The field of ion mobility mass spectrometry (IM-MS) has developed rapidly in recent decades, with new fundamental advances underpinning innovative applications. This has been particularly noticeable in the field of biomacromolecular structure determination and structural biology, with pioneering studies revealing new structural insight for complex protein assemblies which control biological function. This perspective offers a review of recent developments in IM-MS which have enabled expanding applications in protein structural biology, principally focusing on the quantitative measurement of collision cross sections and their interpretation to describe higher order protein structures.

1 | INTRODUCTION

There is no doubt that mass spectrometry (MS) has had an enormous impact in biological research. In particular, the emergence of proteomics has led to major technological advances and workflow designs that allow for identification and quantification of ever larger sets of proteins, which in turn has revolutionized our understanding of the proteome and its diverse roles in biological function. Such advances have not only benefitted high-throughput proteomics, but have also expanded MS into the arena of molecular and structural biology. Today, MS methods are increasingly utilized to describe the three-dimensional structures and binding interactions of large biomolecules, with the aim to improve our understanding of biomolecular assemblies and their function.

In parallel with progress in biological MS, developments in ion mobility (IM) in recent years have seen increasing applications of this technology in biological fields. Strong synergies between MS and IM exist due to their ability to describe complementary information for gas-phase ions. MS separates ions based on their mass-to-charge \( m/z \) ratio, which gives details of the identity and molecular composition of an analyte. In contrast, IM separates ions based on their differential mobility through a buffer gas, and therefore provides an additional dimension of separation that may enable resolution of otherwise indistinguishable ions (such as isobaric structural isomers) or can determine structural and dynamic information by measurement of the rotationally averaged collision cross section (CCS). Although the coupling of IM and MS was first demonstrated as early as 1961,\(^1\) routine use of hybrid IM-MS instrumentation has largely only become possible in recent decades due to improved methods for confining, focusing and transmitting ions across the variable pressure regimes of the two separation technologies.

IM-MS is generally utilized in one of three ways; as an added dimension of separation for increasing peak capacity, as an extra metric for analyte identification, and as a geometric constraint for generation of structural models. The latter application explicitly relies on converting the IM measurement (commonly drift time) to a quantitative measurement of CCS in order to represent the fundamental size and shape preferences of analytes in the gas phase. While it is clear that the coupling of IM with MS can significantly enhance analytical experiments as defined by a range of measures, including speed, sensitivity, selectivity and dynamic range, this perspective focuses on the advances in IM-MS technology enabling quantitative CCS measurement of proteins and protein complexes, and recent efforts to interpret this data for the generation of structural models. In particular, it highlights a number of important considerations to ensure that the CCS value is meaningful and informative in biomacromolecular structure determination to reinforce the role and potential of IM-MS in structural biology.

2 | BASIC PRINCIPLES OF CCS MEASUREMENT

In general terms, IM separates ions based on the rate at which they migrate through a buffer gas under the influence of an external electric field. Importantly, the mobility of an ion is related to structural features
such as charge and CCS, the latter of which can be used to infer information about three-dimensional conformations. Modern IM-MS instrumentation utilizes this basic separation principle in a variety of ways, and in combination with a range of MS platforms. For biomacromolecules, the IM separation technologies available for quantitative size determination are essentially of four types; drift tube, travelling wave, trapped ion and differential mobility analyzers. Aspects of these IM-MS techniques and their applications have been described in detail in several recent notable reviews. Consequently, only a brief summary of these IM separation approaches is provided here.

2.1 Drift tube ion mobility

Time-dispersion forms the basis of separation in the oldest and conceptually simplest form of IM spectrometry, namely drift tube ion mobility spectrometry (DTIMS). Here, ion packets are gated into a drift tube (with lengths of single linear drift tubes typically ranging from a few centimeters up to two meters), where they are propelled by a static uniform low electric field. Usually a neutral, inert gas such as helium, argon or nitrogen at pressures of 1–15 mbar is used to fill the drift tube, which restricts ion motion. The velocity of ions is related to their mobility in the buffer gas, which, in the ‘low field limit’ where the ratio between electric field strength and gas density is small ($\leq 2 \times 10^{-17}$ V cm$^2$), is proportional to the ion CCS ($\Omega$) according to the Mason-Schamp equation:

$$\Omega = \frac{3z e \sqrt{2\pi}}{16N \mu k_B T} \frac{1}{K_0}$$

where $K_0$ is the reduced mobility (measured at standard temperature and pressure), $z$ is the charge state of the ion, $e$ is the elementary charge, $N$ is the number density of the drift gas, $\mu$ is the reduced mass of the ion-neutral drift gas pair, $k_B$ is the Boltzmann constant and $T$ is the gas temperature.

