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Interrogating membrane protein structure and lipid interactions by native mass spectrometry

*Dietmar Hammerschmid [1,2], Jeroen F. van Dyck [2], Frank Sobott[2,3,4], Antonio N. Calabrese[3,4]**

[1]) Proteinchemistry, Proteomics and Epigenetic signalling (PPES)
Department of Biomedical Sciences, University of Antwerp
Universiteitsplein 1, 2610 Wilrijk, Belgium

[2]) Biomolecular & Analytical Mass Spectrometry group
Chemistry Department, University of Antwerp
Groenenborgerlaan 171, 2020 Antwerp, Belgium

[3]) Faculty of Biological Sciences
University of Leeds
Leeds LS2 9JT, United Kingdom

[4]) Astbury Centre for Structural Molecular Biology
University of Leeds
Leeds LS2 9JT, United Kingdom

*corresponding author: a.calabrese@leeds.ac.uk

Summary:

Native mass spectrometry and native ion mobility-mass spectrometry are now established techniques in structural biology, with recent work developing these methods for the study of integral membrane proteins reconstituted in both lipid bilayer and detergent environments. Here we show how native mass spectrometry can be used to interrogate integral membrane proteins, providing insights into conformation, oligomerization, subunit composition/stoichiometry and interactions with detergents/lipids/drugs. Furthermore, we discuss the sample requirements and experimental considerations unique to integral membrane protein native mass spectrometry research.

Key words: Native mass spectrometry; ion mobility; membrane proteins; detergent micelles; lipids

1. Introduction

Native mass spectrometry (MS) has shown promise in structural studies of integral membrane proteins (IMPs), and IMP interactions with lipids (1-3), and other small molecules, e.g. drugs (4). More generally, native MS has shown promise in generating structural information for flexible, intrinsically disordered (5, 6), heterogeneous and/or polydisperse proteins and complexes, providing data when traditional high-resolution techniques (e.g. X-ray crystallography, NMR spectroscopy or cryo-electron microscopy) have failed (7-11). Native MS measurements are relatively fast (although optimization of sample and mass spectrometer conditions can be a bottleneck), require low sample quantities (nano- to picomole) and enable co-existing conformational and assembly states to be examined without ensemble averaging (12). Information obtained from native MS measurements include, for example, oligomeric states, protein subunit interactions, protein self-assembly, and the binding of drugs, metals and cofactors (13-16). A range of other MS-based structural methods can be used to interrogate IMP structure, and provide further information regarding binding interfaces, conformation and dynamics (17), but these are beyond the scope of this chapter.

More than 60 % of drug targets are IMPs (18), notably the G-protein coupled receptors. IMPs, however, have proved difficult to structurally characterize, representing < 3 % of protein structures in the Protein Data Bank (PDB). The reasons behind this paucity of structural data are many-fold. For example, IMPs can be difficult to isolate, express recombinantly, and purify. They may require specific lipids or detergents for their solubilization, and they may need to be reconstituted into an environment (a suitable membrane mimetic) which contains all the necessary interaction partners and co-factors to adopt a conformationally defined state (19). Often, IMPs are extracted/solubilized from their native membrane environment using detergents but these can be perturbing, resulting in oligomerization and/or structural distortions (20). Therefore, alternative methods of IMP solubilization, such as amphipols (21), nanodiscs (22), and styrene maleic acid lipid particle (SMALPs) (20) have been developed with the aim of better mimicking biological membranes.

2. Native mass spectrometry-based structural biology

Unlike proteomics, which requires samples to be proteolyzed prior to MS analyses, native MS measurements are performed on intact proteins and protein complexes under sample conditions which preserve higher-order structure (conformations and interactions), i.e. typically aqueous buffered solutions *ca.* pH 7. The terms non-denaturing or non-covalent mass spectrometry are also used to describe this approach. Native MS relies on using gentle ionization and favorable MS pressure and voltage regimes in the instrument to maintain non-covalent interactions, including intermolecular interactions, in the gas phase. The methodology for the study of IMPs was adapted from studies of water soluble, globular proteins (23, 24), so we will begin by briefly discussing key aspects of native MS approaches as applied to water soluble proteins and how these must be altered to study IMPs.

2.1. Ionization and charge state distributions

For native MS experiments, "soft" ionization techniques are utilized that allow the analyte to be introduced into the gas phase without perturbing the non-covalent interactions that stabilize tertiary/quaternary structure. Electrospray ionization (ESI) is the primary technique utilized, although laser induced liquid bead ion desorption (LILBID) (25) and desorption electrospray ionization (DESI) (26) have recently been employed to study IMPs. In ESI, droplets are generated by applying a potential difference between a capillary containing the sample, and the sample inlet of the mass spectrometer. Positive and negative ions can be generated by ESI, with positive ion mode typically used for proteins. In positive ion mode, positive charges (mostly H^+ ions) accumulate at the tip of the ESI capillary, resulting in the spray of a fine mist of charged droplets (27, 28). The solvent from the droplets evaporates, and when the attractive surface tension holding the droplets together (Rayleigh limit) is overcome by the repulsive Coulombic forces from the accumulated charge, droplet fission occurs (Figure 1A). The process of solvent evaporation and droplet fission continues until the protein, some residual solvent or detergent/lipid, and possibly non-volatile components of the solution such as salt adducts, remain. The "charge" of this final droplet is transferred to the protein, generating a multiply charged protein ion (29). Typically, a miniaturized version of ESI, termed nanoESI is used (30). In nanoESI, lower flow rates (nL/min rather than µL/min for conventional ESI) and sample volumes (as low as 1 µL) are required. nanoESI is more tolerant of buffer contaminants and often results in improved desolvation efficiency. For native MS, static flow nanoESI devices are typically used to infuse samples without prior online chromatographic separation (unlike typical instrument configurations for peptide liquid chromatography-MS experiments) (Notes 1 and 2).

