.CO;2-F)

50 van den Heuvel, R. H. et al. 2006 Improving the performance of a quadrupole time-of-flight instrument for macromolecular mass spectrometry. *Anal. Chem.* **78**, 7473–7483. (doi:10.1021/ac061039a)

51 Wysocki, V. H., Joyce, K. E., Jones, C. M. & Beardsley, R. L. 2008 Surface-induced dissociation of small molecules, peptides, and non-covalent protein complexes. *J. Am. Soc. Mass Spectrom.* **19**, 190–208. (doi:10.1016/j.aps.2007.11.005)

52 Jones, C. M., Beardsley, R. L., Galhena, A. S., Dagan, S., Cheng, G. & Wysocki, V. H. 2006 Symmetrical gas-phase dissociation of noncovalent protein complexes via surface collisions. *J. Am. Chem. Soc.* **128**, 15 044–15 045. (doi:10.1021/ja064586m)

53 Geels, R. B. J., van der Vies, S. M., Heck, A. J. R. & Heeren, R. M. A. 2006 Electron capture dissociation as structural probe for noncovalent gas-phase protein assemblies. *Anal. Chem.* **78**, 7191–7196. (doi:10.1021/ ac609606p)

54 Xie, Y., Zhang, J., Yin, S. & Luo, J. A. 2006 Top-Down ESI-ED-FT-ICR mass spectrometry localizes noncovalent protein–ligand binding sites. *J. Am. Chem. Soc.* **128**, 14 432–14 433. (doi:10.1021/ja063197p)

55 El-Faramawy, A., Guo, Y., Verkerk, U. H., Thomson, B. A. & Shi, K. W. M. 2010 Infrared irradiation in the collision cell of a hybrid tandem quadrupole/time-of-flight mass spectrometer for declustering and cleaning of nanoelectrosprayed protein complex ions. *Anal. Chem.* **82**, 9878–9884. (doi:10.1021/ac102351m)

56 Shukla, A. K. & Futrell, J. H. 2000 Tandem mass spectrometry: dissociation of ions by collisional activation. *J. Mass Spectrom.* **35**, 1069–1090. (doi:10.1002/1096-9888(200009)35:9<1069::AID-JMS54>3.0.CO;2-C)

57 Benesch, J. L. P. 2009 Collisional activation of protein complexes: picking up the pieces. *J. Am. Soc. Mass Spectrom.* **20**, 341–348. (doi:10.1016/j.jasms.2008.11.014)

58 McKay, A. R., Ruotolo, B. T., Ilag, L. L. & Robinson, C. V. 2006 Mass measurements of increased accuracy resolve heterogeneous populations of intact ribosomes. *J. Am. Chem. Soc.* **128**, 11 433–11 442. (doi:10.1021/ja061468q)

59 Tolić, L. P., Bruce, J. E., Lei, Q. P., Anderson, G. A. & Smith, R. D. 1998 In-trap cleanup of proteins from electrospray ionization using soft sustained off-resonance irradiation with Fourier transform ion cyclotron resonance mass spectrometry. *Anal. Chem.* **70**, 405–408. (doi:10.1021/ac970828c)

60 Ruotolo, B. T. & Robinson, C. V. 2006 Aspects of native proteins are retained in vacuum. *Curr. Opin. Chem. Biol.* **10**, 402–408. (doi:10.1016/j.cbpa.2006.08.020)

61 Ruotolo, B. T., Hyung, S. J., Robinson, P. M., Gates, K., Bateman, R. H. & Robinson, C. V. 2007 Ion mobility-mass spectrometry reveals long-lived, unfolded intermediates in the dissociation of protein complexes. *Angew. Chem. Int. Ed. Engl.* **46**, 8001–8004. (doi:10.1002/anie.200702161)

62 Felitsyn, N., Kitova, E. N. & Klässen, J. S. 2001 Thermal decomposition of a gaseous multiprotein complex studied by blackbody infrared radiative dissociation. Investigating the origin of the asymmetric dissociation behavior. *Anal. Chem.* **73**, 4647–4661. (doi:10.1021/ ac103975)

63 Jurchen, J. C. & Williams, E. R. 2003 Origin of asymmetric charge partitioning in the dissociation of charged protein homodimers. *J. Am. Soc. Mass Spectrom.* **14**, 121–127.