The CCS parameter represents the rotationally averaged surface area of the ion which is available for interaction with the buffer gas; a smaller CCS results in fewer collisions with the buffer gas which otherwise impede ion motion, and therefore a shorter drift time through the drift tube. Consequently, measuring the drift time or arrival time distribution of a population of ions provides gas-phase structural information (Figure 1A). Although DTIMS offers comparatively high IM resolving power (often defined in terms of the centroid arrival time...
of the IM peak, normalized to peak width, i.e. \( t / \Delta t \) it suffers from poor ion transmission efficiency, which, until more recent developments, limited applications in structural biology. Newer generation DTIMS-MS instruments have encompassed noteworthy innovations to the basic drift tube design, including the use of electrostatic and electrodynamic fields to accumulate ions before the drift cell\(^{10} \) and focus radially diffuse ions.\(^{11-13} \) Tandem DTIMS has also been used to interrogate mobility-selected ions in a manner analogous to MS/MS experiments, and assess gas-phase stability and microconformational states.\(^{14,15} \)

2.2 Travelling wave ion mobility

Travelling wave ion mobility spectrometry (TWIMS), first reported by Giles and co-workers in 2004\(^{16} \) offers an alternative approach for time-dispersive IM. In a TWIMS device, a series of planar ring electrodes are arranged orthogonally to the ion transmission axis, and radiofrequency (RF) voltages of opposite phases are applied to adjacent electrodes to radially confine the ions. A transient direct current (DC) voltage is then superimposed on the RF, applied to each electrode in succession to induce a series of potential hills or so-called ‘traveling waves’. Mobility separation is achieved as interaction with the buffer gas present in the TWIMS sector gives rise to a drag force, meaning those ions with greater CCS (low mobility) slip behind the wave front more frequently (also known as ‘roll over’) and therefore take longer to traverse the mobility cell (Figure 1B).

As the applied electric field is not constant in TWIMS, the direct relationship between CCS and mobility is no longer applicable, and hence CCS cannot be measured empirically. Attempts have been made to describe the fundamentals of TWIMS using \textit{a priori} theory and numerical simulations of ion dynamics;\(^{17,18} \) however, these approaches are not yet applicable to macromolecular ions. Consequently, drift time is correlated to CCS through an exponential relationship, derived by calibration of the IM cell using reference ions of known CCSs under defined conditions (gas type/pressure, travelling wave speed/height, etc.).\(^{19} \) Nevertheless, the early commercialization of the TWIMS technology, and its suitability for observing high m/z ions mean that the majority of IM-MS datasets for biomacromolecules generated to date have used these TWIMS instruments.

2.3 Trapped ion mobility

Trapped ion mobility spectrometry (TIMS) coupled with MS is a relatively recent addition to the field. Rather than moving ions through a stationary gas, as in DTIMS, ion separation in TIMS is achieved by holding ions stationary in a moving column of gas, and selectively ejecting these ions based on differences in mobility. A TIMS device is a modified ion funnel, through which ions are propelled by a gas flow in the presence of an axially variable, opposing electric field (Figure 1C). An ion with a mobility of \( K \) is trapped at the point where the electric field strength is such that the ion drift velocity equals the carrier gas flow velocity. Following the trapping event, ions are sequentially eluted with ascending mobilities by gradually reducing the electric field strength. A key advantage of this approach is that the physical dimensions of the IM cell can be significantly reduced (approximately 5 cm) while achieving high resolving power (\( R \sim 300 \)), duty cycle (100%), and efficiency (\( \sim 80\% \)).\(^{6} \) Importantly, the fundamental physics of TIMS is the same as that in DTIMS and the relationship between mobility and CCS still applies; hence, it is possible to measure ion CCS using first principles for structural characterization.