Native ESI of proteins/complexes results in charge-state distribution (CSDs) of proteins and protein complexes that often resemble statistical, i.e. Gaussian-like, distributions as a result of the solvent conditions and instrument settings (Figure 1B) (31). The average charge state adopted by a protein closely scales with its solvent accessible surface area (SASA) (32). Compact, folded species exhibit a narrow CSD at higher *m/z* values (low charge), whereas elongated or unfolded structures can accommodate considerably more charge per unit of mass and thus appear in a lower m/z region (often around m/z 1000-2000), and over a much broader range of charge states. Multimodal CSDs can indicate that several distinct conformations of a protein co-exist in solution. Combined, the CSD can be used to provide structural information, and rapidly test if a protein is (un)folded.

Native MS is not restricted to monomeric proteins. The non-covalent interactions responsible for retaining the native fold a protein are of comparable strength to those which maintain the oligomeric state of protein assemblies. Therefore, ESI can be used to transfer non-covalent oligomeric protein assemblies into the gas phase, even those which are largely stabilized by hydrophobic interactions (although some protein-ligand interactions may not survive the ESI process) (33, 34). Thus, in its simplest form native MS allows the determination of mass (confirming the identity and oligomeric state) and SASA of a protein/protein complex, given that globular proteins of defined shape and SASA adopt predictable charge states and collision cross sections (CCS) determined by ion mobility-MS (see Section 2.3).

2.2. Collision induced dissociation

Gas-phase dissociation of protein complexes inside the mass spectrometer can also provide useful structural information. The most common gas-phase dissociation technique is collision-induced dissociation (CID), which is also used for peptide sequencing (35, 36). In CID, the analytes are accelerated into a gas-filled collision cell where they undergo thousands of collisions with inert gas atoms/molecules, such as Ar or N_2. This results in a comparatively slow buildup of internal energy ("heating") causing the thermodynamically weakest bonds to be broken (37). CID of protein complexes can be recognized in mass spectra by the ejection of a highly charged subunit from the complex, where the ejected subunit takes a disproportionately large amount of charge with it (Figure 1C) (Note 3). This asymmetric dissociation is charge-driven and results in highly charged ejected monomer subunits at low *m/z* (similar to unfolded

monomer in solution), leaving the residual complex behind at *m/z* values higher than the precursor (8, 38-40) (Figure 1).

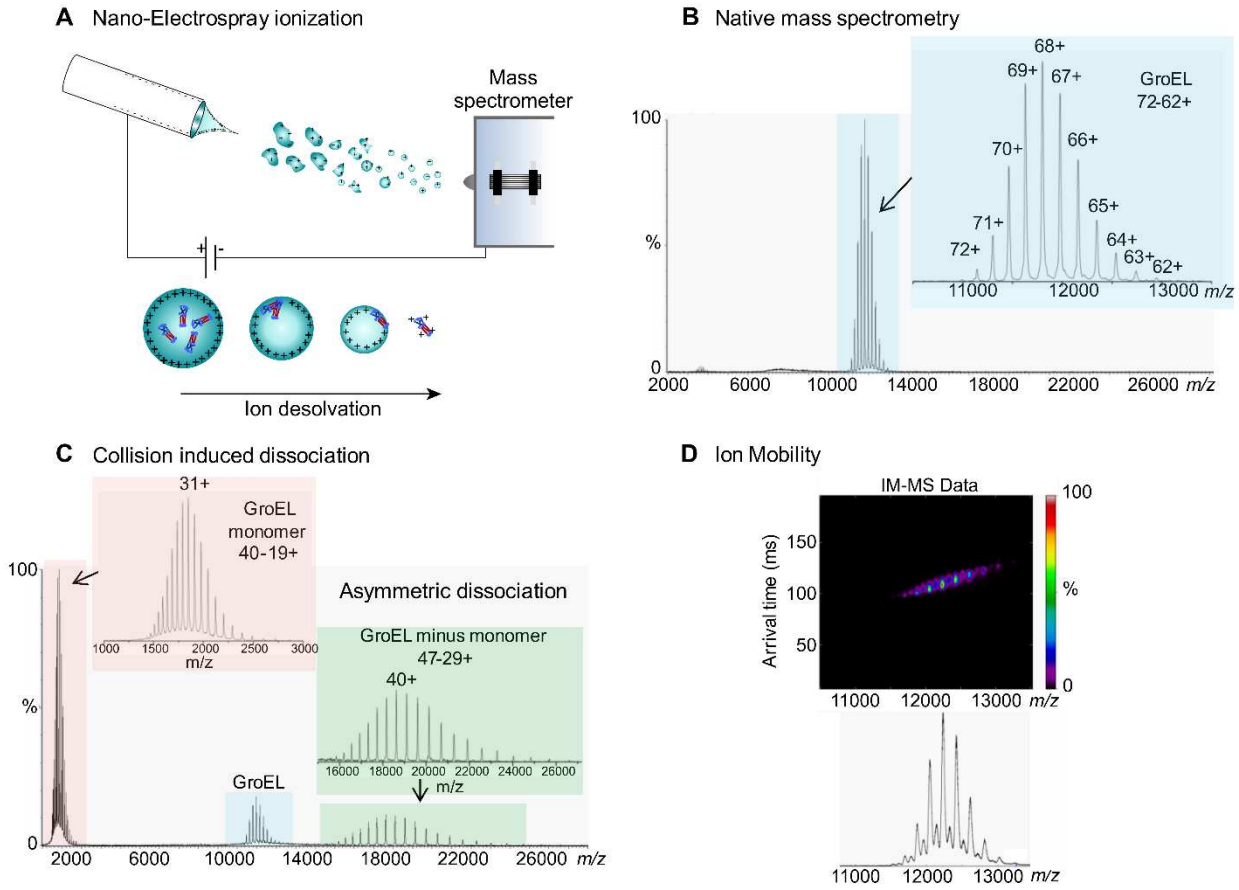

Figure 1: Scheme highlighting different aspects of native MS analysis. A) The first phase is the ionization of the sample by ESI. B) Native MS of the soluble 800 kDa GroEL protein complex containing two heptameric rings built from 57 kDa subunits. The CSD of GroEL (14 mer, 800 kDa) is compact and Gaussian-like, with charge states from 62+ to 72+ (see inset blue box). C) Increased collisional activation causes the GroEL complex to undergo asymmetric dissociation, with one monomer dissociating, taking up to half the total charge (red boxes) and leaving the charge-stripped residual complex (green boxes). The high charge taken by the monomer is due to this subunit unfolding first during collisional activation, with the corresponding increase in surface area allowing the accommodation of more charges. D) MS and ion mobility plot (upper half) (see Section 2.3) of GroEL 14mer. Arrival times are used for calculation of collisional cross sections.