64 Benesch, J. L. P., Aquilina, J. A., Ruotolo, B. T., Sobott, F. & Robinson, C. V. 2006 Tandem mass spectrometry reveals the quaternary organization of macromolecular assemblies. *Chem. Biol.* **13**, 597–605. (doi:10.1016/j.chembiol.2006.04.006)

65 Benesch, J. L. P., Aquilina, J. A., Ruotolo, B. T., Sobott, F., & Robinson, C. V. 2006 Tandem mass spectrometry reveals the quaternary organization of macromolecular assemblies. *J. Am. Soc. Mass Spectrom.* **18**, 2242–2253. (doi:10.1016/j.jasms.2007.09.022)

66 Benesch, J. L. P., Aquilina, J. A., Ruotolo, B. T., Sobott, F. & Robinson, C. V. 2010 Quadrupole-time-of-flight mass spectrometer modified for higher-energy dissociation reduces protein assemblies to peptide fragments. *Anal. Chem.* **81**, 1270–1274. (doi:10.1021/ac901950u)

67 Boeri Erba, E., Ruotolo, B. T., Barsky, D. & Robinson, C. V. 2010 Ion mobility-mass spectrometry reveals the influence of subunit packing and charge on the dissociation of multiprotein complexes. *Anal. Chem.* **82**, 9702–9710. (doi:10.1021/ ac101778c)

J. R. Soc. Interface (2012)
68 Pagel, K., Hyung, S. J., Ruto, B. T. & Robinson, C. V. 2010 Alternate dissociation pathways identified in charge-reduced protein complex ions. *Anal. Chem.* **82**, 5363–5372. (doi:10.1021/ac101211r)

69 Beardsley, R. L., Jones, C. M., Galhena, A. S. & Wysocki, V. H. 2009 Noncovalent protein tetramers and pentamers with ‘n’ charges yield monomers with n/4 and n/5 charges. *Anal. Chem.* **81**, 1347–1356. (doi:10.1021/ac801885k)

70 Blackwell, A. E., Dodds, E. D., Bandarian, V. & Wysocki, V. H. 2011 Revealing the quaternary structure of a heterogeneous noncovalent protein complex through surface-induced dissociation. *Anal. Chem.* **83**, 2862–2865. (doi:10.1021/ac200452b)

71 Yost, R. A. & Enke, C. G. 1978 Selected ion fragmentation with a tandem quadrupole mass-spectrometer. *J. Am. Chem. Soc.* **100**, 2274–2275. (doi:10.1021/ja00475a072)

72 McLafferty, F. W. 1980 Tandem mass spectrometry (MS/MS): a promising new analytical technique for specific component determination in complex mixtures. *Acc. Chem. Res.* **13**, 33–39. (doi:10.1021/ar0106001)

73 Jennings, K. R. 2000 The changing impact of the collision-induced decomposition of ions on mass spectrometry. *Int. J. Mass Spectrom.* **200**, 479–793. (doi:10.1016/S1387-3806(00)00325-0)

74 Aquilina, J. A., Benesch, J. L. P., Bateman, O. A., Sling-sby, C. & Robinson, C. V. 2003 Polyspecificity of a mammalian chaperone: mass spectrometry reveals the population of oligomers in alphaB-crystallin. *Proc. Natl Acad. Sci. USA* **100**, 10 611–10 616. (doi:10.1073/pnas.1932958100)

75 Wolyne, P. G. 1995 Biomolecular folding in vacuo!!([?]). *Proc. Natl Acad. Sci. USA* **92**, 2422–2427. (doi:10.1073/pnas.92.7.2426)

76 Lengqvist, J., Svensson, R., Evergren, E., Morgenstern, Yost, R. A. & Enke, C. G. 1978 Selected ion fragmentation with a tandem quadrupole mass-spectrometer. *J. Am. Chem. Soc.* **100**, 2274–2275. (doi:10.1021/ja00475a072)

77 Konermann, L., Collings, B. A. & Douglas, D. J. 1997 Cytochrome c folding kinetics studied by time-resolved electrospray ionization mass spectrometry. *Biochemistry* **36**, 5554–5559. (doi:10.1021/bi970014d)

