The application of ion-mobility mass spectrometry for structure/function investigation of protein complexes

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Abstract

Ion-mobility mass spectrometry (IM-MS) is an approach that can provide information on the stoichiometry, composition, protein contacts and topology of protein complexes. The power of this approach lies not only in its sensitivity and speed of analysis, but also in the fact that it is a technique that can capture the repertoire of conformational states adopted by protein assemblies. Here, we describe the array of available IM-MS based tools, and demonstrate their application to the structural characterization of various protein complexes, including challenging systems as amyloid aggregates and membrane proteins. We also discuss recent studies in which IM-MS was applied towards investigations of conformational transitions and stabilization effects induced by protein interactions.

Introduction

Proteins are inherently dynamic entities that sample multiple conformational states for their functional activities [1,2]. Therefore, a complete understanding of the structure–function relationships of proteins requires experimental methods that can capture the spread of the conformational states they adopt. However, this l complexity can present a significant challenge to many of the 'classical' high-resolution structural biology tools as X-ray crystallography [3], Nuclear Magnetic Resonance (NMR) [4] and Electron Microscopy (EM) [5]. Here, we will focus on a structural technique that can capture the conformational dynamics of a system, Ion-Mobility Mass Spectrometry (IM-MS). While IM-MS does not provide atomic resolution structures , it has the advantage that co-existing populations of a given assembly can be detected within a single spectrum.

IM-MS is a method that couples MS measurements with IM separation (recent reviews include [6–13]. By means of this method, the time it takes for a protein (or its various populated structural states) to transverse a weak electrical gradient in a gas-filled chamber is measured. The drift time depends not only on the mass and charge, but also on the shape of the analyzed protein complex. Larger ions collide more frequently with the neutral gas, hindering their progress and therefore increasing their drift time relative to more compact ions [14] (Figure 1a). This offers a way to distinguish between conformational states of the same protein. As the number of collisions is proportional to the surface area of the protein, the drift time measurement can be used to determine the rotationally averaged collision

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cross-section (CCS) value, indicating the three-dimensional shape of the protein. For intact proteins and protein complexes introduced from non-denaturing solutions (native IM-MS), this approach provides insights into the stoichiometry, composition, connectivity, topology and conformational heterogeneity of the analyzed biomolecules.

Different methods for applying the electric field and introducing the buffer gas gave rise to various IM-MS platforms, such as drift tube (DT) [11], traveling wave (TW) [10], differential mobility [15], transversal modulation [16], overtone [17], field asymmetric [18] and trapped IM-MS [19]. While each method has proven its worth, here we will focus on the two most common types of mobility techniques DT and TW. In DT, a homogeneous, linear electric field is used, and CCS values are determined directly from the measured drift time and the experimental conditions applied [11]. In TW, on the other hand, potential waves continually propagate through the drift tube [10]. Manipulation of the travelling wave frequency and height enables ion separation. Although this method enhances the duty cycle, absolute CCS values cannot be determined, and an indirect calibration procedure is required [20,21]. To date, both DT and TW IM-MS instruments utilizing a quadrupole time-of-flight platform (QTOF) are commercially available, and the application of the technique to structural studies is rapidly increasing [22,23].

Overall, when applying IM-MS, multiple features can be extracted, enabling in-depth analysis (Figure 1b), among them:

i. *Drift Time* – Separating ions according to their drift time adds an extra dimension to conventional MS measurements, yielding a three-dimensional spectrum containing information regarding the mass-to-charge (*m/z*) ratio, abundance of ions, and drift time. Consequently, the ion mobility capability not only provides structural information, but also enables to separate overlapping peaks by distributing the data into a third dimension, thereby allowing analysis of heterogeneous or polydisperse complexes of markedly similar composition.

ii. *Arrival Time Distribution* – The structural heterogeneity of proteins and complexes is reflected in the full width at half maximum of the arrival time distributions (ATDs). Sharp peaks indicate a single conformation, while broader peaks are consistent with multiple states, indicating conformational flexibility of the protein assembly in solution. Thus, the impact of protein interactions and/or substrate binding on the conformational spread of the analyzed protein species may be determined.