2.3. Ion mobility-mass spectrometry

For more in-depth structural studies, native MS can be combined with ion mobility (IM, Figure 2). In its simplest form, IM separation is accomplished by propelling the ions through a He or N_2 gas-filled cell using a constant electric field (41, 42) in an experiment akin to gas-phase electrophoretic mobility. The time taken for an ion to traverse the drift cell depends on its mass, charge, shape and size. Ions with an extended structure will experience more collisions with gas molecules and therefore take longer to arrive at the drift

cell exit than more compact ions, which experience relatively fewer collisions (Figure 2A,B,E). IM-MS data are often presented in a so-called "drift plot" (Figure 1D), where the drift or arrival time is plotted against *m/z*, with the intensity of each peak indicated in the third dimension. IM-MS enables different protein conformations, or different topologies of a complex, to be observed as they result in different arrival times, even if they adopt the same charge state (single peak in the *m/z* domain). IM-MS can also be used to separate and resolve homo-oligomers which have overlap in m/z but have different mass and charge (Figure 2C,D,E).

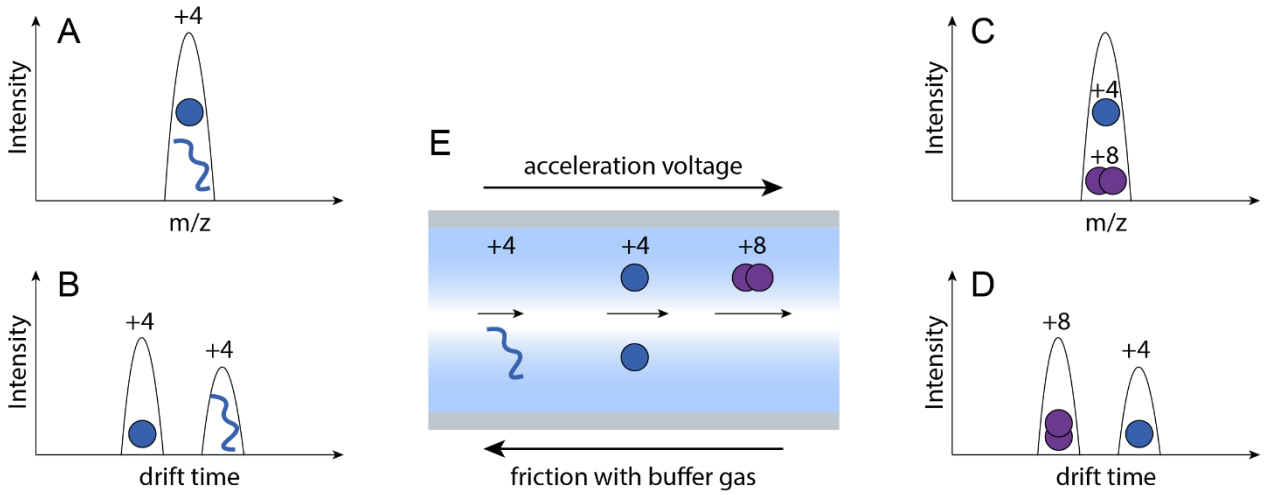

Figure 2: Principle of ion mobility. Friction of ions with buffer gas molecules allows the separation of ions according to their mass, charge, and shape. Conformers with the same m/z (A) (e.g. folded structures (sphere) and extended forms (string)) can be separated as they have different drift times (B). Overlapping oligomers with different mass and charge (C) can also be separated by ion mobility (D). (E) Schematic representation of IM separation.

The measured drift times can be converted to collision cross sections (CCS), directly when using a conventional IM device (43), or, if using a travelling wave IM-enabled mass spectrometer, after calibration using a series of ions of known CCS (44) (Note 4**)**. The CCS, typically given in $Å^2$ or nm^2, is a physical property of a molecule that corresponds to the rotationally averaged "shadow" of a protein or complex. CCS variations can indicate that a conformational change has occurred, e.g. due to effect of a bound co-factor or ligand, or that the protein is unfolded. Changes in CCS as low as 1 % can be considered significant (45). Various software packages (46-48) can be used to predict the CCS of a protein based on a high-resolution structure, or low-resolution models. This enables the experimentally determined CCS values to be compared with those calculated from structural data, which can be implemented in modelling pipelines (49). Alternatively, conformational changes upon ligand binding, or some other structural perturbation, can be monitored by the relative change in CCS (50).

Proteins can also be unfolded by gentle collisional activation, so-called collision-induced unfolding (CIU), with conformational changes detected by the resultant increase in CCS. It has been shown that the domain architecture of proteins directs their mechanism of unfolding in the gas-phase (51), therefore this technique can be used to inform on protein structure. CIU can also be performed on protein complexes, as activation results in unfolding of a subunit, prior to its ejection from the complex. This CIU approach is complementary to top-down CID fragmentation which can inform on the protein sequence (see Section 5.2). The CCS of a protein ion can be plotted against the collision energy voltage applied in the instrument (52), allowing the gas phase unfolding trajectory of the ion to be visualized. Typically, CIU results in stepwise changes in CCS once critical energy thresholds are reached, whilst at intermediate voltages the CCSs remain stable (53). Analysis of such unfolding trajectories allows the gas phase stability to be quantified (Note 5) (54). Quantitative comparison of CIU trajectories enables (de-)stabilization of protein structure as a result of the addition ligand binding to be detected (55). In the case of membrane proteins the stabilizing effects of different detergents or lipids have been examined, and even been used to predict appropriate conditions for membrane protein crystallization (56, 57).