2.4 Differential mobility analyzers

Differential mobility analyzers (DMAs) operate by introducing ions into a stream of buffer gas flowing with a fixed velocity in the region of an external magnetic field, causing the ions to follow trajectories dependent on their mobility. In a simple geometry, a uniform electric field is applied between two parallel plates, with the ion velocity normal to the electric field. Ions are introduced via an inlet slit and exit through an offset aperture at a fixed distance downstream. The voltage between the two plates is scanned to sequentially sample ions of selected mobility (Figure 1D). Consequently, rather than measuring ion drift time, DMAs measure the voltage required to transmit the ions, which is inversely proportional to mobility. Conversion of the DMA voltage measurement to CCS requires a rigorous calibration methodology.\(^{20-22} \) Nevertheless, DMAs have been utilized for size measurement of a range of protein assemblies,\(^{22-24} \) although many of these have been in the absence of coupled MS measurements. The performance of DMAs with nanometer-size objects is severely limited by Brownian motion; however, resolving powers in excess of 50 have recently been described using high-transmission parallel-plate DMAs,\(^{25} \) which can be coupled as a front-end device to almost any mass spectrometer with an atmospheric pressure ion source. Consequently, unlike most other IM separations, the DMA can be used to measure the mobility of electrosprayed ions without the need for energetic confinement or declustering, or even vacuum interfaces.

2.5 IM-MS for the masses

To date, both DTIMS and TWIMS IM-MS instruments have been commercialized, utilizing quadrupole time-of-flight platforms (QTOF) for mass analysis. TWIMS was released as a commercial system in 2006 by Waters (Synapt HDMS) and this resulted in an escalation in the use of IM-MS for the study of proteins and protein assemblies. This instrument has since undergone multiple design revisions to alter both the traveling potential waveform and the drift region pressure, thereby increasing IM resolution (Synapt G2), and the source and ion transfer optics to improve ion transmission (Synapt G2-S and G2-Si). Agilent Technologies released the first commercial high-performance drift tube IM-MS instrument in 2014 (6560 IM-QToF), which offers an ion mobility resolving power of \( \sim 70 \) (close to the theoretical diffusion limit\(^{26} \)) and allows for intrinsic measurement of CCS to a precision better than 0.5%. TIMS-MS was commercialized by Bruker, with the first instrument launched only in 2016, and consequently the impact of this technology is yet to be fully established.

Other types of IM-MS technology have yet to be utilized in the measurement of CCS for proteins and other biomolecules. For example, high-field asymmetric waveform ion mobility spectrometry (FAIMS) interfaces are commercially available and are among the
highest resolving devices attainable. However, FAIMS separation relies on differences in mobilities at high and low electric field, and the theory of this separation is currently quite poorly understood with the exact characteristics controlling the mobility of an ion at high field apparently independent of \( m/z \) and the low-field CCS. Consequently, structural dimensions such as CCS cannot currently be directly and reliably extracted from the data. Other high-resolution IM technologies are emerging, including overtone IM\(^{27} \) and cyclotron-type DTIMS analyzers.\(^{28} \) While these are yet to be utilized for the description of biomacromolecular structure, they offer exciting possibilities for enhanced analysis in future.

An interesting review of CCS measurements in the scientific literature has been made recently, surveying the over 24,000 CCS values reported from 1975 to 2015, to provide both a historical and an analytical context for studies to date and insight into future directions of the field.\(^{29} \) This work highlights the surge of publications since 2010 which use quantitative IM measurements, attributed to the relatively recent commercialization of IM-MS instrumentation which combines the advantages of both analytical strategies. With regard to instrument type, 87% of measurements were conducted using DTIMS, probably due to the direct relationship between drift time and CCS, with 9% recorded on TWIMS instruments and the remainder from other IM techniques such as DMA and TIMS. Furthermore, it is noted that 70% of the CCS values reported are for peptides and proteins, reiterating the importance of this technology in analytical biochemistry.