78 Sharon, M., Ilag, L. L. & Robinson, C. V. 2007 Evidence for micellar structure in the gas phase. *J. Am. Chem. Soc.* **129**, 8740–8746. (doi:10.1021/ja070820h)

79 Barrera, N. P. & Robinson, C. V. 2008 Micelles protect membrane complexes from solution to vacuum. *Science* **321**, 243–246. (doi:10.1126/science.1159292)

80 Ilag, L. L., Ubarreta-Xenida, I., Tate, C. G. & Robinson, C. V. 2004 Drug binding revealed by tandem mass spectrometry of a protein–micelle complex. *J. Am. Chem. Soc.* **126**, 14 362–14 363. (doi:10.1021/ja0450307)

81 Barrera, N. P. et al. 2009 Mass spectrometry of membrane transporters reveals subunit stoichiometry and interactions. *Nat. Methods* **6**, 585–587. (doi:10.1038/nmeth.1347)

82 Wang, S. C., Politis, A., Di Bartolo, N., Bavo, V. N., Tucker, S. J., Booth, P. J., Barrera, N. P. & Robinson, C. V. 2010 Ion mobility mass spectrometry of two tetrameric membrane protein complexes reveals compact structures and differences in stability and packing. *J. Am. Chem. Soc.* **132**, 15 468–15 470. (doi:10.1021/ja104312e)

83 Zhou, M. et al. 2011 Mass spectrometry of intact V-type ATPases reveals bound lipids and the effects of nucleotide binding. *Science* **334**, 380–385. (doi:10.1126/science.1210148)

84 Loo, J. A., Edmonds, C. G., Udseth, H. R. & Smith, R. D. 1990 Effect of reducing disulfide-containing proteins on electrospray ionization mass spectra. *Anal. Chem.* **62**, 693–696. (doi:10.1021/ac00206a009)

85 Chothia, C. R., Katta, V. & Clait, B. T. 1990 Probing conformational changes in proteins by mass spectrometry. *J. Am. Chem. Soc.* **112**, 9012–9013. (doi:10.1021/ja00180a074)

86 Vis, H., Heinemann, U., Dobson, C. M. & Robinson, C. V. 1998 Detection of a monomeric intermediate associated with dimerization of protein Hu by mass spectrometry. *J. Am. Chem. Soc.* **120**, 6427–6428. (doi:10.1021/ja9811187)

87 Leblanc, J. C. Y., Beuchemin, D., Siu, K. W. M., Guvermont, R. & Berman, S. S. 1991 Thermal-denaturation of some proteins and its effect on their electrospray mass-spectra. *Org. Mass Spectrom.* **26**, 853–859. (doi:10.1002/omss.19910260403)

88 Levy, E., Dresch, T., Giersch, L. M. & Kaltashov, I. A. 1999 Unfolding dynamics of a β-sheet protein studied by mass spectrometry. *J. Mass Spectrom.* **34**, 1289–1295. (doi:10.1002/(SICI)1096-9888(199912)34:12<1289::AID-JMS882>3.0.CO;2-U)

89 Dobo, A. & Kaltashov, I. A. 2001 Detection of multiple protein conformational ensembles in solution via deconvolution of charge-state distributions in ESI MS. *Anal. Chem.* **73**, 4763–4773. (doi:10.1021/ac010713f)

90 Konermann, L., Collings, B. A. & Douglas, D. J. 1997 Cytochrome c folding kinetics studied by time-resolved electrospray ionization mass spectrometry. *Biochemistry* **36**, 5554–5559. (doi:10.1021/bi970014d)

91 Hernández, H. & Robinson, C. V. 2007 Determining the stoichiometry and interactions of macromolecular assemblies from mass spectrometry. *Nat. Prot.* **2**, 715–726. (doi:10.1038/nprot.2007.73)

92 Levy, E. D., Boeri, E., Robinson, C. V. & Teichmann, S. A. 2008 Assembly reflects evolution of protein complexes. *Nature* **453**, 1262–1265. (doi:10.1038/nature06942)