iii. *Collision Cross-Section* – By measuring the ATD of each charge state, it is possible to calculate, either directly (DT) or indirectly (TW), CCS values [11,20,21]. The derived CCS represents the effective area of the gas-phase ion that can interact with the drift gas, averaged over all orientations. Thus, it can be related to structural features of the ion. Considering that theoretical CCS values can be calculated for protein structure models [24,25], experimentally determined CCS can be utilized to help discriminate between different possible models. These *in silico* calculations are based on a variety of approximations as to what happens experimentally. The earliest developed [26] and most commonly

used, is the projection approximation algorithm (PA), which essentially considers the 'shadow' that a given molecule would cast on a flat surface as representative of its collision cross section. In order to apply this approach to real molecules, and to the real effect of collisions on their rugged surfaces, empirical scaling factors derived from experiments have been introduced [27]. More accurate treatments calculate the momentum transferred as well as the effect of the interaction potential between the analyte ion and the buffer gas [24,25,28], but such more rigorous approaches require high computational power and are less well trained on large protein complexes.

iv. *Collision Induced Unfolding* – The conformational stability and domain organization of intact proteins can be analyzed by collision induced unfolding (CIU) experiments. In this type of experiment, the collision energy (CE) is elevated in a stepwise manner, causing protein activation that may consequently induce conformational change or partial unfolding. As the activation step takes place before the drift tube, CCS values of compact and extended structures can be obtained for a single *m/z* value, enabling detection and quantification of conformational changes. Usually, the collision voltage at which the transitions between conformations occur, and the CCS values of the intermediate states, are evaluated and compared. Effects of ligand binding or association with protein partners may be visualized by changes in the CIU fingerprint. Similarly, protein isoforms that originate from various diversification mechanisms (i.e., alternative splicing, gene duplication, post-translational modifications) are expected to yield distinct CIU trajectories, providing insights into their different structural characteristics.

Recent studies demonstrating how the IM-MS toolbox might be harnessed for studying the structural properties of protein complexes are detailed below:

Characterization of protein structure

Understanding how protein structure is affected by a single amino acid substitution [29,30] or post-translational modification [31]•, are among the examples to which IM-MS has made valuable contributions. Such comparative structural studies are further enhanced by applying the CIU protocol, capable of probing the different structural characteristics of species with identical drift times. This was recently demonstrated for antibody isoforms possessing different numbers and/or patterns of disulfide bonding and glycosylation levels [32]. Moreover, by combining IM-MS with surface-induced dissociation (SID), which enables the selective disruption of the weaker interfaces of soluble protein complexes, it is possible to glean information on the native structure of both the intact assembly, and its subcomplexes [33]. The application of SID IM-MS has recently been extended to membrane protein complexes, demonstrating that the structure of such complexes and their generated subproducts, are retained within the mass spectrometer, even after having been liberated from detergent micelles [34]• (Figure 2a). A further advancement came with the realization that IM-MS analysis of recombinant protein complexes can be performed directly on crude

cell lysates, saving the time, cost and labor of protein purification, and paving the way toward throughput IM-MS screening of engineered proteins (Figure 2b) [35]•.

On the whole, IM-MS has proven to be a reliable technique that is capable of detecting even subtle structural differences. Nevertheless, it is important to note that different factors may affect the structure of gas phase ions during analysis, and these must be taken into consideration when performing such measurements, and particularly when extracting CCS values. For example, in TW IM-MS experiments, care must be taken when choosing the appropriate calibrants, as the latter should fit the nature and character of the analyzed proteins [36,37]. Attention should also be given to the phenomenon of gas-phase compaction [38], particularly in instances of elongated, non-globular and intrinsically unstructured proteins, or proteins with flexible hinge regions in between structured domains [39,40]. Temperature constitutes an additional factor that may influence the conformational preferences of proteins [41] and protein complexes [42]•. Overall, when acquiring IM-MS data, especially when uncharacterized protein assemblies are studied, we recommend to define first the optimal experimental conditions, in order to ensure preservation of the native-like protein conformations, as previously described [38].

Analysis of protein interactions

The ability to capture multiple coexisting states within a single spectrum has turned IM-MS into a valuable tool for characterizing protein-protein and protein-ligand interactions. Recent examples include epitope mapping of antibody-antigen interactions [43]•, analysis of the changes in structure and stability imposed by protein-ligand binding [44–46], and real-time monitoring of the effect of small-molecule inhibitors on the conformational distribution and activity of enzymes [47]••.