3. Native mass spectrometry of integral membrane proteins

3.1. The native mass spectrum of IMPs

IMPs adopt, on average, lower charge states relative to water soluble proteins (58). Whilst the charge states acquired by water soluble proteins correlates with their solution-phase conformation (59, 60), this is not necessarily the case for IMPs. The reason for this discrepancy lies in the hydrophobic environment utilized for solubilizing the IMP (see Section 4.1). While the charge state distribution observed for water soluble proteins is finalized during the ionization process, the resulting charge states for IMPs are a product of gas-phase reactions necessary for release. IMPs are introduced into the gas phase in complex with their solubilizing hydrophobic environment, so this entire complex is the final charged species produced upon ESI. The IMP is released into the gas phase by accelerating the IMP-detergent/lipid/polymer complex using elevated voltages and high gas pressures once in the mass spectrometer (Figure 3A). It is likely that the lipid/detergent clusters carry charge when removed, leaving the IMP with lower than average charge compared with a water soluble protein of the same size.

IMPs adopt broader charge state distributions than water soluble proteins, partly because their average charge is low. Consequently, drawing conclusions based on the charge state distribution of IMPs alone might be misleading. However, for both IMPs and water soluble proteins, lower charged species are more compact/native-like and unfolded proteins are indicated by highly charged, low m/z species. Such unfolded IMPs could result from poor stabilization in solution due to the IMP being solubilized using the wrong hydrophobic environment (see Section 4.1), or an outcome of using harsh instrument conditions which leads to collision-induced dissociation/unfolding.

Several crucial instrument parameters must be optimized when analyzing IMPs by native MS. The gentle release of membrane proteins from the utilized hydrophobic environment without protein unfolding requires pressure and accelerating voltages to be tuned in both the source region (initial vacuum stage) and the collision cell of the mass spectrometer. Laganowski et al. have discussed this tuning process on a detergent-solubilized pentameric ligand–gated ion channel (61), highlighting the effect of different settings. In particular, the voltage applied to the sampling cone, drawing the ions into the mass spectrometer, must be raised to assist in desolvation and IMP ejection (from values *ca.* 10 V for water soluble proteins to *ca.* 200 V for IMPs). Energetic collisions using elevated CID voltages (from values *ca.* 10 V for water soluble proteins to *ca.* 220 V for IMPs), and gas pressures can also be used to release the IMP.

When elevated pressures are used, but with only low levels of collisional activation, the native mass spectrum is dominated by a broad, featureless background signal (i.e. a "hump", Figure 3B), due to lipid and/or detergent molecules remaining attached to the complex, making the sample highly heterogeneous. Increased collision energies are required for IMP release, illustrated here for nanodiscs (Figure 3B) (Note 6).

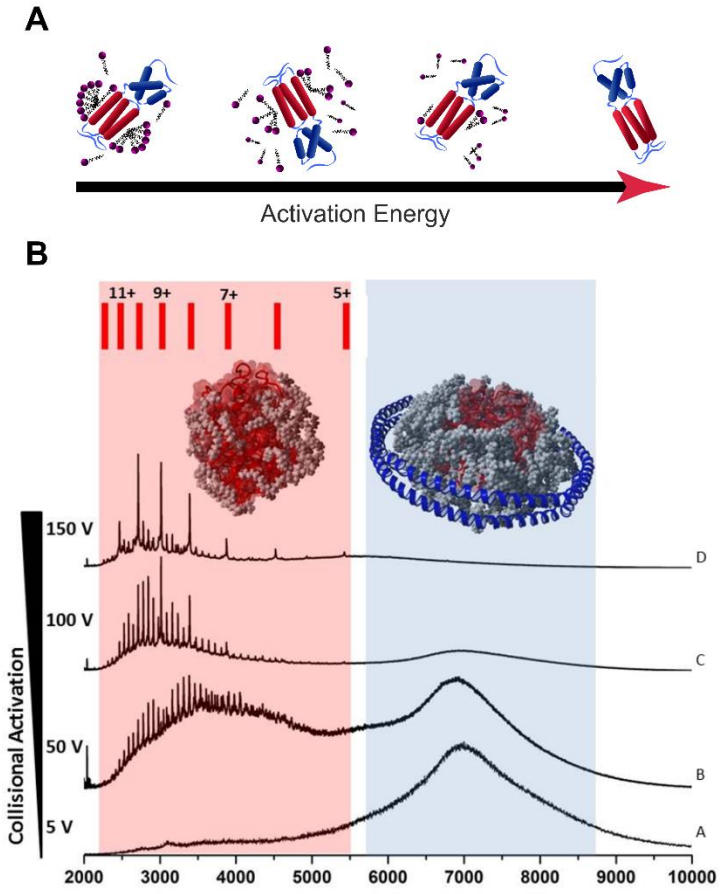

Figure 3: A) Schematic illustration of collision energy dependent IMP release in the gas phase. B) Release of a membrane protein reconstituted in a nanodisc (unpublished own data). The figure illustrates that too low releasing energies yield in a featureless "hump" caused by a wide variation of detergent and/or lipids still attached to the complex. An increase in collision energy leads to a gradual release of membrane protein complexes from the particles.

3.2. Instrumentation for native MS of membrane proteins

Instrumentation for native MS must be specially tailored to the needs of the experiment, in particular, to allow the controlled release of the IMP from the detergent/non-detergent reconstitution system to ensure unwanted unfolding/dissociation of the protein or complex does not occur.