3 | INTERPRETING CCS DATA FOR PROTEIN STRUCTURE DETERMINATION

Generating a description of protein structure from IM-MS data requires significant analysis, much of which relies on supporting computational software. In most IM-MS studies of protein structure, protein connectivities and topologies are defined in different stages on the basis of varying experimental data (Figure 2). First, it is necessary to obtain the identities of the constituent protein(s) within a sample, usually achieved through liquid chromatography (LC)/MS protein identification experiments (‘bottom up’ and ‘top down’ proteomic sequencing), which bring their own set of challenges outside the scope

![Workflow for an IM-MS approach to protein structural biology. Following purification or recombinant production of the protein complex, the sample is subjected to three main streams of analysis. For unknown proteins, denaturation and enzymatic digestion allow for identification through a combination of top down and bottom up proteomics experiments, forming a component list of the protein complex. Mass measurement of the intact complex and sub-complexes formed through solution- or gas-phase disruption gives a subunit interaction map utilizing native MS approaches. CCS measurement by native IM of the complex and sub-complexes gives size information that can refine structures for both multi-domain monomers and protein assemblies, which can be combined with the interaction map to define the topology of the complex. Incorporation of available high-resolution data or structural modelling can finally provide an atomic model.](#)
of this discussion. Mass measurement by native MS then gives an indication of binding interactions and stoichiometries in quaternary structures. Often, however, this is complicated by the decreased mass accuracy and resolution achieved as a result of incomplete desolvation following ionization, although multiple software platforms have been developed for the deconvolution of overlapping charge state distributions in protein electrospray mass spectra, along with automated mass analysis and measurement of relative peak intensities.30,31

Typically for protein assemblies, two-dimensional contact maps are first generated by observation of proteins and sub-complexes produced upon disruption of intact assemblies. Continued focus on gas-phase disruption methods, for example, high-energy collision-induced dissociation following extensive charge reduction or amplification,32 surface-induced dissociation33 and electron-based fragmentation, offers great promise for both top down type identification of protein complexes by MS as well as for the construction of protein interaction maps in IM-MS structural studies. Currently, however, such gas-phase technologies do not provide sufficient information to deduce a complete description of protein complex topologies, and complementary solution-phase disruption methods are utilized to generate sub-complexes for detection by IM-MS. Since the physical principles of these disruption experiments are still poorly understood, it is typically necessary to perform a relatively exhaustive search for optimal solution conditions, such as organic solvent composition, ionic strength and pH, that give rise to appropriate sub-complex generation yet are still compatible with IM-MS analysis.34 It is hoped that ongoing efforts to predict disruption behavior will contribute to reducing this bottleneck and further expand the application of IM-MS to protein structure determination. This stage of analysis can also benefit from quantitative IM-MS measurements to evaluate successful protein contact map generation. For example, CCS measurements were used to confirm the connectivity of a trimeric subcomplex of the 13 protein eukaryotic initiation factor 3 (eIF3), which then allowed the model of the entire assembly to be restrained with high confidence.35

Once individual proteins, complexes and sub-complexes have been identified, distance and size constraints from IM-MS measurements are then integrated (often with other experimental biophysical datasets) with homology or partial atomic-resolution data to describe a three-dimensional structural model. The interpretation of IM data therefore commences with conversion of the IM measurement into a CCS value or range. The accuracy and precision with which this CCS value can be determined have important consequences for both the accuracy of structures determined and the level of detail that can be described. Consequently, more detailed discussion of this aspect is provided in section 4.

Following accurate CCS measurement model structures must be generated, either in silico or by utilizing other available high-resolution structural data such as X-ray and nuclear magnetic resonance (NMR) structures, and their theoretical CCS values calculated for comparison with experimental measurements. Many computational approaches have been developed to predict the CCSs of model structures; however, accurately computing CCSs with explicit consideration of the scattering process is challenging and computationally expensive for large molecules particularly, due to contributions of long-range polarization and multiple and diffuse scattering effects, among other considerations. For this reason, macromolecular structures are commonly modelled using non-explicit methods, such as the ‘projection approximation’ (PA)26,37 and ‘projection superposition approximation’ (PSA),38 or using hard-sphere collisions methods such as in ‘exact hard sphere scattering’ (EHSS).39

