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Review

**Extending native mass spectrometry approaches to
integral membrane proteins**

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Abstract

Recent developments in native mass spectrometry and ion mobility have made it possible to analyze the composition and structure of membrane protein complexes in the gas phase. In this short review, we discuss the experimental strategies which allow to elucidate aspects of the dynamic structure of these important drug targets, such as the structural effects of lipid binding or detection of co-populated conformational and assembly states during gating on an ion channel. As native mass spectrometry relies on nano-electrospray of natively reconstituted proteins, a number of commonly used lipid- and detergent-based reconstitution systems have been evaluated for their compatibility with this approach, and parameters for the release of intact, native-like folded membrane proteins studied in the gas-phase. The strategy thus developed can be employed for the investigation of the subunit composition and stoichiometry, oligomeric state, conformational changes, and lipid and drug binding of integral membrane proteins.

Keywords: native mass spectrometry, membrane proteins, structural biology, lipid binding, detergent micelles.

Introduction

Although it is estimated that around a quarter of human genes encode for integral membrane proteins (IMP), the difficulty of studying the structural aspects of these proteins is underlined by the fact that less than 3% of the current entries in the PDB database represent membrane proteins. Traditional structural biology methods like NMR and x-ray crystallography yield high-resolution structures, yet might be hindered by certain intrinsic properties of membrane proteins, such as the difficulty to overexpress IMPs in order to obtain enough sample, the need to find suitable native reconstitution conditions (solubilization of membrane protein using typical approaches employed for molecular and structural studies, i.e. detergent micelles, bicelles, amphipols or nanodiscs, with the aim of preserving the quaternary structure of the IMP), and the fact that they often form large, sometimes heterogeneous complexes. Other techniques including electron microscopy (EM) are better suited to handle these restrictions, but yield lower-resolution structural information and can also suffer from structural heterogeneity in IMPs.

A relatively new method for the study of membrane protein structure and assembly is native mass spectrometry (MS) combined with ion mobility (IM) spectrometry. In this review we will give a short overview of recent advances using this approach. We will show how native mass spectrometry can be used to investigate the oligomeric state, dynamic structure and lipid binding of IMPs, with particular emphasis on the experimental strategies used to obtain gas-phase particles which retain key characteristics of the membrane protein complexes present in solution.

Keeping it together – in the gas phase

Native mass spectrometry relies on the gentle transfer of folded proteins or complexes from a volatile aqueous buffer into the vacuum of the instrument using nano-electrospray ionization (nano-ESI) (Fenn *et al.*, 1989). Key to this approach has been the introduction of a miniaturized version, of the conventional ESI source, by Wilm and Mann (Wilm and Mann, 1996), which benefits from a much lower flow rate (in the nl/min range) and thus reduced sample consumption (as little as 1-2 μ l of a 20 μ M solution for IMPs) compared to static flow ESI. Most importantly though, the smaller spray tip diameter in nano-ESI yields smaller

droplets, which in turn require less harsh desolvation conditions and thus facilitate the detection of native-like structures. An additional advantage of nano-ESI is the greatly improved sensitivity of the method, which facilitates the use of sample concentrations as low as 0.1 μM , although for membrane proteins typically 10 μM or higher are recommended (Konijnenberg *et al.*, 2013).

In order to maintain native protein structures intact, careful control of the conditions in the sample interface and within the instrument is crucial, to avoid activation of the ions and possible loss of higher-order structure. Within the mass spectrometer, the internal energy of ions (their “temperature”) is dictated by the combination of gas pressures (and thus the number of collisions) and the applied accelerating voltages. In general, native mass spectrometry uses elevated pressure regimes within the instrument to ensure sufficient focusing of the ion beam, while also retaining higher order structure and folded conformations. Although higher pressures lead to more frequent collisions, it is the amount of acceleration due to the applied voltages that dictates the effect of these collisions on the internal energy of the ions. High accelerating voltages induce collisional “heating”, where the internal energy of the ion increases with each collision. On the other hand, when low accelerating voltages are applied, the exact opposite effect takes place and collisional cooling is observed (Chernushevich and Thomson, 2004). Gentle collisions at relatively high gas pressures serve a triple purpose: firstly, they maintain the “temperature” (internal energy) of the ions during the adiabatic expansion of gas from atmospheric pressure into the vacuum of the source region, which would otherwise lead to a strong cooling effect for the ions. Secondly, the collisions allow for remaining solvent, buffer or detergent to dissociate from the protein or complex. Such evaporative dissociation has a cooling effect on the internal energy of the analyte, thus promoting complete desolvation while maintaining native-like conformations. Finally, the increased gas pressure ensures that high m/z ions (e.g. protein complexes) form a focused ion beam in a process called collisional focusing, which is a prerequisite for their transmission and detection in the mass spectrometer (Chernushevich and Thomson, 2004).

Native mass spectrometry of integral membrane proteins

A breakthrough in applying native MS to membrane proteins was demonstrated in a seminal paper by Barrera and Robinson, who used detergent above the critical micelle concentration

(CMC) in order to form micelles and preserve non-covalent interactions in the structurally well-characterized membrane protein BtuC₂D₂ during the critical step of desolvation and ionization by using nano-ESI (Barrera *et al.*, 2008). Previously, it was generally believed that the presence of free detergent in solution at concentrations much higher than those of the protein complex components would suppress ionization, as detergent and other surfactants can occupy the surface of the electrospray droplets and prevent the release of protein (Annesley, 2003). In addition, detergent micelles are found to be quite heterogeneous both in respect to mass and charge, making direct *m/z* analysis of membrane proteins embedded in micelles impossible. Empty micelles formed from non-ionic and zwitter-ionic detergent, without embedded protein, appear to show a very broad range of sizes in ESI-MS, from monomer roughly up to their published aggregation numbers (Borysik and Robinson, 2012*a*, 2012*b*), suggesting that both evaporation of neutral detergent and (to a lesser extent) dissociation of charged detergent molecules play a major role during transfer from solution to the gas phase. While detergent clusters in the gas phase should probably not be called “micelles” anymore, they are still effective in protecting embedded membrane protein against dissociation or unfolding inside the mass spectrometer, as shown by the preservation of non-covalent interactions (Barrera *et al.*, 2008; Borysik *et al.*, 2013) and the determination of collision cross sections that closely resemble those calculated from high-resolution structures for these IMPs (Laganowsky *et al.*, 2014; Konijnenberg *et al.*, 2014).