The investigation of the holdase chaperone Skp, and its interactions with a range of outer membrane proteins (OMPs) differing in size, not only serves as an additional, elegant example of the application of IM-MS, but also underscores the integration of the method with computer modeling and molecular dynamics simulations [48]•• (Figure 3). This study indicated that the CCS distribution of Skp in its free state is smaller than would be estimated by its crystal structure, demonstrating that the assembly collapses in the gas phase, possibly because the central cavity of the chaperone is empty. However, Skp binding to an 8-stranded OMP client resulted in CCS values comparable to that predicated by its crystal structure, suggesting that the client, which is sequestered within the central Skp cavity, prevents collapse of the chaperone in the gas phase. For larger OMPs containing 16-strands, which cannot be fully accommodated in the expanded cavity, it was demonstrated that sequestration is achieved by binding of an additional Skp complex to the substrate. Overall, the study provided a novel understanding of the mechanism by which Skp is able to bind, fold and release substrates that vary dramatically in size.

The ability to monitor the effects of ligand binding on protein stability is not limited to small molecules or globular proteins: indeed a method [49] complemented with software [50]•• for quantitative analysis of lipid binding to membrane proteins was recently reported. In this approach, membrane proteins solubilized in different types of lipids were sprayed into the

mass spectrometer and changes in mass and CIU plots were quantified, to assess the effects of these interactions on protein stability. Results indicated that specific lipids stabilize particular membrane proteins in different ways (Figure 4). Moreover, comparison of the IM-MS approach with solution-phase techniques, as circular dichroism and differential scanning fluorimetry, demonstrated that this method is more sensitive, especially for lipid interactions with membrane proteins.

Amyloid structure and assembly

In the last decade, IM-MS has become an important tool in the investigation of amyloids, which are challenging systems due to their polydispersity, polymorphic, and transient nature. The advantage of IM-MS in studying these systems lies in its ability to distinguish and separate the different oligomeric species, without affecting the equilibrium of the ensemble [51,52]. Several recent examples include investigations into the effect of zinc binding on the aggregation of amylin [53], the mechanism of inhibition of amyloid β aggregation [54], and the regulatory mechanism that controls higher-order oligomerization of the microtubule-associated protein tau during its normal and pathological activities [55]. Another interesting study investigated the aggregation profiles of the homotetrameric protein transthyretin, purified directly from human blood serum. Transthyretin is known to misfold, aggregate, and cause different types of amyloidosis. IM-MS measurements pointed to differences in the assembly state and stability of this protein between healthy individuals and symptomatic patients suffering from this disease [56]•.

IM-MS analysis has also been also used to investigate the formation of heterogeneous pre-fibrillar, oligomeric species produced by the co-incubation of the amyloid-β peptide (Aβ-40) with the human islet amyloid polypeptide [57]. Comparison between the conformations, dynamics and relative gas-phase stabilities of homo- and hetero- assembled species showed that cross-polymerization resulted in the formation of unique structures, with distinct degrees of stability and oligomerization rates. Interestingly, the measured stabilities for the hetero-oligomeric species were found to lie between the values measured for each of the homo-oligomers, suggesting that mixed species may exert significant effects on the progress of fibril formation, and thus may have biological consequences *in vivo.*

Recently, IM-MS analysis has been taken a step further by combining IM-MS with gas-phase infrared (IR) spectroscopy [58]••. The benefit of this hybrid approach is that it enables acquisition not only of information concerning tertiary/quaternary, but also of secondary structures. This method, for example, has been used to resolve the secondary structures of oligomers formed by amyloidogenic peptides. The analysis showed that higher-order oligomers exist in different conformations, with CCS values ranging between compact spherical aggregates through extended oligomers. Conformer-specific IR analysis revealed a correlation between the size increase and β-sheet content, and pinpointed the onset of this structural transition from compact and unordered, to an extended β-sheet structure. Overall, this combined IM-MS technique offers novel means for examining the secondary structural transitions of self-assembled systems.