Simple methods such as the PA essentially consider the ‘shadow’ of spherical model atoms to find the two-dimensional area of the molecule, which is averaged over a defined number of orientations. They are computationally inexpensive and compatible with coarse-grained modelling approaches. Such algorithms have been used effectively for a number of years to correlate IM measurement with protein topologies. Provided an appropriately tuned scaling factor is applied which takes into account the general underestimation of CCS for large molecules by this approach,40 PA methods are well established as accurate representations of the IM analysis of biomolecules and their complexes. The most explicit method for calculating CCSs of large biomolecules currently available, the ‘diffuse trajectory method’,41 uses a hybrid of classical scattering trajectories and stochastic modelling of inelastic scattering to account for energy transfer between the ion and neutral; however, this tends to overestimate CCSs for large biomolecules by approximately 5%.42 Efforts to improve the accuracy and efficiency of CCS calculation for large molecules have resulted in software such as the Ion Mobility Software (IMos) Suite, offering combinations of potential interaction and CCS calculation methods.43 More recently, a trajectory method-based CCS calculator has been described which utilizes parallel processing and optimized trajectory sampling, and implements both He and N2 as collision gas options, to enable detailed IM-MS analysis of large ions to be tractable at a high level of sophistication.44

It is not fully understood how important it is to accurately model the charge distribution of an analyte ion when calculating CCS, and to date this has largely been poorly considered when generating theoretical CCS values for large ions. However, the ion charge density is intimately linked to the contribution of glancing collisions, and a recent investigation suggests accurate structure elucidation on the basis of IM-MS measurements must account for charge distribution when localized charges are present, even for systems as large as 12 kDa.45 This work also provides evidence that accurate structure elucidation is unlikely if CCS values recorded in one buffer gas are converted into other buffer gases when the electronic properties of the two gases differ.

The CCS distribution (reflected by the full width at half maximum of the mobility measurement, provided due consideration has been given to ion diffusion in the drift time domain46) can often be another important experimental indicator not just of the structure, but rather of the structural heterogeneity of proteins and their complexes. Narrow CCS distributions tend to indicate a single predominant conformation, while broader CCS ranges are consistent with conformational flexibility in solution which allows the protein to sample multiple states. Of course, with increased IM resolving power, it is possible to capture in even greater detail the repertoire of often co-existing conformational states adopted by macromolecules. For example, the higher resolution afforded by TIMS allowed, for the first time, the separation of a large number of conformations and delineation of structural transitions for the globular proteins ubiquitin and β-lactoglobulin as a function of charge state.47
4 | CRITICAL EVALUATION OF THE CCS MEASUREMENT

CCS is a macroscopic quantity that, although typically used to describe an intrinsic property of the analyte, is in fact specific to the drift gas as well as the temperature and electric field used during the measurement. The fact that the CCS is not a true molecular cross section, but rather an observational property that averages all geometric orientations and ion-neutral interactions (such as glancing versus head on collisions, single versus multiple collisions within a cavity of the analyte, etc.) across the time frame of the IM separation, means that it is difficult to interpret the true meaning of this parameter. Although IM-MS currently provides the most precise measurement of this parameter, by its nature, the structural information obtained is ‘course-grained’; however, this level of granularity is of less concern when used to probe coarse features such as domain-level protein structures.

The initial requirement for the analysis of proteins and their complexes by IM-MS is the generation of ions. This is largely made possible by soft ionization methods, principally electrospray ionization (ESI), which allows the ions to retain much of their solution-phase structural properties.48,49 However, a major consideration in the quantitative evaluation of CCS determined by IM-MS, particularly where the intent is to compare with solution-relevant structures, is the possible structural rearrangement that can take place over the range of time scales and energies during processes of ESI, desolvation, transport and analysis. For example, a recent systematic study has demonstrated that instrumental parameters in TWIMS can induce substantial variation in CCS measurements arising from both contraction and expansion of observed structures.50 Similarly, gas-phase structures of a range of protein complexes have been shown to be substantially more compact than the corresponding X-ray crystal structure would suggest, often attributed to gas-phase collapse of structures containing open cavities.21 Such reports highlight the importance of measuring the CCS values under conditions that truthfully reflect the native structure in solution, which is arguably not given due consideration in many quantitative IM-MS studies to date.