A successful strategy for the intact characterization of membrane proteins includes therefore: (a) the native reconstitution in solution, (b) transfer of detergent clusters (“micelles”) into the gas phase, and (c) subsequent dissociation to reveal the embedded membrane proteins or complexes (Figure 1). In contrast to the desolvation of soluble proteins, where the most activating conditions (highest acceleration voltages) are typically applied in the source in order to strip remaining water and buffer molecules, membrane proteins require that their detergent cover survives transfer into the gas phase, and that it is only removed, typically using collisional activation, in the collision cell. It is therefore imperative that the source conditions are tuned carefully, in order to maintain interactions between detergent molecules which protect the embedded membrane protein until inside the mass spectrometer, where its controlled release occurs (Laganowsky *et al.*, 2013).

Although this strategy was attempted before (Ilag *et al.*, 2004), higher protein concentrations in combination with the use of increased gas pressures and levels of collisional activation yielded the much sought after results: resolved oligomeric membrane protein complexes in

the gas phase (Barrera *et al.*, 2008). Whereas for soluble proteins, declustering voltages are normally kept to a minimum to prevent unfolding or dissociation, collision-induced dissociation (CID, without precursor peak isolation) was required here in the collision cell to remove the detergent and reveal the embedded IMPs in their native oligomeric state. As such, the release of IMPs from detergent clusters in the gas phase shows parallels with the desolvation of soluble native proteins, which is not complete before the ions enter the mass spectrometer, but continues as a prolonged evaporation event into the first vacuum stages (“source”). The main differences lie however in the essential, protective role which remaining detergent plays until the ions are inside the collision cell (i.e. declustering occurs later), and the usually stronger interaction between detergent molecules and also detergent and protein. Energetic collisions of detergent-embedded IMPs with inert gas molecules are used to strip the detergent molecules and thereby release the intact, charged IMP while conserving its non-covalent interactions. This requires however that the strength of the interactions which define the higher-order structure of the membrane protein (e.g. hydrogen bonds, salt bridges), is greater than the interactions between protein and detergent. This is usually the case for structurally defined IMPs, but not necessarily for some of the more dynamic and transient interactions, such as the ones found in assemblies of pore-forming peptides, which can be difficult to study.

In order to obtain well-resolved peaks with a minimum of residual detergent attached, the acceleration voltages needed for removal of sugar-based detergents such as *n*-dodecyl β -D-maltopyranoside (DDM) in the collision cell are near the maximum of what commercial instruments can provide. Therefore specific modifications, which allow use of higher gas pressures and an increased range of collision energies, are often employed (van den Heuvel *et al.*, 2006; Laganowsky *et al.*, 2013). It is generally believed that the excess energy provided by collisional activation dissipates in the detergent cover first, causing its evaporation (see below). A further increase of the collision energy (where possible) will then lead to unfolding and dissociation of the embedded protein, possibly even top-down fragmentation, similar to what has been described for soluble proteins (Benesch *et al.*, 2009).

Using this strategy, native MS can yield valuable data on the subunit composition and stoichiometry of IMPs in different detergent or lipid environments, which are not easily obtained using other methods, particularly when proteins are structurally heterogeneous (i.e. when different conformational and oligomeric states are co-populated). In cases where well-resolved mass spectra are available, the presence of post-translational modifications

(PTM) and the binding of ligands can also be studied (Barrera *et al.*, 2008). In combination with homology modeling, such data was used in a recent study to build a structural model for the MexB efflux pump (Barrera *et al.*, 2009). In addition to sequence and stoichiometry information, native MS is also used to investigate the stability and organization of membrane protein complexes. Wang *et al.* showed that dissociation thresholds in the collision cell can be related to the spatial organization of a membrane protein complex. By comparing two tetramers with similar sizes – KirBac3.1 and BtuC₂D₂ – they showed how the proportion of the complex that is effectively located inside the micelle influences its stability towards CID. All four subunits of KirBac3.1 have transmembrane segments, whereas for BtuC₂D₂ only two subunits span the membrane, which renders the latter more sensitive to dissociation at comparable collision energy regimes. This type of information can be useful to evaluate the arrangement of subunits in the membrane, or to prove or refute structural models which were proposed on the basis of other data (Hall *et al.*, 2012). Native MS is increasingly used in combination with other biophysical approaches (e. g. electron microscopy) and computational methods. The comparatively low sample consumption of such hybrid approaches compared to crystallography or NMR spectroscopy can make the structural characterization of membrane protein complexes feasible, which is otherwise difficult to obtain..