93 Stengel, F., Baldwin, A. J., Painter, A. J., Jaya, N., Basha, E., Kay, L. E., Vierling, E., Robinson, C. V. & Benesch, J. L. P. 2010 Quaternary dynamics and plasticity underlie small heat shock protein chaperone function. *Proc. Natl Acad. Sci. USA* **107**, 2007–2012. (doi:10.1073/pnas.0910126107)

94 Baldwin, A. J., Loe, H., Robinson, C. V., Kay, L. E. & Benesch, J. L. P. 2011 AlphaB-crystallin polyspecificity is a consequence of unbiased quaternary dynamics. *J. Mol. Biol.* **413**, 297–309. (doi:10.1016/j.jmb.2011.07.016)

95 Fändrich, M., Tito, M. A., Leroux, M. R., Rostom, A. A., Hartl, F. U., Dobson, C. M. & Robinson, C. V. 2000 Observation of the noncovalent assembly and disassembly pathways of the chaperone complex MspGinC by mass spectrometry. *Proc. Natl Acad. Sci. USA* **97**, 14 151–14 155. (doi:10.1073/pnas.240326507)
101 Smith, R. D. & Lightwahl, K. J. 1993 The observation of noncovalent interactions in solution by electrospray- ionization mass-spectrometry—promise, pitfalls and prognosis. *Bioil. Mass Spectrom.* **22**, 493–501. (doi:10.1002/bms.1200220902)

102 Smith, R. D., Lightwahl, K. J., Winger, B. E. & Loo, J. A. 1992 Preservation of noncovalent associations in electrospray ionization mass-spectrometry—multiply charged polypeptide and protein dimers. *Org. Mass Spectrom.* **27**, 811–821. (doi:10.1016/0029-6637(92)80053-G)

103 Hossain, B. M. & Konermann, L. 2006 Pulsed hydrogen/deuterium exchange MS/MS for studying the relationship between noncovalent protein complexes in solution and in the gas phase after electrospray ionization. *Anal. Chem.* **78**, 1613–1619. (doi:10.1021/ac051687e)

104 Lane, L. A., Ruotolo, B. T., Robinson, C. V., Favrin, G. & Benesch, J. L. P. 2009 A Monte Carlo approach for assessing the specificity of protein oligomers observed in nanoelectrospray mass spectrometry. *Int. J. Mass Spectrom.* **283**, 169–177. (doi:10.1016/j.ijms.2009.03.006)

105 Sun, J., Kitova, E. N., Sun, N. & Klassen, J. S. 2007 Method for identifying nonspecific protein–protein interactions in nanoelectrospray ionization mass spectrometry. *Anal. Chem.* **79**, 8301–8311. (doi:10.1021/ac0709347)

106 Ding, J. M. & Anderegg, R. J. 1995 Specific and nonspecific dimer formation in the electrospray- ionization mass-spectrometry of oligomucleotides. *J. Am. Soc. Mass Spectrom.* **6**, 159–164. (doi:10.1016/1044-0355(94)00102-6)

107 Cunniff, J. B. & Vousos, P. 1995 False positives and the detection of cyclodecetrin inclusion complexes by electrospray mass-spectrometry. *J. Am. Soc. Mass Spectrom.* **6**, 437–447. (doi:10.1016/1044-0355(95)00053-G)

108 Robinson, C. V., Chung, E. W., Krugelund, B. B., Knudsen, J., Aplin, R. T., Poulsen, F. M. & Dobson, C. M. 1996 Probing the nature of noncovalent interactions by mass spectrometry: A study of protein–CoA ligand binding and assembly. *J. Am. Chem. Soc.* **118**, 8646–8653. (doi:10.1021/ja960211x)

109 Daniel, J. M., Fries, S. D., Rajagopalan, S., Wendent, S. & Zenobi, R. 2002 Quantitative determination of noncovalent binding interactions using soft ionization mass spectrometry. *Int. J. Mass Spectrom.* **216**, 1–27. (doi:10.1016/S1387-3860(02)00585-7)

110 Rich, C., Baer, S., Jecklin, M. C. & Zenobi, R. 2010 Probing the hydrophobic effect of noncovalent complexes by mass spectrometry. *J. Am. Soc. Mass Spectrom.* **21**, 286–289. (doi:10.1016/j.jasms.2009.10.012)