One important area of research in this regard is the stabilization of protein structures for gas-phase analysis, which has been demonstrated by a range of methods including chemical cross-linking, charge manipulation (especially charge reduction), addition of small molecule additives, and variations in solution salt content. The determination of CCS by DTIMS relies on simple algebraic relationships, while DMA-type and TWIMS analyzers require robust calibration approaches. Therefore, while it is relatively straightforward to derive size measurements from primary data, the drift-time CCS relationship must be determined experimentally during TWIMS data collection and hence is heavily dependent on the availability of high-quality calibrant ions with reference CCS values. This has been aided by recent efforts to expand the available CCSs database for a large set of denatured peptides, denatured proteins, native-like proteins, and native-like protein complex ions that spans over two orders of magnitude.13

Further challenges are associated with comparison of CCS values measured across different platforms. For example, reports have indicated that ions can be susceptible to heating in the high intensity electrical fields of TWIM-MS55 although more recent results suggest that this effect does not influence CCS accuracy or precision, at least in second-generation TWIMS instruments.56 Proteins investigated using DMA technology have appeared to be more compact in the gas phase than previously thought,21 although a broad and critical evaluation of the implications of this data has not yet been carried out. Attention must also be paid to the buffer gas, since ion-neutral interactions are important for determination of CCS. Helium was traditionally used in IM analysis of small analytes, which formed the basis for the development of CCS calculation algorithms. However, other gases such as nitrogen are commonly utilized for IM-MS studies of proteins and protein complexes, and, notably, it has been shown that different buffer gases yield different CCSs for identical conformations of a given ion. Furthermore, different gases can influence the conformations presented by gas-phase ions, particularly for natively folded forms.57 A correct interpretation of IM data on any protein must therefore reconcile these effects, particularly when considering comparison across platforms or between experimental and theoretical values.

5 | HIGHLIGHTS OF QUANTITATIVE ION MOBILITY IN STRUCTURAL BIOLOGY

Characterization of proteins, and in particular the dynamic protein interactions that govern most critical cellular processes, is an ongoing goal in structural biology. High-resolution methods such as X-ray crystallography, NMR spectroscopy and, more recently cryo-electron microscopy (cryoEM) have dominated this field and have been highly successful in providing atomic level insight into a large number of protein complexes and interaction networks. However, intrinsic limitations of these techniques associated with factors such as protein flexibility and heterogeneity highlight the need to develop new approaches aimed at multi-protein structure determination, preferably on a high-throughput scale with broad scope. IM-MS offers promise to be a more sensitive, rapid and universal alternative to traditional
structural biology methods, and can bridge the gap between generation of high-throughput data on protein interactions from proteomics and the structural biology field determining structures for isolated functional macromolecular complexes.

5.1 | Large protein assemblies

Early IM-MS studies of biomacromolecules were largely used to improve understanding of the relationship between solution- and gas-phase structures, by measurement of CCS values for isolated, small proteins. As it became clear that IM-MS can contribute important physiologically relevant details of conformation and dynamics, this method rapidly developed to also study larger and more heterogeneous assemblies. However, a major challenge for IM-MS in structural biology is the generation of accurate model structures for systems with limited or incomplete high-resolution data, and, ideally, from IM-MS constraints alone. First steps towards addressing this challenge have been demonstrated by Robinson and co-workers, through development of a computational approach that combines incomplete atomic structures with experimental constraints derived from CCS measurements to generate complete models of multiprotein complexes. In this work, coarse-grained and homology modelling was used to ‘fill-in’ missing details from high-resolution experimental structures at both subunit and oligomer levels. Topological models were then constructed by determining the packing arrangements via known archetypal shapes and/or crystal symmetries that provide the best fit to the experimental CCS values (Figure 3A). The method therefore refines each building block using experimental data before larger oligomeric complexes are sequentially constructed.

The success of this approach was illustrated by application to multimeric protein complexes within the Escherichia coli replisome. For example, a significant portion (~75%) of the DnaB helicase protein, the primary enzyme in E. coli which coordinates DNA replication by opening double-stranded DNA, was absent from high-resolution structures in the Protein Data Bank (PDB), and the structure of the functional hexamer was not available. Sufficient homology with the corresponding complex from Bacillus subtilis models could be used as a building block for screening archetypal geometries to best fit measured CCSs. Further consideration of structural homologues and IM CCS restraints allowed for construction of a tail-to-tail model of the DnaB dimer, and confirmed that the functional unit of DnaB forms a hexameric ring-like arrangement (Figure 3B). Similar success was achieved with other homo- and heterogeneous complexes of the E. coli replisome, and is likely to become more commonplace as the accuracy and precision of CCS measurements continue to improve.