The use of ion mobility to study conformation and assembly topology of membrane proteins

While hydrophobic interactions are well known to play a crucial role for the structure and stability of membrane proteins, in particular with regard to interactions with their molecular environment, the specific folding and assembly of the protein itself also relies on intra- and intermolecular hydrogen bonds and other types of attractive forces, which are retained or even strengthened in the gas phase (Jarrold, 2007). The hydrophobic effect on the other hand, which is one of the key forces driving micelle assembly, vanishes in the water-free environment of the gas phase. The question therefore arises how we can know whether a membrane protein maintains its native-like fold in the mass spectrometer, under the chosen experimental conditions? Although narrow charge state distributions of soluble protein in nano-ESI suggest that a compact, well-folded structure is maintained, and this observation can be used as an indicator for sufficiently “native” conditions during the experiment, it should be noted that charge states are a product of the electrospray process itself and therefore not

sufficient to define the subsequent conformational change which proteins, particularly unstructured and membrane-bound ones, might undergo. Both the average charge and the width of the charge state distribution are believed to be representations of the surface area of a protein or complex in its state during the final stages of the electrospray process (Grandori, 2003; Hall and Robinson, 2012). For membrane proteins, ESI charge states have been shown to correlate with the surface area of the soluble, detergent-free domains of a membrane protein, and are generally found to be lower than for soluble proteins (Barrera *et al.*, 2013). As charging occurs during electrospray, the observed charge state distributions do not necessarily reflect the folding state of proteins released from detergent clusters later in the process.

A more suitable method to study the conformational behavior and assess the structural integrity of proteins is ion mobility (IM) spectrometry. Native IM-MS is a gas-phase ion separation technique, where ions are separated based on their mobility (influenced by compactness, mass and charge) through a gas-filled drift cell (Figure 2). Under the influence of a low electric field, the ions are accelerated through an inert gas until they reach a constant speed (drift velocity) due to the friction they experience – somewhat similar to a gas-phase electrophoretic mobility. More extended conformations will undergo more collisions with this gas compared to compact structures, and will thus drift more slowly. The time an ion spends in the ion mobility cell (i.e. drift time) is characteristic for its rotationally averaged collision cross section (CCS), measured in \AA^2 . Although the structural information thus obtained is limited, the separation power of ion mobility is sufficiently high to differentiate co-existing protein conformations or assembly topologies, if their CCSs differ by at least 2-5% (Utrecht *et al.*, 2010; Konijnenberg *et al.*, 2014). In particular, we can test structural models, either generated *in silico* or derived from protein structures solved by NMR spectroscopy or x-ray crystallography, by calculating the theoretical CCS and comparing them with observed experimental values computed from drift time profiles (Shvartsburg and Jarrold, 1996; Shvartsburg *et al.*, 2007; Bleiholder *et al.*, 2011, 2013). One of the principal strengths of the IM-MS approach is its ability to observe, in an unbiased way, co-existing species which differ in mass/charge or ion mobility, instead of just providing aggregate values over the whole ensemble (see below). As such, IM-MS provides a powerful tool to assess whether membrane proteins maintain their native conformation in the gas-phase, and to study their folding state and assembly topology in response to the detergent and lipid environment. For a more detailed description of the principles of ion mobility, instrumentation and application, the

reader is redirected to several excellent reviews (Utrecht *et al.*, 2010; Jurneczko and Barran, 2011; Konijnenberg *et al.*, 2013; Lanucara *et al.*, 2014).

Detergent protects membrane proteins in the gas phase

The observation that natively reconstituted membrane proteins in detergent micelles could not only survive the transfer to the gas phase, but also the seemingly harsh conditions required to release them from their detergent cover, was a significant one. The energy regime that is applied in order to set free IMPs from detergent such as DDM would be sufficient to unfold most soluble proteins. So how is it possible that weak, non-covalent interactions in membrane proteins are maintained, whereas soluble protein complexes would already undergo dissociation under similar conditions? Studies of the dissociation behavior of micelles have revealed that the detergents are ejected by a process called detergent evaporation (Borysik and Robinson, 2012a, 2012b). With increasing energy, the detergent micelle is shrunk down in size and charge as detergent molecules, often in pairs, are ejected from the remaining cluster. This process - although taking place at a different pressure regime and stage in the mass spectrometer - shows clear similarities to the desolvation of soluble proteins, where collisional activation in the source would slowly increase the internal energy of the system (“heating”), if evaporative cooling of solvent did not counter this effect (Bagal *et al.*, 2009). For soluble proteins, it is well documented that “native” peaks are inevitably rather broad, often showing distinctive tailing which is ascribed to residual solvent, buffer and (counter-)ions (Przybylski and Glocker, 1996; Loo, 1997; Sobott *et al.*, 2005; McKay *et al.*, 2006; Lössl *et al.*, 2014), whereas narrow m/z signals can only be achieved under more activating conditions which may already cause some partial unfolding and/or dissociation of native protein.

Similar to how residual solvent is often found to remain bound under conditions which maintain the native character of a soluble protein in native MS (Patriksson *et al.*, 2007; Steinberg *et al.*, 2007), the attempt to completely remove detergents bound to membrane proteins can lead to unwanted gas-phase unfolding. Figure 3a-d shows the effect of an increased declustering potential on the mechano-sensitive ion channel of large conductance (MscL). This pentameric channel was natively reconstituted into triton X-100 detergent, and the compact form observed under gentle conditions (Figure 2a-b) matches the CCS calculated from the x-ray structure of the closed state reasonably well (Konijnenberg *et al.*, 2014). Under

conditions which favor increased declustering, at higher collision energies, narrower peaks are obtained due to a reduced number of detergents bound (Figure 2c), but at the same time a second, more extended conformation appears in the ion mobility plot (Figure 2d), indicating that a fraction of the ions is already activated to an extent that they partially unfold. In another study, the native form of the PagP aquaporin was only observed for protein with a number of DDM molecules bound, whereas less adducted peaks corresponded to a partial collapse of a flexible loop of the membrane protein (rather than unfolding). The authors of this work conclude that evaporative cooling caused by detergent release, rather than the presence of detergent itself, seems to actually prolong the lifetime of the native structure in the gas-phase (Borysik *et al.*, 2013). Investigations into the nature and behavior of detergents in the gas phase also gave insight into the charge states of detergent micelles, which lie well below the Rayleigh limit for spherical objects (Borysik and Robinson, 2012a, 2012b). This might explain why most membrane proteins pick up less charge than would be predicted from observations made on soluble proteins (Konijnenberg *et al.*, 2013; Barrera *et al.*, 2013). Lower charge states might be beneficial for preserving native-like conformations in the gas phase, as recently reported for charge-reduced membrane proteins which displayed more compact structures (Laganowsky *et al.*, 2014; Mehmood *et al.*, 2014). Similar to observations for soluble proteins, IMPs seem to undergo expansion due to Coulombic effects for higher charge states. This is the reason why for ion mobility studies, the lowest charge state is typically selected as it is believed to represent the most native-like conformation (Jurneczko and Barran, 2011).