111 Liu, L., Bagal, D., Kitova, E. N., Schnier, P. D. & Klassen, J. S. 2009 Hydrophobic protein- ligand interactions preserved in the gas phase. *J. Am. Chem. Soc.* **131**, 15980–15981. (doi:10.1021/ja9060454)

112 Sharon, M. & Robinson, C. V. 2011 A quantitative perspective on hydrophobic interactions in the gas-phase. *Curr. Proteomics.* **8**, 47–58. (doi:10.2174/157016411794697363)

113 Barrera, N. P. & Robinson, C. V. 2011 Advances in the mass spectrometry of membrane proteins: from individual proteins to intact complexes. *Annu. Rev. Biochem.* **80**, 247–271. (doi:10.1146/annurev-biochem-062309-093307)

114 Breuker, K. & McLafferty, F. W. 2008 Stepwise evolution of protein native structure with electrospray into the gas phase, 10-(12) to 10(2) s. *Proc. Natl Acad. Sci. USA* **105**, 18145–18152. (doi:10.1073/pnas.0807005105)

115 Benesch, J. L. P., Ruotolo, B. T., Simmons, D. A. & Barrera, N. P., Morgner, N., Wang, L., Saibil, H. R. & Robinson, C. V. 2010 Separating and visualising protein assemblies by means of preparative mass spectrometry and microscopy. *J. Struct. Biol.* (doi:10.1016/j.jsb.2010.03.004)

116 Szu¨dak, G., Bothner, B., Yeager, M., Brugidou, C., Fauquet, C. M., Hoey, K. & Chang, C. M. 1996 Mass spectrometry and viral analysis. *Chem. Biol.* **3**, 45–48. (doi:10.1016/S1074-5521(96)80083-6)

117 Ouyang, Z., Takats, Z., Blake, T. A., Gologan, B., Guyonno, A. J., Wiseman, J. M., Oliver, J. C., Jo Davison, V. & Cunniff, J. B. 1995 False positives and the association between noncovalent protein complexes in solution and in the gas phase after electrospray ionization. *Anal. Chem.* **78**, 1613–1619. (doi:10.1021/ac051687e)

119 Oomens, J., Polfer, N., Moore, D. T., van der Meer, L., Marshall, A. G., Eyer, J. R., Meijer, G. & van Helden, G. 2005 Charge-state resolved mid-infrared spectroscopy of a gas-phase protein. *Phys. Chem. Chem. Phys.* **7**, 1345–1348. (doi:10.1039/b502323)

120 Meyer, T., de la Cruz, X. & Orozco, M. 2009 An atomistic view to the gas phase proteome. *Structure* **17**, 88–95. (doi:10.1016/j.str.2008.11.006)

121 Scarf, C. A., Thallassinos, K., Hilton, G. R. & Scrivens, J. H. 2008 Travelling wave ion mobility mass spectrometry studies of protein structure: biological significance and comparison with X-ray crystallography and nuclear magnetic resonance spectroscopy measurements. *Rapid Commun. Mass Spectrom.* **22**, 3297–3304. (doi:10.1002/rcm.3737)

122 Shelimov, K. B., Clemmer, D. E., Hudgins, R. R. & Jarrold, M. F. 1997 Protein structure in vacuo: gas-phase confirmation of BPTI and cytochrome c. *Proc. Natl Acad. Sci. USA* **94**, 6057–6060. (doi:10.1073/pnas.94.12.6057)

123 Baldwin, A. J., Graham Cooks, R., Davis, V. & Davisson, V. & Graham Cooks, R. 2003 Preparing protein microarrays by soft-landing of mass-selected ions. *Science.* **301**, 1351–1354. (doi:10.1126/science.1088776)

124 Badman, E. R., Hoaglund-Hyzer, C. S. & Clemmer, D. E. 2004 MS/MS of oligonucleotides. *Anal. Chem.* **76**, 1351–1354. (doi:10.1021/ac049808d)

125 Koeniger, S. L., Merenbloom, S. I. & Clemmer, D. E. 2006 Evidence for many resolvable structures within conformation types of electrosprayed ubiquitin ions. *J. Phys. Chem. B* **110**, 7017–7021. (doi:10.1021/jp056165h)