5.2 | Membrane proteins

Structure determination of membrane protein assemblies in particular continues to define a substantial challenge in structural biology. Membrane proteins constitute approximately 30% of a typical genome, and more than half of all membrane proteins are predicted to be pharmaceutical targets. However, they contribute less than 1% of the structures currently deposited in the PDB. Relatively recent developments in native MS have seen renewed interest in the analysis of membrane proteins by this method, with the realization that membrane proteins can be transferred to the gas phase while encapsulated in lipid or detergent environments, before being
thermally activated to strip away the solubilizing lipid/detergent environment and enable mass or IM separation. While the influence of this activating stage must be carefully considered and controlled in order to minimize structural perturbations that would contribute to flawed CCS measurement, recent examples highlight the exciting possibilities in utilizing IM-MS for structural characterization of membrane protein complexes and the structural changes associated with lipid and other small molecule interactions.

5.3 | Amyloid structure and assembly

Unlike many other biophysical techniques that provide an averaged structure, IM-MS is capable of addressing questions related to structural and dynamic heterogeneity. This represents a unique advantage of the method to monitor conformational transitions and structurally diverse populations, which, for proteins in particular, are often critical to biological function. One clear application in this regard is the understanding of inter- and intramolecular interactions and conformational dynamics that underpin protein folding, misfolding and aggregation, such as in the investigation of amyloid forming proteins. IM-MS is able to report on co-existing conformations and oligomeric populations of such aggregating proteins in a single spectrum, without affecting the ensemble equilibria. Furthermore, many studies have investigated the folding and unfolding of proteins in the gas phase, in principle revealing intrinsic properties of the protein in the absence of solvent.

One of the earliest studies demonstrating the use of CCS measurements to describe amyloid formation was an analysis of early aggregation states of the amyloid beta 42 peptide (Aβ42), the putative neurotoxic species implicated in Alzheimer's disease. Supported by molecular modelling, the results of this investigation were in good agreement with labor-intensive photochemical cross-linking experiments, and follow-up studies gave unique insight into the formation of an Aβ42 dodecamer proposed to be the primary toxic species in Alzheimer's disease. Since this time, many research groups have additionally investigated the fibril-forming properties of amyloid beta peptides by IM-MS (further reviewed in Bleiholder and Blower), although this and other biophysical characterization approaches have to date failed to unambiguously clarify the structural and mechanistic detail by which this process takes place, demonstrating the extreme complexity of the amyloid problem. As an additional example, measurement of experimental CCS values for oligomeric states of the β2 microglobulin protein has given unique insights into their structural characteristics and dynamic transitions, enabling production of a map of the events occurring in the early stages of fibril formation (Figure 4), of critical interest in dialysis-related amyloidosis. IM-MS has further been applied to the study of a wide range of amyloid-forming proteins, as well as their interactions with chaperones, small molecules and metals, demonstrating the potential of IM-MS as a powerful complementary structural biology method for investigation of these challenging systems.

6 | CHALLENGES AND PERSPECTIVES

It is clear that integration of IM with MS offers substantial opportunity to increase the information content of such analyses, whether from the perspective of increased analytical space as a result of complementary separation methods, or, as described in detail here, by quantitative evaluation of the CCS measurement. In this latter regard, IM-MS and the measurement of CCS has clearly been established as a valuable tool in structural biology, as demonstrated by the variety of studies across diverse biological systems presented here.