Gas-phase unstable detergent clusters facilitate IMP release under gentle conditions

Despite the obvious success of native MS with membrane proteins, questions arose on the use of such high collision energies to remove detergent (Laganowsky *et al.*, 2013). Was it possible to guarantee native structures in the gas-phase under such highly energetic conditions? Even though protein complexes might maintain their oligomeric state, this does not immediately imply that they have also retained their complete native fold. Although ion mobility can help to solve such questions, by comparison of an experimentally determined CCS with the theoretical value derived from an x-ray structure, such an approach cannot be taken when there is no high-resolution structure available yet. Especially for membrane

proteins which are too large or complex for study by NMR spectroscopy or resist crystallization, the structural restraints that ion mobility can generate might be much sought after for the generation or validation of computational models (Politis *et al.*, 2013, 2014).

Recent studies have highlighted the role which the gas-phase properties of detergent clusters play for the gentle release of membrane proteins in the mass spectrometer (Laganowsky *et al.*, 2014; Konijnenberg *et al.*, 2014). While stable micelles are formed in detergent solutions above the CMC, thanks to a combination of attractive forces and the hydrophobic effect, the ability of detergent molecules to form intermolecular hydrogen bonds was found to be decisive for the gas-phase stability of detergent clusters (Konijnenberg *et al.*, 2014). Micelles which are stabilized due to an extensive hydrogen bond network between detergent molecules are much more resistant to dissociation in the mass spectrometer, whereas the hydrophobic effect is lost as a stabilizing factor *in vacuo* (Figure 1). Triton X-100 is such a detergent with low hydrogen bond capacity. Its micelles are mainly hydrophobically driven and not stable in the gas phase, allowing release of protein such as MscL pentamers at only 10 V collisional activation, in contrast to detergents such as DDM which form extensive hydrogen bond networks and require much higher levels of activation for disassembly (Konijnenberg *et al.*, 2014). While the choice of detergent will of course mainly depend on the need to natively reconstitute the specific IMP in a detergent or lipid environment which preserves its higher-order structure, the use of detergent which forms clusters unstable in the gas phase (when possible) provides a robust platform for the gentle release and study of folded membrane proteins.

The use of such gas-phase-unstable detergent clusters has proven to be invaluable for the study of the dynamic structures of IMPs, with IM-MS revealing changes in the conformational ensemble of IMPs or changes in their stability upon lipid binding in the gas-phase (Laganowsky *et al.*, 2014; Konijnenberg *et al.*, 2014). In an attempt to elucidate the gating mechanism of the mechano-sensitive ion channel of large conductance (MscL), Konijnenberg *et al.* used the ability of a number of charged drug molecules covalently bound in the pore to effect structural transitions which imitate membrane tension-induced channel opening. In this way, defined opening states could be prepared that are suitable for analysis by electron microscopy, electron paramagnetic resonance, and native MS. Using the ability to not only detect the oligomeric state of MscL, but also separate and characterize different conformations simultaneously (Figure 4A), the authors could detect stepwise opening of the channel with increased numbers of charged drug molecules bound in the pore, at the same

overall charge state. When comparing the different hetero-oligomers – each with a number of drug binding sites defined by the number of Cys mutant subunits – the CCS is found to increase with the number of possible binding sites. Interestingly, the CCS increases via population of well-defined states of increasing size, thus suggesting that MscL gating occurs stepwise on its way to a fully open conformation (Konijnenberg *et al.*, 2014). Comparing the experimentally determined CCS values with those obtained from molecular dynamics simulations of MscL gating revealed that the opening proceeds via an iris-like rotation of the transmembrane spanning helices (see insets in Figure 4A).

In recent years, it has become increasingly apparent that the interactions of proteins with specific lipids in the bilayer membrane are often crucial for their structure and function. Mass spectrometry studies of detergent-solubilized IMPs may also reveal lipids which remain bound under both solution (reconstitution) and gas-phase (detergent stripping) conditions. The observation that membrane proteins maintain interactions with lipids even when removed from the with an excess of detergents (Barrera *et al.*, 2013; Zhou *et al.*, 2014), points towards a specific role for their presence. Recent, exciting work by Laganowsky *et al.* demonstrated convincingly using native IM-MS how specific lipids can stabilize membrane proteins. By screening the interactions of the ammonium channel AmbT with a range of different lipids, using gas-phase unfolding studies, phosphatidylglycerol (PG 18:1,16:1) was found to have a significantly higher stabilizing effect (Figure 4B,C). The importance of this approach is further highlighted by the successful use of this lipid for protein crystallization. The x-ray structure of AmbT shows how a specific loop interacts with PG, further illustrating the stabilizing effect of the lipid. In the same study, the authors were also able to identify lipids which stabilize the MscL and aquaporin Z channels and are known to be important for their function. Taken together, these observations show how native IM-MS can not only be used to study aspects of dynamic structure of membrane proteins, but also as an important screening tool for specific lipid interactions with IMPs, thus also furthering chances for successful membrane protein crystallization.