After ionization at atmospheric pressure, ions are drawn into the mass spectrometer and the ion beam is focused in the first vacuum stage of the instrument by a combination of increased gas pressure and declustering voltages (62, 63). Maintaining a high pressure in this region results in an increased number of low energy collisions, decelerating the ions and focusing the ion beam (called collisional cooling, which is essential for measuring protein complexes in their native state) (63, 64). For reconstituted membrane proteins however, gentle activation by these collisions may provide the initial activation necessary for ejecting the protein from the protective detergent or lipid environment (63).

Having reached the vacuum of the mass spectrometer and with the detergent/lipid/polymer partially removed, the IMP then travels to ion optical elements (e.g. ring electrodes and quadrupole) which allow further declustering and focusing of the ion beam. A quadrupole ion guide can also be used for mass selection of ions (Figure 4 and 5), for example in tandem mass spectrometry experiments. However, IMPs may not be fully released from the detergent/lipid for individual peaks to be mass-resolved, limiting the ability to select individual m/z species for MS/MS (CID or CIU) (65). Instruments for native MS of IMPs are typically configured with an extended mass range (e.g. 32 000 m/z) quadrupole (62, 66), as native proteins, in particular IMPs, are relatively lowly charged.

Subsequently, the ions can enter a collision cell for CID, where the IMP can be fully released from bound detergent or lipid by collisional activation (unless this has already happened earlier in the source, see above). The released protein ions can then be detected using a mass analyzer, typically a time-of-flight (ToF) or Orbitrap device (see below).

3.3.1 Q-ToF based instruments for native MS

Quadrupole-time of flight (Q-ToF) instruments are particularly useful tools to interrogate large protein complexes, given that ToF analyzers have a theoretically unlimited *m/z* range. However, the quadrupole must be modified (such that it has an increased range up to *m/z* 32,000), and the pressures accessible in

the source region and collision cell must be increased, along with the declustering/collision voltages (62, 67) (Figure 4).

The Synapt mass spectrometer (68) (Waters, Wilmslow, UK) (Note 7) is a popular Q-ToF instrument, where the collision cell is replaced with a so-called 'Triwave', comprising of a travelling-wave IM cell flanked by two collision cells (termed 'trap' and 'transfer'). This configuration enables IM (Figure 2) measurements on native-like IMPs that have been released from detergent in the trap cell.

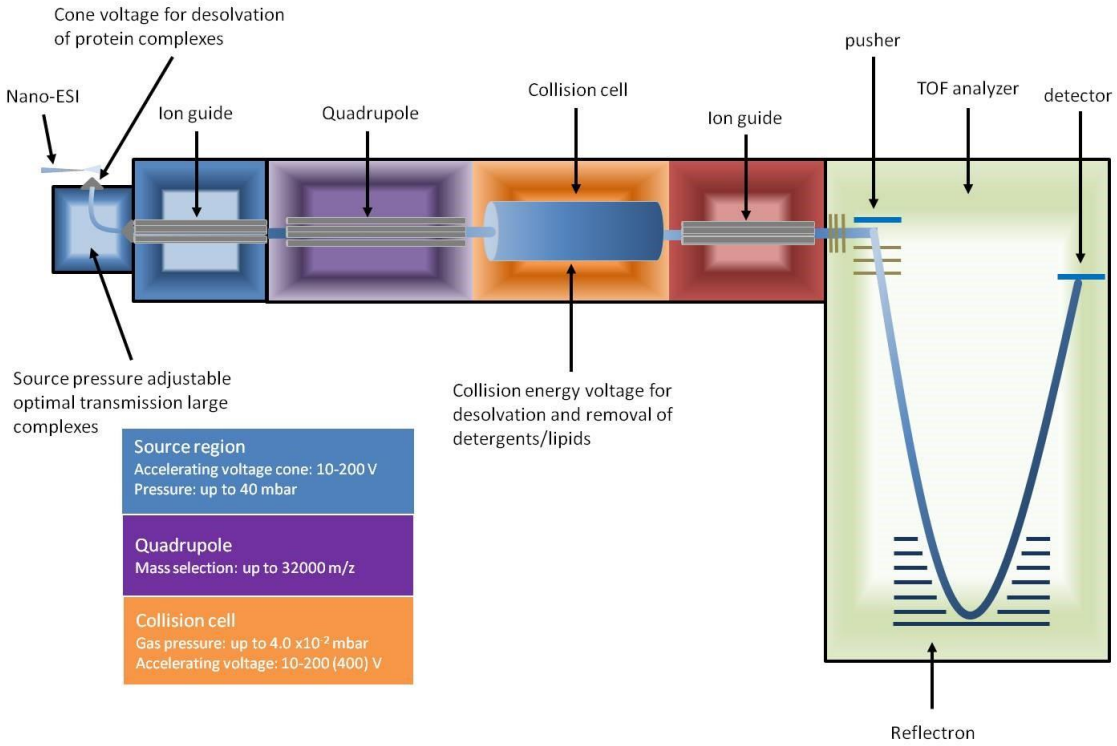

Figure 4: Schematic of a conventional Q-ToF instrument with key settings requiring optimization for transmission of large protein complexes. Ions are generated by nanoESI and enter the first vacuum stage in the source region (blue) where increased pressures provide for collisional cooling to improve the transmission of large complexes. Collisional activation, which is necessary for desolvation and removal of detergent/lipid clusters, can be initiated by an increased cone voltage (up to 200 V). The quadrupole (purple) mass filter can be used to select a specific ion of interest or a broader m/z region. In the subsequent collision cell, energetic collisions with inert gas molecules (argon or SF_6) may be used to fully liberate the IMP from the detergent/lipid cluster. The resulting ion populations are then analyzed in the time-of-flight (ToF) analyzer according to their m/z ratio.