126 Ruotolo, B. T., Giles, K., Campuzano, I., Sandercock, A. M., Bateman, R. H. & Robinson, C. V. 2005 Evidence for macromolecular protein rings in the absence of bulk water. *Science.* **310**, 1658–1661. (doi:10.1126/science.1120177)

127 Zhou, M. et al. 2008 Mass spectrometry reveals modularity and a complete subunit interaction map
159 Hogan Jr, C. J. & de la Mora, J. F. 2011 Ion mobility measurements of nonadenated 12–150 kDa proteins and protein multimers by tandem dwell mass spectrometry (DAMA-MS). *J. Am. Soc. Mass Spectrom.* **22**, 158–172. (doi:10.1007/s13361-010-0014-7)

160 Wright, P. J. & Douglas, D. J. 2009 Gas-phase H/D exchange and collision cross sections of hemoglobin mono- mers, dimers, and tetramers. *J. Am. Soc. Mass Spectrom.* **20**, 484–495. (doi:10.1016/j.jsams.2008.11.006)

161 Giles, K., Pringle, S. D., Worthington, K. R., Little, D., Wildgoose, J. L. & Bateman, R. H. 2004 Applications of a travelling wave-based radio-frequency-only stacked ring ion guide. *Rapid Commun. Mass Spectrom.* **18**, 2401–2414. (doi:10.1002/rcm.1641)

162 Giles, K., Williams, J. P. & Campuzano, I. 2011 Enhancements in travelling wave ion mobility resolution. *Rapid Commun Mass Spectrom.* **25**, 1559–1566. (doi:10.1002/rcm.5013)

163 Pringle, S. D., Giles, K., Wildgoose, J. L., Williams, J. P., Slade, S. E., Thalassinos, K., Bateman, R. H., Bowers, M. T. & Scrivens, J. H. 2007 An investigation of the mobility separation of some peptide and protein ions using a new hybrid quadrupole/travelling wave IMS/oa-ToF instrument. *Int. J. Mass Spectrom.* **261**, 1–12. (doi:10.1016/j.ijms.2006.07.021)

164 Ruotolo, B. T., Benesch, J. L. P., Sandercock, A. M., Hyung, S. J. & Robinson, C. V. 2008 Ion mobility-mass spectrometry analysis of large protein complexes. *Nat. Protoc.* **3**, 1139–1152. (doi:10.1038/nprot.2008.78)

165 Smith, D. P., Knappman, T. W., Campuzano, I., Malham, R. W., Berryman, J. T., Radford, S. E. & Ashcroft, A. E. 2009 Deciphering drift time measurements from travelling wave ion mobility spectrometry-mass spectrometry studies. *Eur. J. Mass Spectrom.* **15**, 113–190. (doi:10.1255/ejms.947)

166 Thalassinos, K., Grabenauer, M., Slade, S. E., Hilton, G. R., Bowers, M. T. & Scrivens, J. H. 2009 Characterization of phosphorylated peptides using travelling wave-based and drift cell ion mobility spectrometry. *Anal. Chem.* **81**, 248–254. (doi:10.1021/ac801916l)

167 Bush, M. F., Hall, Z., Giles, K., Hoyes, J., Robinson, C. V. & Ruotolo, B. T. 2010 Collision cross sections of proteins and their complexes: a calibration framework and database for gas-phase structural biology. *Anal. Chem.* (doi:10.1021/ac1022953)

168 Campuzano, I., Bush, M. F., Robinson, C. V., Beaumont, C. C., Richardson, K. S., Kim, H. & Kim, H. I. 2011 Structural characterization of drug-like compounds by ion mobility mass spectrometry: comparison of theoretical and experimentally derived nitrogen collision cross sections. *Anal. Chem.* (doi:10.1021/ac2026256)

169 Valentine, S. J., Counterman, A. E. & Clemmer, D. E. 1999 A database of 660 peptide ion cross sections: use of intrinsic size parameters for bona fide predictions of cross sections. *J. Am. Soc. Mass Spectrom.* **10**, 1188–1211. (doi:10.1016/S1044-0335(99)00079-3)

170 Shvartsburg, A. A. & Smith, R. D. 2008 Fundamentals of travelling wave ion mobility spectrometry. *Anal. Chem.* **80**, 9689–9699. (doi:10.1021/ac8016295)