Detergent-free native mass spectrometry of membrane proteins

Although detergents have long been used to solubilize and study membrane proteins in micelles, there is always a chance that they introduce a structural bias, as they can only approximate the native, planar lipid bilayer environment of IMPs. Several alternative

approaches were recently introduced, but only nanodiscs, bicelles and amphipols have so far been reportedly tested for their applicability in mass spectrometry. A key requirement for native MS, as for other biophysical and structural methods, is that the protein is soluble and its higher-order structure stable in the chosen detergent, amphipol or lipid-based reconstitution platforms.

Amphipols are amphiphatic, short polymers which wrap around the hydrophobic trans-membrane region of IMPs (Zoonens and Popot, 2014). The number of amphipol molecules bound depends on the size of the transmembrane domain of the protein or complex (Della Pia *et al.*, 2014). Amphipols bind membrane proteins rather tightly, albeit non-covalently, and solubilize them in a stable environment without the need for detergents or lipids (Calabrese *et al.*, 2014). The tight binding of amphipols can however frustrate experiments which aim to elucidate the higher-order structure of the embedded membrane proteins (Hopper *et al.*, 2013), and IM-MS studies are further complicated due to the fact that amphipols are polydisperse in length (Leney *et al.*, 2012). Recent work compared the effectiveness of amphipols in maintaining the native conformation of monomeric IMPs with the commonly used detergent DDM (Calabrese *et al.*, 2014). The CCS observed for alpha-helical proteins was found to be close to their theoretical values, suggesting that amphipols might be at least as good at maintaining the native conformation of membrane proteins as detergents. The lower charge states obtained with amphipols were suggested to be an important indicator for non-denatured conformations (Calabrese *et al.*, 2014). Research on oligomeric IMPs solubilized with amphipols however indicates that they might not be effective in maintaining the correct oligomeric state of membrane proteins released in the gas phase (Hopper *et al.*, 2013), possibly because interactions of amphipols with the protein are strong compared to the interactions between the protein subunits themselves.

While lipid bilayers themselves form large heterogeneous structures and are therefore difficult to handle in mass spectrometry, bicelles and nanodiscs are examples of more defined, membrane-like reconstitution systems which are now increasingly being used in structural studies. Bicelles are lipid bilayer discs (figure 5A) consisting of a mixture of long-chain lipids (dimyristoyl-phosphatidylcholine (DMPC)) which make up the bilayer, and short-chain detergent (3-(cholamidopropyl) dimethylamminio-2-hydroxy-1-propanesulfonate (CHAPSO)) or short-chain lipid (dihexanoyl-phosphatidylcholine (DHPC)) which form the edges around the hydrophobic tails of the lipids (Sanders and Prosser, 1998). Using a DMPC/CHAPSO mixture yields discs varying in diameter from 20-40 nm and with a thickness of around 4 nm

(Sanders and Prosser, 1998). The diameter of the bicelles depends on the bicelle concentration present in solution, with larger diameters at lower concentrations (Rodríguez *et al.*, 2010). The native state of oligomeric proteins is stabilized by bicelles of a larger diameter due to the increased space and reduced curvature of the lipid bilayer (Lee, 2004). Bicelle-reconstituted IMPs have been successfully analyzed by native MS (Hopper *et al.*, 2013). Diacylglycerol kinase (DgkA) released from bicelles by CID was found to be in a compact state, implicated by the low and narrow charge state distributions for monomer and trimer (2+ to 4+). When comparing these charge states to DgkA which was solubilized in amphipol or detergent, where mainly monomer (3+ to 9+) and a small population of trimer (for amphipol, 5+ to 7+) was detected, the use of bicelles seems to give rise to a more native-like state of the protein (Hopper *et al.*, 2013).

Nanodiscs on the other hand are, somewhat similar to bicelles, lipid bilayer discs, but stabilized by a pair of stacked membrane scaffolding proteins (MSP) (Figure 5A). MSPs are all alpha-helical amphiphatic proteins which wrap around the edge of the discs, thus protecting the hydrophobic tails of the lipids. Nanodiscs are fairly monodisperse in diameter, ranging from 9.8 to 12.9 nm depending on the length of the MSP used (Bayburt and Sligar, 2010), and provide a suitable platform for IMP reconstitution. Native MS experiments on “empty” nanodiscs have confirmed their remarkable homogeneity, and the number of lipids they contain (depending on the size of the nanodiscs) was found to correlate very well with the expected number (Marty *et al.*, 2012) (Figure 5B). In order to structurally characterize embedded membrane proteins, they have to be released from the nanodiscs using collisional activation (CID), as the varying number of lipids and charges does not allow to resolve peaks of the intact particles. Nanodiscs are reported to be very stable, and medium levels of collision energy (200 V) lead to ejection of only a small number of lipids. Further increase of the collision energy, to 360-400 V, causes disassembly of the MSP dimers and the lipids fully dissociate, finally also releasing the embedded IMP (Hopper *et al.*, 2013). Using DgkA again, oligomeric protein was detected at relatively low charge states (6+ to 7+) (Hopper *et al.*, 2013). The fact that low charge states and the expected oligomeric sizes of IMPs are observed when using nanodiscs highlights the potential of lipid bilayers as a platform for membrane protein studies in native MS. Unfortunately, no ion mobility data of proteins released from bicelles or nanodiscs has been published yet. It is therefore difficult at the moment to say which reconstitution system is more favorable for use in native MS, depending on the type of protein under study. While charge state distributions of released IMPs are not sufficient to

judge the “nativeness” of an approach (see above), they suggest nevertheless that lower charge states are likely to correspond to more native-like, compact structures. A recent ion mobility spectrometry centered study (Calabrese *et al.*, 2014), which focuses on the effectiveness of amphipols and detergents in protecting the native-like conformations of IMPs in the gas-phase, indeed suggests that this relationship still holds: at least for α -helical membrane proteins, amphipols yielded lower charge states which also had more compact CCSs.