Conclusions

The variety of studies presented herein, covering diverse biological systems, demonstrate the strength of IM-MS. This method may be applied not only to investigations of soluble protein complexes, but also to analysis of challenging systems that are often resistant to crystallization, such as membrane proteins and amyloid aggregates. Considering that, by means of this technique, all co-existing populations can be probed in a single spectrum, its main advantages lie not only in examining complex samples containing mixtures of proteins, but also in its ability to probe binding assays and assembly pathways, while quantifying the conformational heterogeneity and relative stabilities of each species identified. Owing to the ease with which this method can be implemented, and the recent development of multiple software packages such as PULSAR [50], Amphitrite [59], ORIGAMI [60] or CIUsuite [61], all of which enable automated analysis of large and complex datasets, we anticipate that the applications of IM-MS will greatly increase.

Moreover, it is expected that in the future, the IM-MS method will be expanded to include high resolution instruments such as FTICR and Orbitrap platforms, paving the way toward defining mobility times of different post-transnationally modified species of a single protein assembly, alongside MS^n analyses [62,63]. This task is not trivial, however, due to the slow acquisition rate of these instruments, in comparison to the IM separation time. Nevertheless, progress in this direction is already being made [64]. An additional, promising direction involves the integration of IM-MS with other gas-phase techniques that can provide complementary structural information, such as the use of IM-MS as a pre-selection tool to perform gas phase IR [58,65] or FRET [66] measurements, and we expect that additional applications will arise.

Acknowledgements

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Highlights

- IM-MS provides valuable tools for structural characterization of protein complexes.

- The spread of conformational states adopted by a protein can be captured by IM-MS

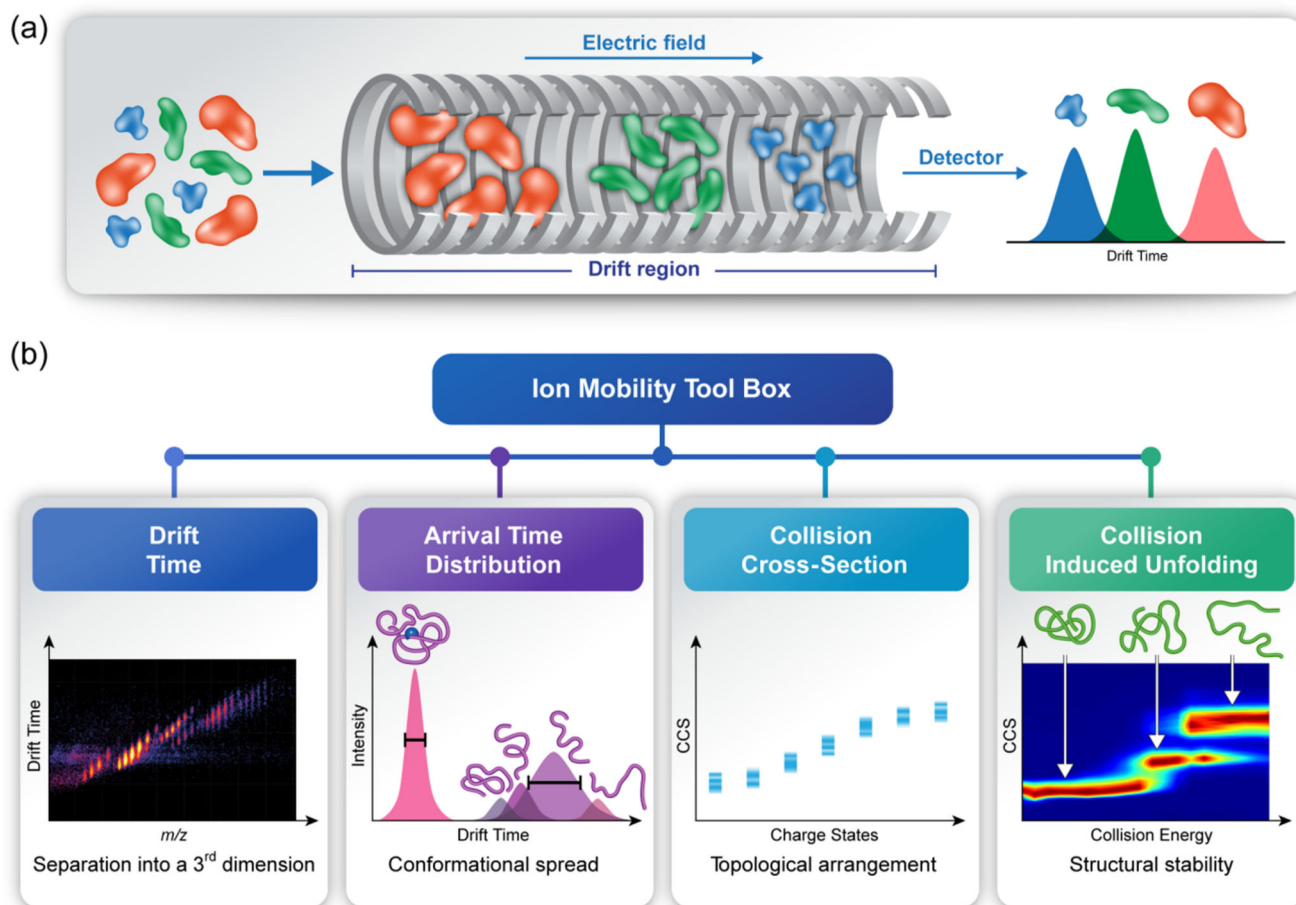- We discuss the application of IM-MS for investigating diverse protein systems