Advances in instrumentation in recent decades have provided increased IM and MS resolution, as well as high transmission efficiency and sensitivity that benefit analysis of large biomolecules. In addition to continued fundamental progress in the IM domain, including design of novel instrument geometries which allow longer separation paths such as Structures for Lossless Manipulations (SLIM) modules and cyclic traveling wave devices currently in development, it is expected that quantitative IM methods will be more routinely coupled with high-resolution MS measurement using, for example, Fourier transform ion cyclotron resonance (FTICR) and orbitrap mass analyzers. IM devices for coupling with orbitrap mass analyzers already exist, including the recent release of FAIMS-Orbitrap instrumentation. While the task of coupling DTIMS is not trivial due to the slow MS acquisition rates of these mass analyzers in comparison with IM separation times, recent reports suggest this may be soon

FIGURE 4  Model of early aggregation states of β2 microglobulin. Monomeric β2 microglobulin co-populates distinct native-like, partially folded, and unfolded states at acidic pH, while the dimer is a highly dynamic species. Initial association of the monomer to the dimer results in a dynamic trimer that undergoes structural change to a more stable species. The tetramer evolves in a similar manner, first forming with a dynamic structure that subsequently becomes stabilized. Further association proceeds by stacking elongated monomeric subunits. Reproduced with permission from Smith et al
achievable, and would enable the ability to measure subtle variations in CCS for heterogeneous systems of similar mass and size, such as post-translationally modified proteins. A very recent report has even demonstrated the possibility of measuring protein CCSs without the need for an IM cell, by measuring the decay rate of a particular m/z signal in an orbitrap analyzer across the operating pressure range of the instrument. While this approach does not currently match the analytical considerations of dedicated IM-MS methods, it may contribute to an ever greater integration of CCS measurements in structural biology.

An additional promising direction for IM-MS involves coupling with other gas-phase technologies to further enhance the structural information that can be afforded in a single experiment. IM-MS has recently been integrated with gas-phase infrared spectroscopy for protein analysis to provide additional insight at the secondary structure level. Similarly, gas-phase conformations of amyloidogenic peptides have been studied by a combination of fluorescence resonance energy transfer (FRET) and IM, and it can be expected that additional applications will arise. The general trend towards integrative structural biology, whereby data from multiple biophysical methods are combined to provide a more complete description of the system of interest, also provides further drive to integrate IM-MS with other MS-based structural methods such as chemical cross-linking and hydrogen/deuterium exchange as well as other structural biology tools such as cryo-EM.

Outside the academic environment, the pharmaceutical industry represents a commercial sector with potential applications requiring quantitative IM-MS structural characterization of biomolecules. Biopharmaceutically relevant molecules such as monoclonal antibodies and antibody-drug conjugates represent difficult structural targets due to their intrinsic flexibility, which contributes to a lack of full high-resolution coordinate files. Furthermore, a large amount of gas-phase collapse has been demonstrated for monoclonal antibodies, challenging structural interpretation. Consequently, to date most biopharma applications involving proteins have utilized IM-MS in a manner aimed at resolution of structural isomers rather than direct CCS determination, leaving an underexplored application of CCS measurement, for example in evaluation of batch-to-batch variation. Collision-induced unfolding experiments monitored by IM-MS also offer a complementary approach to distinguish subtle differences in large biotherapeutics (and other biomolecules), such as between IgG subclasses with different disulfide bonding patterns.

Despite the current successes and future promise, a number of challenges remain in the quantitative analysis of CCS values from IM-MS experiments, primarily in the area of computational data interpretation. As described above, many sources of potential error still exist, from the measurement of absolute CCS values (particularly for TWIMS) and ensuring physiological relevance of the gas-phase measurement, to the generation of predicted CCS for theoretical structures to enable accurate comparison with experimental constraints. In general, broad agreement has been observed between IM CCS values recorded for proteins and protein complexes with corresponding published X-ray structures. However, while this holds true for the currently studied protein analytes, it is yet to be seen whether existing approaches are suitable to describe the multitude of diverse structures predicted across the proteome. Furthermore, a significant improvement is required in the computational prediction of protein structures or coarse-grained approximations in the absence of X-ray or NMR datasets in order to increase the scope and throughput of structure determination by IM-MS.

In summary, the measurement of protein CCS values by IM-MS must be carefully considered and evaluated. However, it can provide a valuable experimental constraint to define protein structures, often for systems which elude traditional structure biology approaches. With continued technological advancement, and motivation to stretch this technology to ever more complex systems, it is expected that the applications of quantitative IM-MS in structural biology will greatly increase in coming years.

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