Outlook

Although MS has been used before to study intact membrane proteins for some time (Whitelegge, 2013), recent evidence suggests that aspects of their higher-order structure, such as oligomeric distribution, conformational transitions or specific lipid binding, can be examined by using native MS. This finding was a great breakthrough for membrane protein studies, and has put IM-MS approaches in the focus of considerable recent attention. Obtaining information on the oligomeric states of the protein is one of the strengths of this approach, particularly when the protein is polydisperse or structurally dynamic, while difficult to achieve with other methods. The ability to detect and investigate specific lipid binding to IMPs opens a window to an important field of protein-lipid interaction studies. Initial native MS experiments were performed with detergent micelles as a reconstitution platform, and so far the bulk of published work still uses this well-established approach, confirming its popularity. Detergents though might not always provide the ideal environment for the preservation of native structure in solution and in the MS experiment. Alternative reconstitution systems such as amphipols, bicelles, and nanodiscs have shown to hold great promise for membrane protein studies, particularly also in native MS where the protein is set free from its membrane-like environment just prior to analysis. The most important factor however remains, despite all the recent exciting developments in the use of native mass spectrometry, that each different membrane protein has its own individual character and dictates which native reconstitution system is best suited to ensure correct folding and assembly for further *in vitro* or *in vacuo* structural investigation.

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Tables and figures

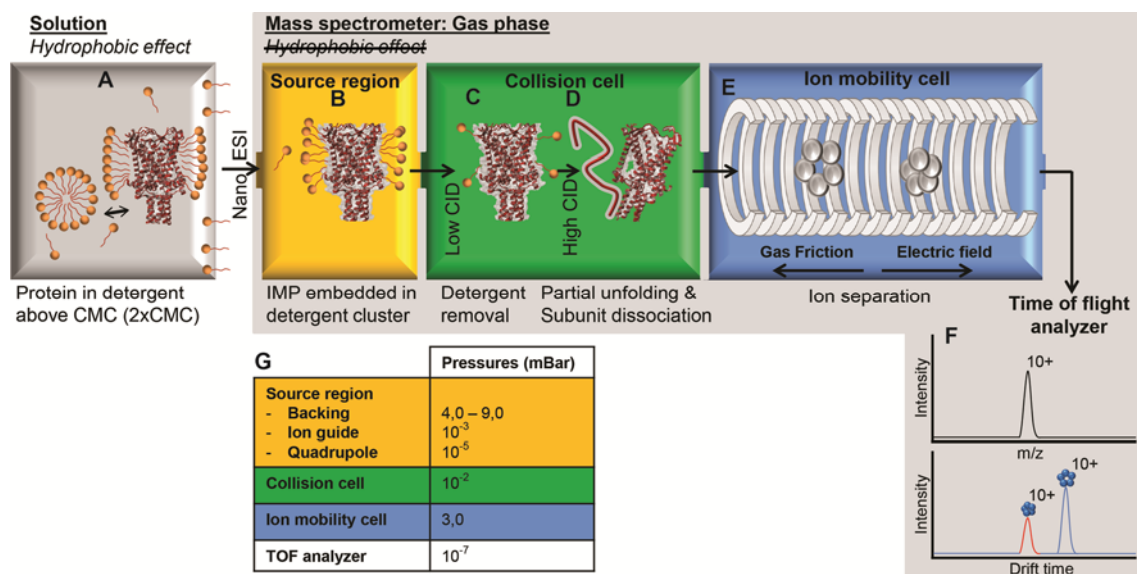


Figure 1 Schematic view of the fate of reconstituted IMPs in detergent micelles under non-denaturing MS conditions.

After release from solution by nano-ESI (A) the analyte enters the vacuum (B) in the source region (ion beam focusing, poss. mass/charge selection in quadrupole). In the collision cell (C), increasing amounts of collisional activation (CID) lead to the disassembly of detergent clusters. The amount of energy needed depends on the detergent used, where triton x-100 releases at low energy (10-50 V), while DDM needs higher activation (up to 100-200 V). Using excess energy (D) to release IMPs may result in the start of unfolding. The ion mobility cell (E) separates the protein complexes according to their mass, charge and global structure (rotationally averaged size and shape, also called "collision cross section"). The time it takes for a protein to move through the cell is related to its mobility, which can be converted into a collision cross section. Due to the friction with gas, more extended ions will travel more slowly even at the same charge and molecular mass (Da), granting information regarding structural heterogeneity within the same charge state (F). Typical pressures in the different compartments are shown in table (G).

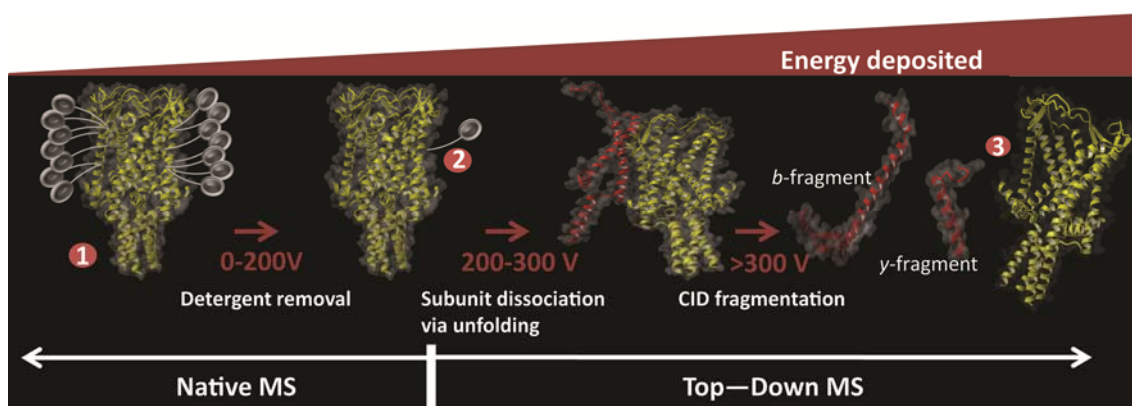


Figure 2 Effect of different energy regimes used in mass spectrometry of membrane proteins under native conditions when using sugar-based detergents such as DDM.