Figure 1. Structural characterization of protein complexes by IM-MS.
(a) Ion mobility is a method that measures the time it takes ions to travel through a gas-filled chamber, across which a weak electric field is applied. In the drift tube, ions experience two counter forces of electrostatic pulling through the cell and collisions with the buffer gas, which delay their movement. Ions with a larger surface area will experience more collisions with the buffer gas and, as a result, will take longer to traverse the drift tube, in comparison to smaller, more compact ions of the same molecular mass and charge, which will undergo fewer collisions with the buffer gas, and hence will display greater mobility and a shorter drift time. (b) IM-MS measurements enable the extraction of various structural properties. For example, mobility-based separation adds a third dimension to native MS analyses, and resolves heterogeneous samples that contain closely related species. Measurements of arrival time distributions reflect the conformational range of the examined species, and can be converted to CCS values. When measured over a range of collision energies, as is characteristic of CIU protocols, CCS can reflect the stabilities and intermediate folding states of the different protein species while they undergo in vacuo unfolding.
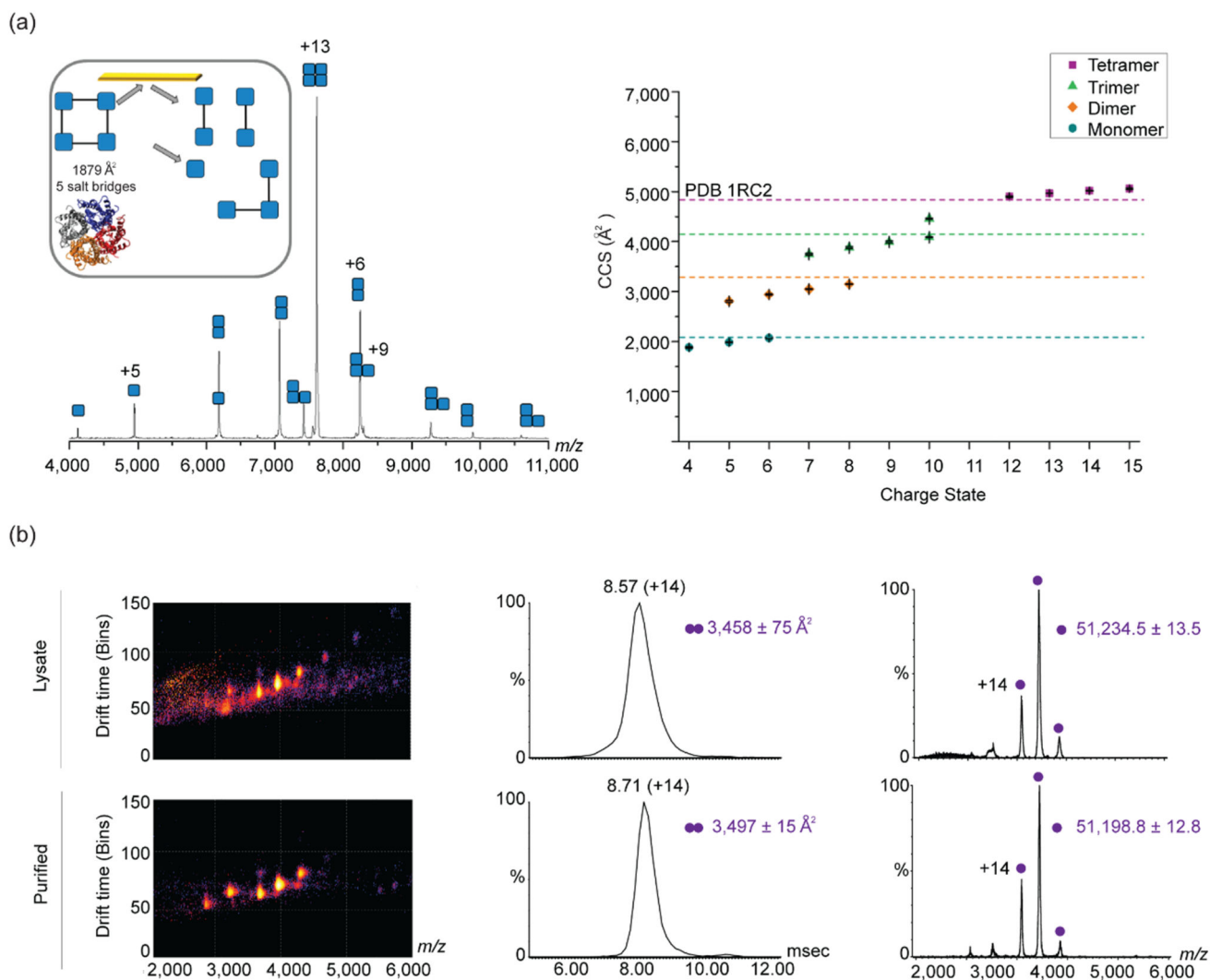
Figure 2. Applying IM-MS for structural characterization of soluble and membrane proteins.
(a) Application of SID with IM-MS for the study of membrane proteins is shown for AqpZ.
The SID spectrum for the 13+ charge state of AqpZ, shows the different subcomplexes that
were dissociated after collision with the surface. The inset represents the structure of AqpZ
and the interfacial analysis, as determined from the PISA software. The crystal structure
(PDB 1RC2) is shown below. The right graph shows the CCS distribution of the different
sub-complexes of AqpZ. Theoretical CCS values of the different structures, represented as
dashed lines, were determined using MOBCAL and the scaled PA. Adapted with permission
from [21]•. (b) IM-MS analysis of recombinant proteins directly from crude cell lysates
produces comparable information to that obtained from purified proteins, as exemplified for
Hsp31. The three-dimensional IM-MS spectra of the crude cell lysate and purified samples
are shown on the left, while the arrival time distribution and two dimensional spectra of *m/z*
over intensity are shown on the middle and right panels, respectively. Adapted with
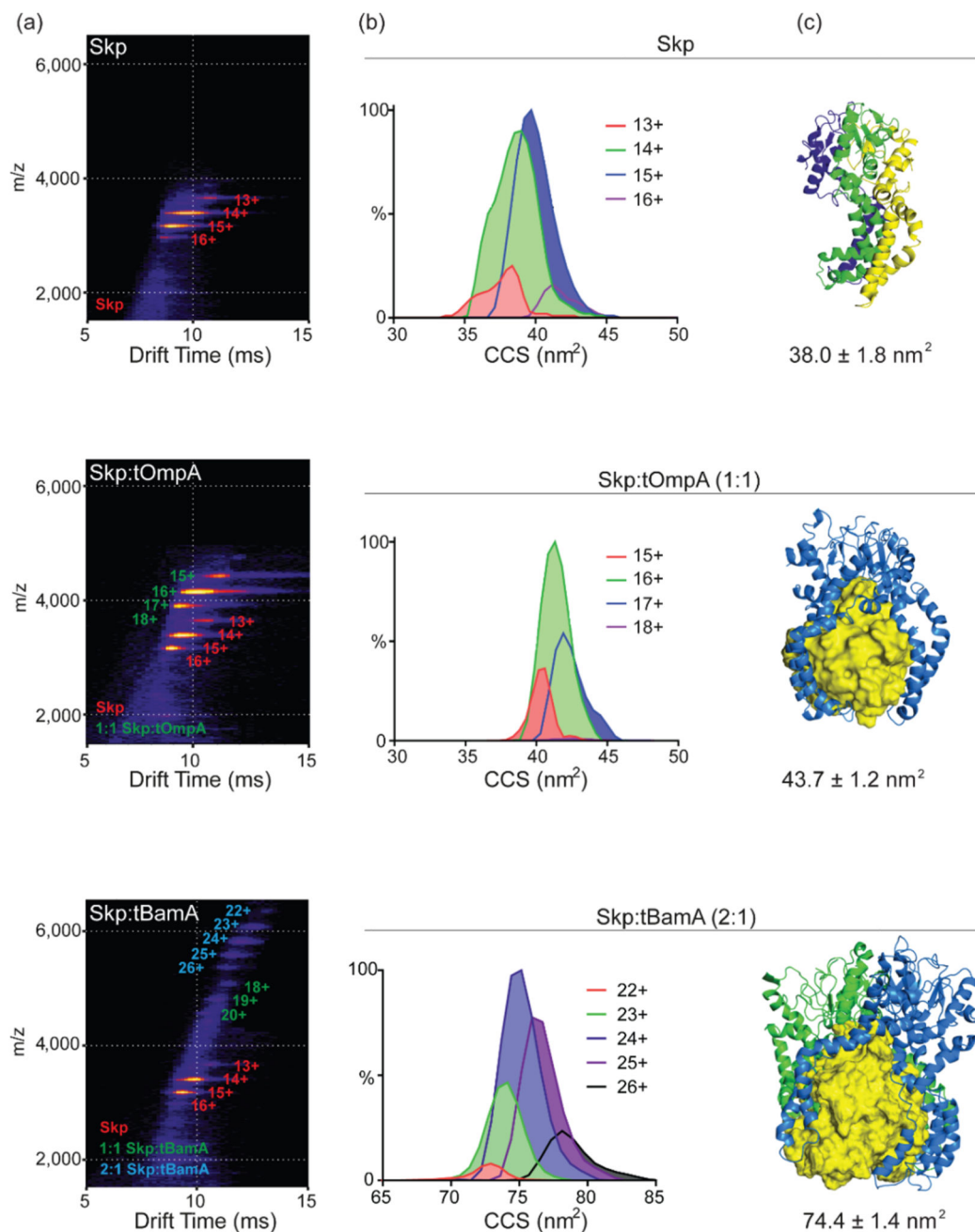permission from [22]•.