3.3.2 Orbitrap instruments for native MS

Orbitrap mass spectrometers with extended or ultra-high mass range (EMR, UHMR) capabilities have recently been developed based on the Q-Exactive platform (Figure 5) (Thermo Fisher Scientific, Waltham/MA, USA). The high resolving power of the Orbitrap analyzer and the enhanced desolvation capability of the instrument enables high-resolution mass measurements of protein complexes (Note 8). The instrument allows, through so-called in-source trapping (S-Lens region), the release of membrane proteins using high energies in the early vacuum stages of the instrument, enabling subsequent precursor selection in the quadrupole. Since the instrument has been shown to preserve non-covalent interactions (69, 70), albeit without ion mobility capabilities, the high resolving power enables the effect of different lipids and lipid mixtures on protein assembly and stability to be studied (57, 71).

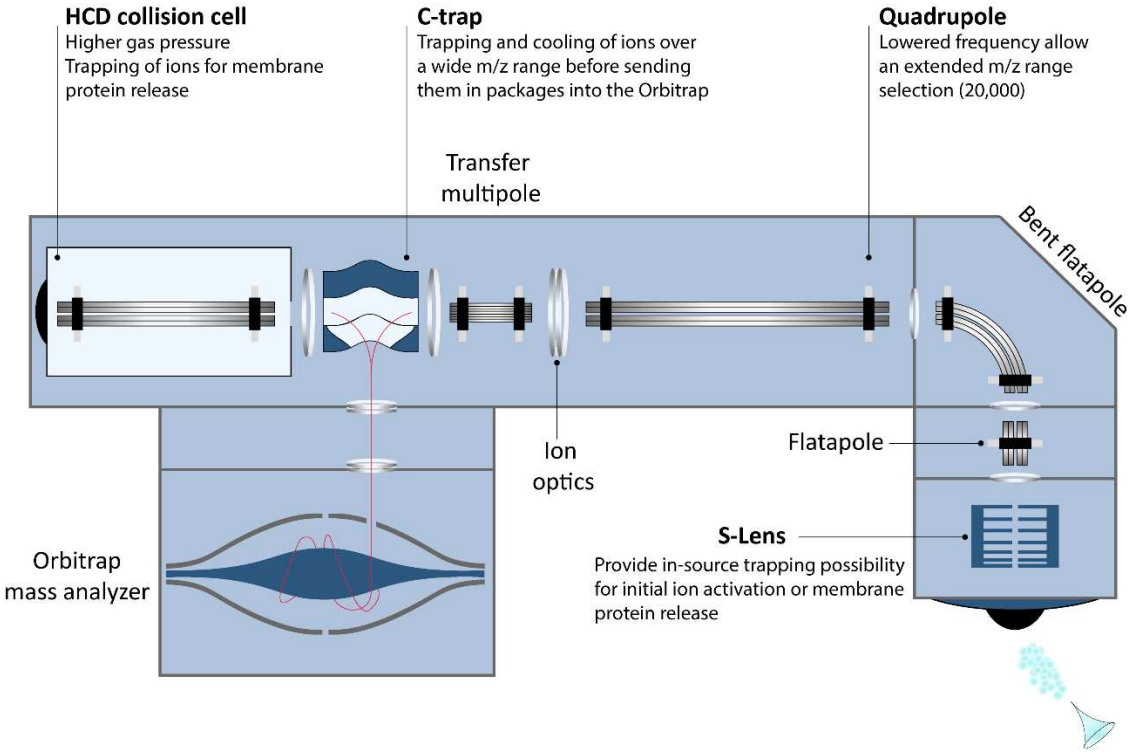

Figure 5: Schematic of a Q-Exactive EMR Orbitrap mass spectrometer (Thermo Fisher Scientific). Extended or ultra-high mass range (EMR, UHMR) modifications allows the investigation of large protein complexes which makes it of particular interest for IMP characterization. The possibility of collisional activation and IMP release in the S-lens (in-source trapping) enables MS/MS with ion selection in the quadrupole mass filter and further fragmentation in the HCD collision cell. It is also possible to preserve the embedded membrane protein complex until it reaches the HCD cell, where the IMP can be liberated. For analysis, the resulted ions get trapped in the C-trap from where ion packages are sent to the Orbitrap (drawing is not to scale) where orbiting ions create frequencies which are converted into m/z signals.

4. Buffers and IMP reconstitution for native MS

Samples for native MS must be relatively pure, comparable to the requirements for EM, NMR or X-ray crystallography (although MS has the advantage of being able to handle heterogeneous protein samples). Typically, only sub-mM concentrations of non-volatile biochemical buffers (e.g. Tris-HCl or phosphate) can be tolerated by ESI, as they do not evaporate during the ESI process and can form extensive clusters with the analyte and result in ion suppression (72, 73). Instead, protein samples are typically prepared in volatile solutions, such as ammonium acetate at mM to M concentrations with the pH adjustable by adding ammonia or acetic acid (23) (Note 9).

4.1. The "right" hydrophobic environment – Crucial for IMPs to adopt their conformationally defined states

Many reconstitution approaches have been developed to solubilize IMPs to enable their structural interrogation, whilst providing a native-like hydrophobic environment in an aqueous medium. To date, detergent micelles are used for most structural work, although detergent-free reconstitution systems [43] such as amphipols (21), bicelles (74), nanodiscs (75), and SMALPs (20) have also proven useful. Given the role that lipids are thought to play in regulating both the structure and function of IMPs, reconstitution systems that better mimic the native bilayer are being increasingly utilized.

In order to observe IMPs by native MS, the IMP embedded in an appropriate hydrophobic environment must first be introduced into the gas phase, as the detergent/lipid protects the structural integrity of the IMP (76). Once in the gas phase, the collisional activation must be applied to liberate the IMP from its hydrophobic environment (see Section 3.1).