The apparent protective ability of detergent micelles in the gas-phase allow non-covalent interactions to be maintained for membrane proteins at levels (up to 200 V) at which soluble protein complexes would already undergo significant dissociation. Increasing the collision energy higher than necessary for membrane protein release leads to collision induced unfolding, followed by dissociation and even fragmentation, yielding sequence information. Collision energies reported are for illustrational purposes and will vary based on the protein and detergents used. Reproduced from (Konijnenberg *et al.*, 2015).

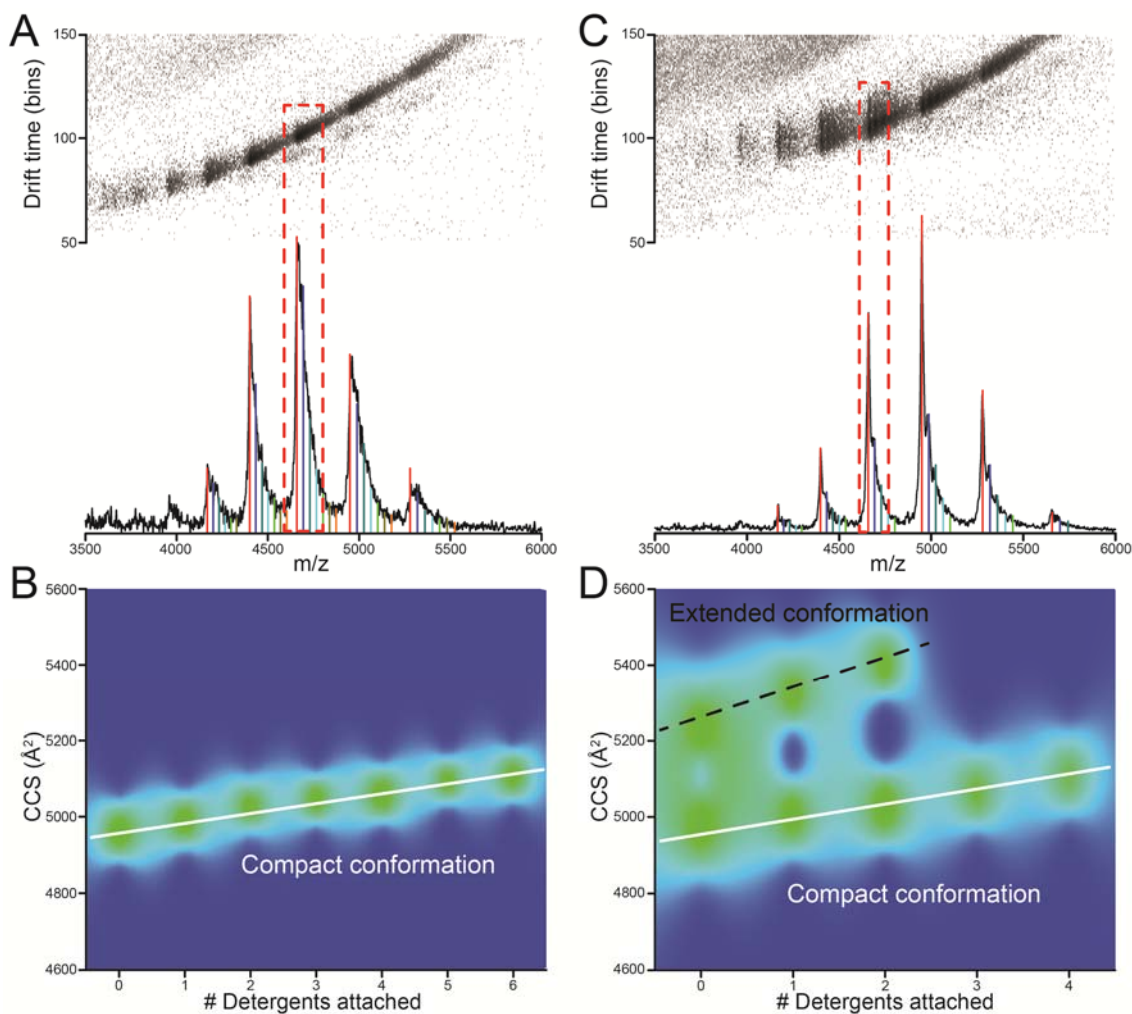


Figure 3 (a) Residual detergent molecules guarantee the native conformation of pentameric MscL in the gas-phase. Although triton X-100 detergent clusters are not stable in the gas-phase, up to six triton X-100 molecules remain bound to the protein and are resolved at 10 V trap collision energy (colored lines under each peak). (b) Under this mild declustering regime, the 17+ charge state of MscL is present in a single, compact and native conformation over the whole range of detergent adducts, with only a slight increase in ccs observed due to the number of bound detergent molecules (c) and (d). Increasing the trap collision energy to 50 V does not only lead to further detergent stripping and thus sharper peaks, but also induces some partial protein unfolding. The additional, slightly extended conformation visible in (d) no longer resembles the native solution structure of the closed MscL channel as determined by x-ray crystallography (Konijnenberg *et al.*, 2014).