171 Blechholzer, C., Wytenbach, T. & Bowers, M. T. 2011 A novel projection approximation algorithm for the fast and accurate computation of molecular collision cross sections (I). Method. *Int. J. Mass Spectrom.* **308**, 1–10. (doi:10.1016/j.ijms.2011.06.014)

172 Mesleh, M. F., Hunter, J. M., Shvartsburg, A. A., Schatz, G. C. & Jarroll, M. F. 1996 Structural information from ion mobility measurements: effects of the long-range potential. *J. Phys. Chem.* **100**, 16082–16086. (doi:10.1021/jp961623v)

173 von Helden, G., Hsu, M. T., Gotts, N. & Bowers, M. T. 1993 Carbon cluster cations with up to 84 atoms—structures, formation mechanism, and reactivity. *J. Phys. Chem.* **97**, 8182–8192. (doi:10.1021/jp01033a011)

174 Williams, J. P., Lough, J. A., Campuzano, I., Richardson, K. & Sadler, P. J. 2009 Use of ion mobility mass spectrometry and a collision cross-section algorithm to study an organometalllic ruthenium anticancer complex and its adducts with a DNA oligonucleotide. *Rapid Commun. Mass Spectrom.* **23**, 3563–3569. (doi:10.1002/rcm.4285)

175 Mack, E. 1925 Average cross-sectional areas of molecules by gaseous diffusion methods. *J. Am. Chem. Soc.* **47**, 2468–2482. (doi:10.1021/ja01687a007)

176 Shvartsburg, A. A. & Jarroll, M. F. 1996 An exact hard-spheres scattering model for the mobilities of polyatomic ions. *Chem. Phys. Lett.* **261**, 86–91. (doi:10.1016/0009-2614(96)00941-4)

177 Shvartsburg, A. A., Schatz, G. C. & Jarroll, M. F. 1998 Mobilities of carbon cluster ions: critical importance of the molecular attractive potential. *J. Chem. Phys.* **108**, 2416–2423. (doi:10.1063/1.475625)

178 Jurneczko, E. & Barran, P. E. 2011 How useful is ion mobility mass spectrometry for structural biology? The relationship between protein crystal structures and their collision cross sections in the gas phase. *Analyst* **136**, 20–28. (doi:10.1039/c0an0373e)

179 Ayed, A., Krutchinsky, A. N., Ens, W., Standing, K. G. & Duckworth, H. W. 1998 Quantitative evaluation of protein–protein and ligand–protein equilibria of a large allosteric enzyme by electrospray ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **12**, 339–344. (doi:10.1002/(SICI)1097-0231(19980415)12:7<339::AID-RCM163>3.0.CO;2-6)

180 Konermann, L. & Liu, J. J. 2011 Protein–protein binding affinities in solution determined by electrospray mass spectrometry. *J. Am. Soc. Mass Spectrom.* **22**, 408–417. (doi:10.1016/s1336-1010-00521-1)

181 Fraser, G. W. 2002 The ion detection efficiency of micro-channel plates (MCPs). *Int. J. Mass Spectrom.* **215**, 13–30. (doi:10.1016/S1387-3806(01)00553-X)

182 Zheng, J. & Shamsi, S. A. 2003 Combination of chiral capillary electrophromatography with electrospray ionization mass spectrometry: method development and assay of warfarin enantiomers in human plasma. *Anal. Chem.* **75**, 6295–6305. (doi:10.1021/ac0301983)

183 Ketch, C. A., Hernandez, H., Sterling, A., Baumann, M., Allen, M. H. & Robinson, C. V. 2003 Use of a microchip device coupled with mass spectrometry for ligand screening of a multi-protein target. *Anal. Chem.* **75**, 4937–4941. (doi:10.1021/ac304251c)

184 Zhou, M. & Robinson, C. V. 2010 When proteomics meets structural biology. *Trends Biochem. Sci.* **35**, 522–529. (doi:10.1016/j.tibio.2010.04.007)

185 Sharon, M., Mao, H., Boeri Erba, E., Stephens, E., Zheng, N. & Robinson, C. V. 2009 Symmetrical modularity of the COP9 signalosome complex suggests its multifunctionality. *Structure* **17**, 31–40. (doi:10.1016/j.str.2008.10.012)