4.1.1. Detergent-based reconstitution systems

Detergents are the most commonly utilized reconstitution system for native MS. When added to aqueous solutions above the critical micelle concentration (CMC), detergent molecules spontaneously form micelles. For native MS, detergents are typically added to IMP solutions at a concentration *ca.* double the CMC. Micelles comprise a hydrophobic inner core made up of the hydrophobic tails of detergent molecules, and are hydrophilic on the outside where the detergent head groups are facing the aqueous

solvent (Figure 6). Detergent micelles form around the hydrophobic regions or the whole of an IMP, thereby maintaining its solubility and usually, its structure.

An important factor in the selection of detergents for native MS is the amount of energy required to liberate the protein from the micelle after transfer into the gas phase (Note 10). Some detergent micelles are stabilized mostly by hydrophobic interactions, while others rely on the H-bonding network between the detergent molecules. Where electrostatic interactions are present between the detergent molecules, high levels of collisional activation may be required to dissociate the micelles, which may induce unwanted dissociation of the complex or collisional unfolding during its release. This will not be of concern in the case where the secondary interactions stabilizing a protein's structure are stronger than those between detergent molecules, but the amount of exposed (soluble) protein structure not embedded in the micelle may also play a role, as these are not protected from collisional activation (77, 78).

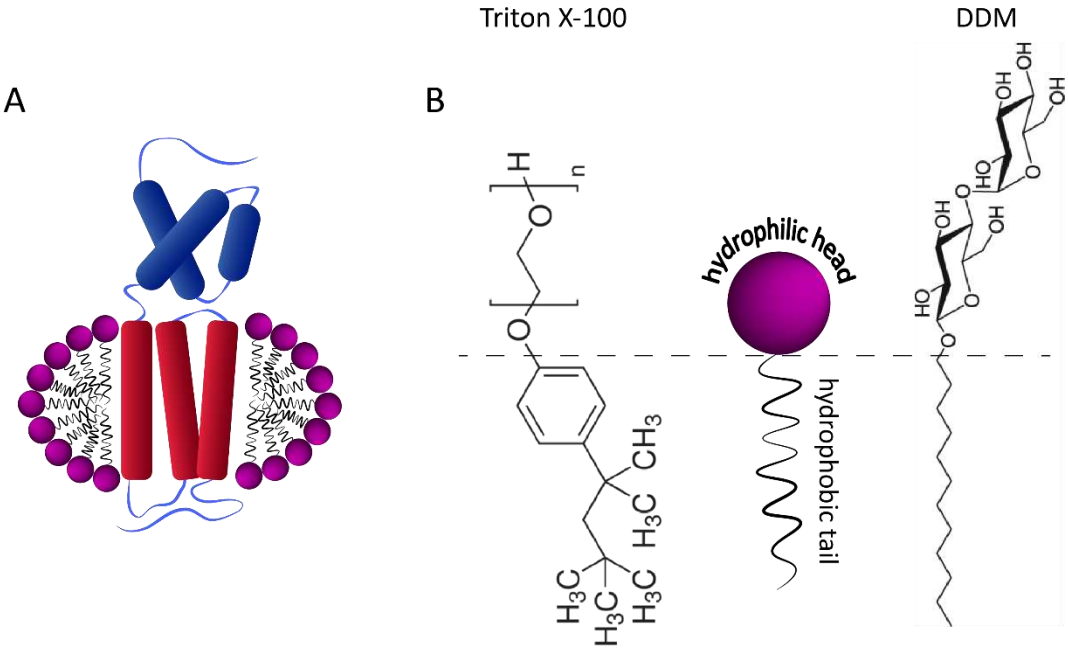

Figure 6: A) Reconstitution of the transmembrane domain (red) of an IMP in a detergent micelle (purple). The soluble domain of the protein lies outside of the micelle (blue). B) Structure of Triton X-100 (n typically around 9.5) and DDM with a schematic illustration of their hydrophilic head group (top) and their hydrophobic tail (bottom).

For each IMP, the detergent that preserves the native fold and assembly state must be determined (61, 79, 80). Finding the most suitable detergent can be a major bottleneck in structural biology pipelines generally. For native MS, a detergent that structurally stabilizes the IMP, but is also compatible with release of the IMP in the gas phase must be found. Native MS can be used to screen the effect of detergents on the folding and assembly state of a protein. For example, in the case of the mechanosensitive channel of large conductance (MscL), whilst n-Dodecyl-β-D-maltoside (DDM)

maintained the channel's structure in solution (50), detergent removal in the gas phase resulted in dissociation of the pentamer due to the activation necessary to disassemble the micelle. Lauryldimethylamine oxide (LDAO) did not maintain the native channel (oligomeric state) in solution, whereas Triton X-100 not only stabilized the native state in solution, but also allowed detergent removal in the gas phase to liberate the intact pentameric channel (Figure 7) (50, 81).

Some commonly used detergents are not well suited for native MS due to their strong electrostatic interactions which are hard to dissociate without affecting the protein inside, with non-ionic or zwitterionic detergents recommended, depending on the protein (78). The most commonly used detergent for native MS, as for many other structural techniques, is still the nonionic DDM. However, alternatives such as C8E4, C12E8 and Triton X-100 have also recently been shown to display favorable properties, as highlighted in a recent comparative study (82).