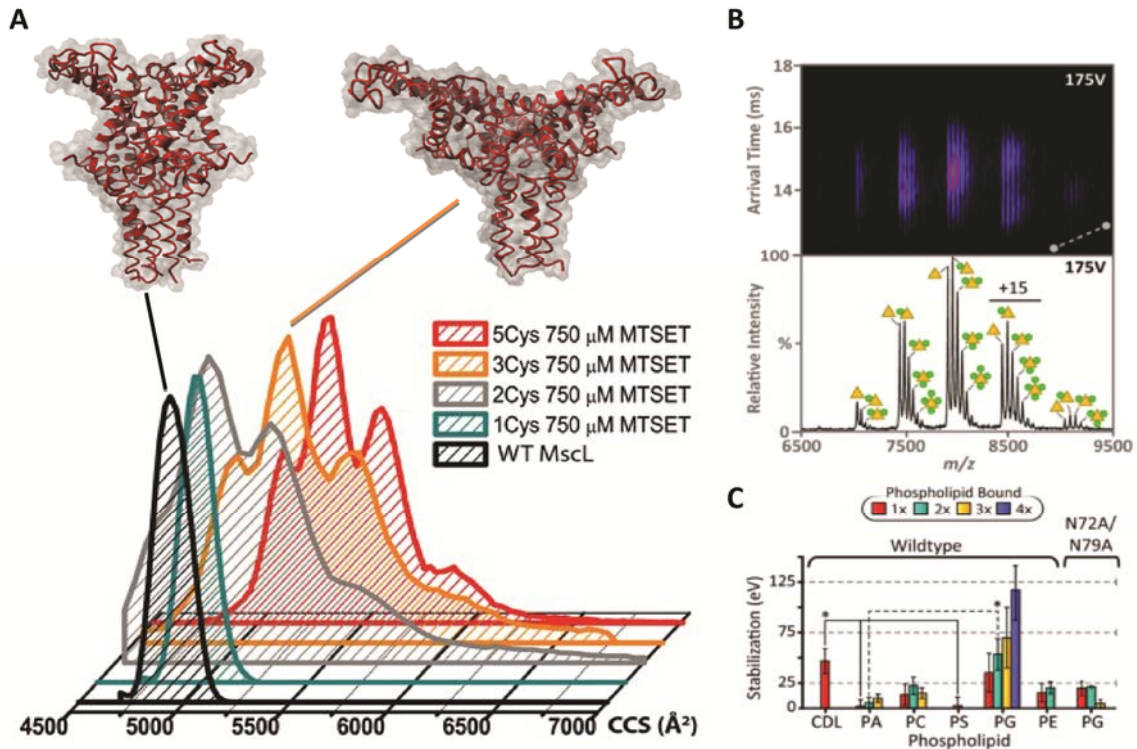


Figure 4 In combination with native MS, ion mobility is a powerful tool for the study of dynamic structure of integral membrane proteins.

(A) By using a charged compound (MTSET) which binds to cysteine residues in mutant subunits which form heteropentameric MscL with w.t. subunits, up to 5 charges are placed inside the pore which causes gradual opening and concomitant flattening of the structure similar to the effect of membrane pressure. Distinct intermediate opening states can be characterized under gentle MS conditions (10 V CE, collision energy) by their collision cross section, and matched with structural models generated by molecular dynamics (inset) indicating that opening of MscL occurs in an iris-like rotation. (B) Ion mobility spectrum of AmtB with 0-4 PG lipids bound at 175 V CE. Increasing the amount of collisional activation allows to follow unfolding of AmtB in the gas-phase. (C) Stabilizing effect of different lipids on AmtB. PG was the only lipid that required significant higher collision energies to unfold AmtB, thus indicating a specific interaction. PG binding also facilitated crystallization of AmtB, and the x-ray structure shows that a loop comprised of residues 70-81 interacts with the lipid. As expected, mutating residues N72 and N79 to alanine abolished this effect. Reproduced with permission from (Laganowsky *et al.*, 2014).

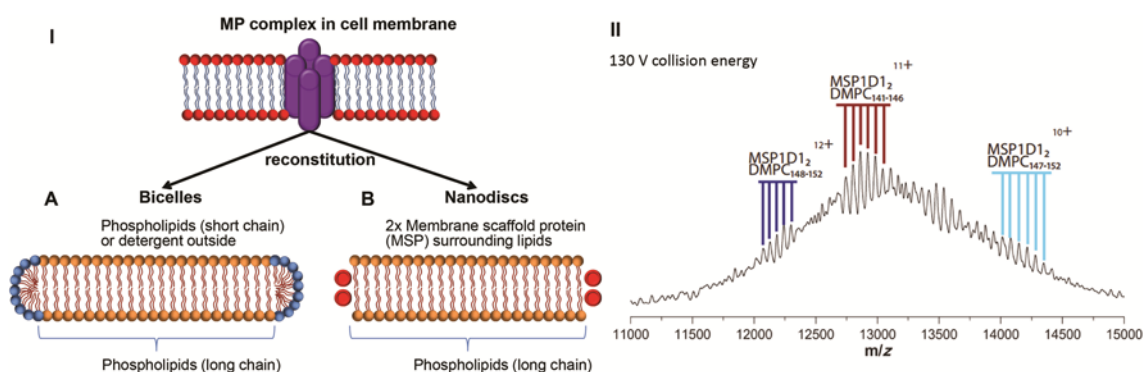


Figure 5 Membrane protein reconstitution into bilayers and nanodiscs is compatible with native MS.

(IA) Bottom left: cartoon representation of the cross section of a bicelle with a phospholipid bilayer (orange) and detergent cover (blue) surrounding the lipid disc protecting the hydrophobic tails of the lipids. Bottom right: in nanodiscs, the phospholipid bilayer (orange) is protected by two rings of membrane scaffold proteins (MSP, red circles) which shield the hydrophobic lipid tails and ensure relatively monodisperse particles. (IB, II) Mass spectrometry of nanodiscs under activating conditions reveal different charge states (10+ to 12+) of the intact nanodiscs which consist of two copies of MSP1D1 (membrane scaffold protein D1) with a variable number of DMPC lipids. The sizes of lipid clusters observed are in close agreement with the theoretical value of 160 DMPC lipids per nanodisc.