186 Wu, S., Lorette, N. M., Tolic, N., Zhao, R., Robinson, E. W., Tolmachev, A. V., Smith, R. D. & Pasa-Tolic, L. 2009 An integrated top-down and bottom-up strategy for broadly characterizing protein isoforms and modifications. *J. Proteome Res.* **8**, 1347–1357. (doi:10.1021/ pr800720d)

187 Cox, J. & Mann, M. 2011 Quantitative, high-resolution proteomics for data-driven systems biology. *Annu. Rev.*
Biochem. **80**, 273–299. (doi:10.1146/annurev-biochem-061308-093216)

188 Nilsson, T., Mann, M., Aebersold, R., Yates 3rd, J. R., Bairoch, A. & Bergeron, J. J. 2010 Mass spectrometry in high-throughput proteomics: ready for the big time. *Nat. Methods.* **7**, 681–685. (doi:10.1038/nmeth0910-681)

189 Pagel, K., Kupser, P., Bierau, F., Polfer, N. C., Steill, J. D., Oomens, J., Meijer, G., Koksch, B. & von Helden, G. 2009 Gas-phase IR spectra of intact α-helical coiled coil protein complexes. *Int. J. Mass Spectrom.* **283**, 161–168. (doi:10.1016/j.ijms.2009.02.026)

190 Hill, H. H., Dwivedi, P., Wu, C., Matz, L. M., Clowers, B. H. & Siems, W. F. 2006 Gas-phase chiral separations by ion mobility spectrometry. *Anal. Chem.* **78**, 8200–8206. (doi:10.1021/ac0608772)

191 Seibert, M. M., Ekeberg, T., Maia, F. R., Svenda, M., Andreasson, J., Jonsson, O. *et al.* 2011 Single mimivirus particles intercepted and imaged with an X-ray laser. *Nature* **470**, 78–81. (doi:10.1038/nature09748)

192 Robinson, C. V., Sali, A. & Baumeister, W. 2007 The molecular sociology of the cell. *Nature* **450**, 973–982. (doi:10.1038/nature06523)

193 Griffiths, W. J. & Wang, Y. 2009 Mass spectrometry: from proteomics to metabolomics and lipidomics. *Chem. Soc. Rev.* **38**, 1882–1896. (doi:10.1039/b618553n)

194 Moini, M. & Nguyen, A. 2008 Analysis of major protein–protein and protein–metal complexes of erythrocytes directly from cell lysate utilizing capillary electrophoresis mass spectrometry. *Anal. Chem.* **80**, 7169–7173. (doi:10.1021/ac801158q)

195 Ramsey, J. M., Mellors, J. S., Jorabchi, K. & Smith, L. M. 2010 Integrated microfluidic device for automated single cell analysis using electrophoretic separation and electrospray ionization mass spectrometry. *Anal. Chem.* **82**, 967–973. (doi:10.1021/ac902218y)

196 Takats, Z., Wiseman, J. M., Gologan, B. & Cooks, R. G. 2004 Mass spectrometry sampling under ambient conditions with desorption electrospray ionization. *Science* **306**, 471–473. (doi:10.1126/science.1104404)

197 Ferguson, C. N., Benchaaar, S. A., Miao, Z., Loo, J. A. & Chen, H. 2011 Direct ionization of large proteins and protein complexes by desorption electrospray ionization-mass spectrometry. *Anal. Chem.* **83**, 6468–6473. (doi:10.1021/ac201390w)

198 McLean, J. A. 2009 The mass-mobility correlation redux: the conformational landscape of anhydrous biomolecules. *J. Am. Soc. Mass Spectrom.* **20**, 1775–1781. (doi:10.1016/j.jasms.2009.06.016)

199 Cowieson, N. P., Kobe, B. & Martin, J. L. 2008 United we stand: combining structural methods. *Curr. Opin. Struct. Biol.* **18**, 617–622. (doi:10.1016/j.sbi.2008.07.004)

200 Russel, D., Lasker, K., Phillips, J., Schneiderman-Duhovny, D., Velazquez-Muriel, J. A. & Sali, A. 2009 The structural dynamics of macromolecular processes. *Curr. Opin. Cell Biol.* **21**, 97–108. (doi:10.1016/j.ceb.2009.01.022)