Figure 3. Investigation of the multivalent chaperone of outer membrane proteins.
(a) IM-MS plots of Skp alone, Skp bound with an 8-stranded (tOmpA) or with a 16-stranded (tBamA) OMP, the latter is larger than the Skp central cavity. Charge states corresponding to Skp, 1:1 Skp:OMP and 2:1 Skp:OMP are depicted on the plots in red, green and blue, respectively. (b) Representative CCS distributions of the major populations in each sample. Peak heights were normalized to MS peak intensities. (c) Representative structures from MD simulations obtained after 10 ns of in vacuo simulation. Skp is represented in cartoon format and OMPs are depicted as yellow surfaces. In the top panel, the different Skp subunits are

colored green, blue and yellow. In the bottom panel, two Skp complexes, colored in blue and green are in complex with the OMP. Theoretical CCS values obtained by the molecular dynamics simulations are shown below. Adapted with permission from [48]••.
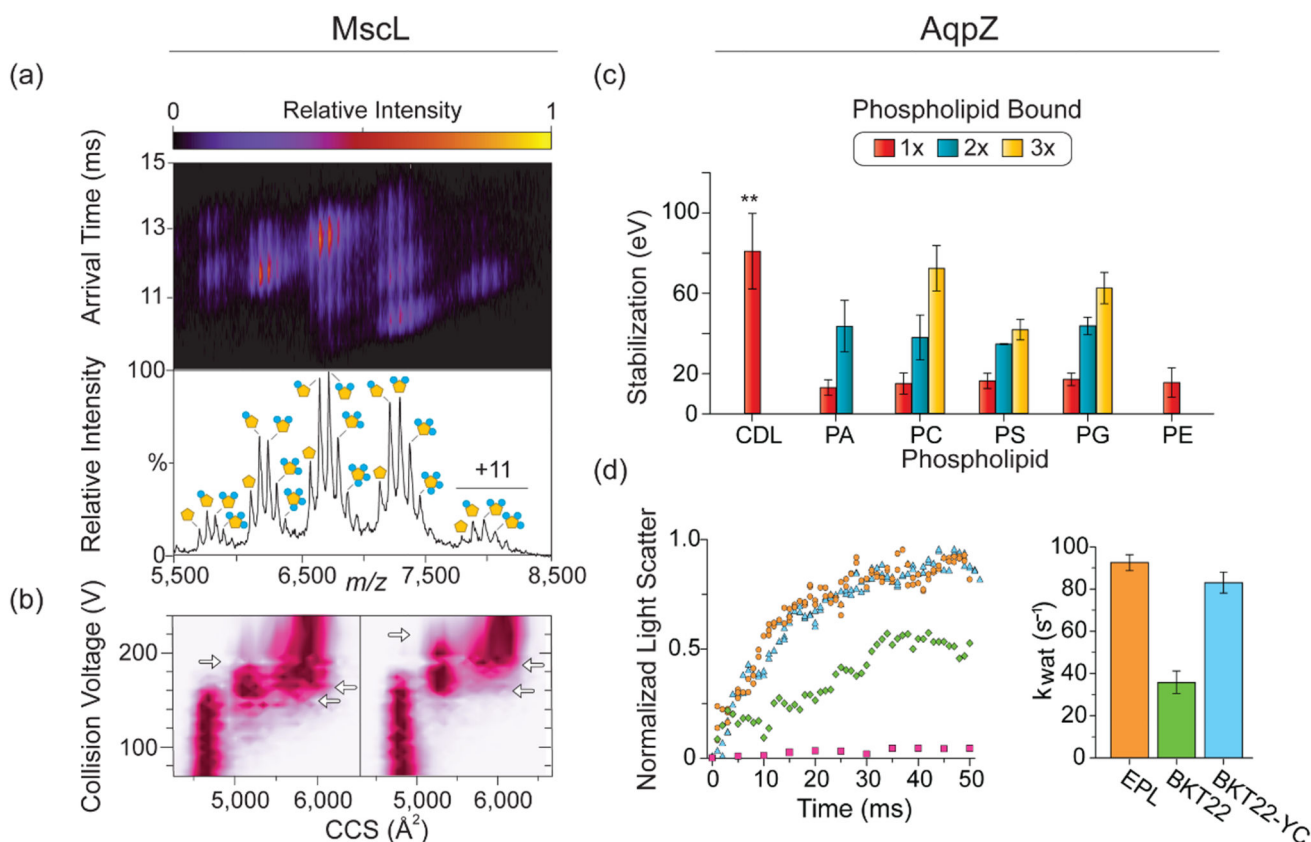
Figure 4. Quantification of the stabilizing effects of protein-lipid interactions by IM-MS.
(a) IM-MS reveals that several phosphatidylinositol phosphate (PI) lipids bind to MscL. The corresponding mass spectrum is shown below. (b) CIU diagrams of collision voltage, plotted against CCS, for the +12 ions of apo MscL (left), and MscL bound to 4 PI molecules (right), show the differences in gas-phase stability of the unfolding intermediates. Collision voltages at which transitions occur are shown by the horizontal arrows. (c) Quantification of AqpZ stabilization by various lipids, as calculated from CIU parameters. Results show that cardiolipin (CDP) has the strongest stabilizing effect on AqpZ. (d) The stabilization effect of CDP was validated by a functional assay that probed the water permeability of AqpZ reconstituted into liposomes containing various lipids: total polar lipid extracts from *E. coli* (EPL) (orange); cardiolipin-deficient lipid extracts (BKT22) (green); lipid extracts in which cardiolipin was restored (BKT22-YC) (cyan); and empty liposomes (EPL) (pink). The calculated rate constants of water transport (K_{wat}) ± SE are shown on the right. Adapted with permission from [49].