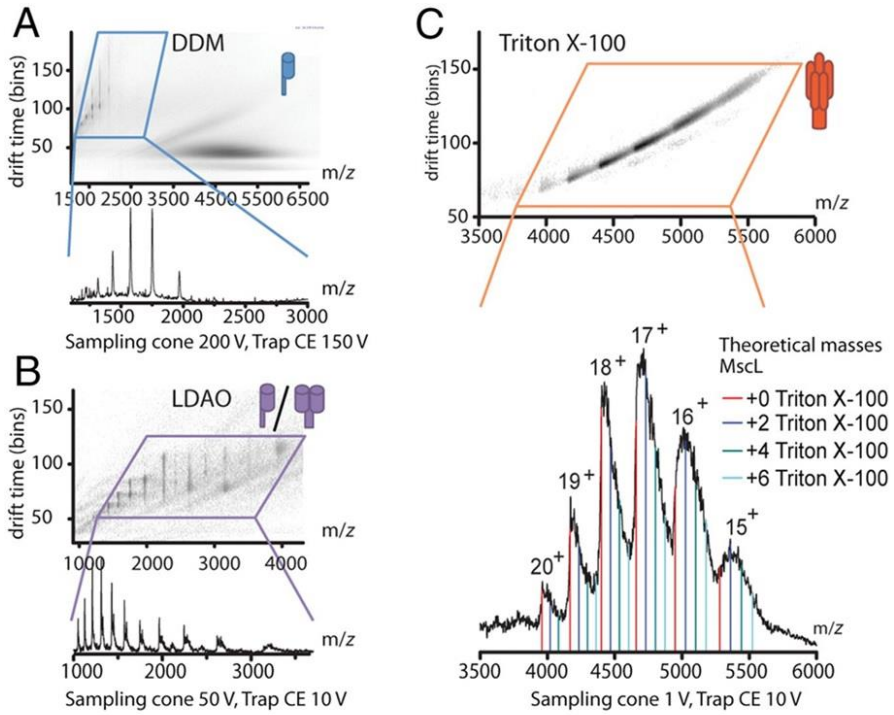

Figure 7: Native IM-MS spectra of MscL. Comparison of three different detergents: A) MscL released from DDM showed only monomers, but the protein is pentameric in solution (50). B) In LDAO only unfolded monomeric and dimeric MscL was observed, due to the non-native solution environment. C) The pentameric MscL complex was observed when liberated from Triton X-100 micelles which needs only low collisional activation (1 V sampling cone and 10 V trap CE) and results in compact conformations in the drift plot. Under the drift plot is the corresponding mass spectrum with charge states in the 15+ to 20+ range retaining up to 6 Triton X-100 molecules (vertical lines).

4.1.2. Detergent-free reconstitution systems

Detergent micelles may structurally perturb membrane proteins (83), due to the detergent being an artificial replacement of the lipid bilayer, and the curvature of the micelle (84). Therefore, detergent-free systems, which incorporate lipids or amphiphatic polymers (amphipols), are increasingly becoming preferred for structural studies of IMPs by all methods, since they are less structurally perturbing, and may better mimic native membranes. Bicelles, nanodiscs and SMALPs are different types of lipid bilayer discs encased with either short chain lipids/detergents (bicelles), an amphipathic α-helical membrane scaffold protein (MSP; nanodiscs) or a styrene maleic acid (SMA) co-polymer (SMALPs) shielding the hydrophobic tails of the lipids on the sides of the disc and keeping it “in shape” (Figure 8).

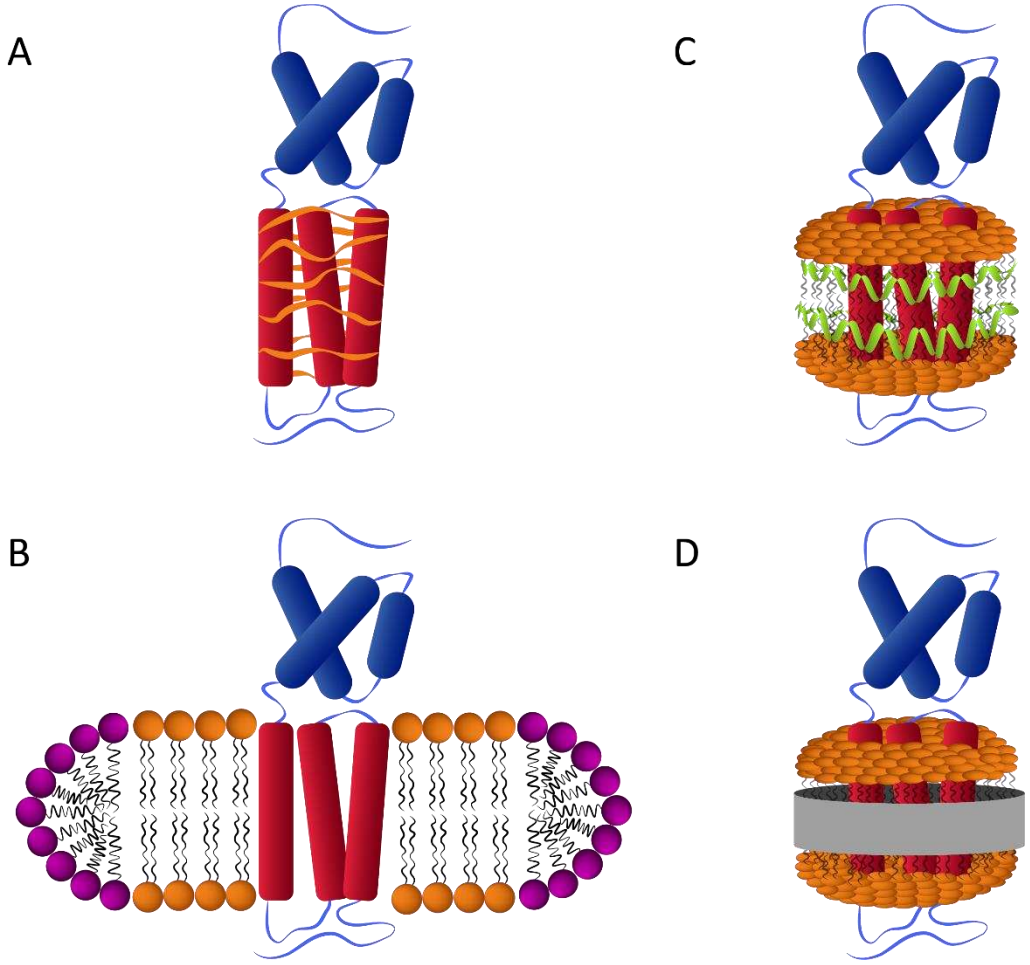

Figure 8: Detergent-free reconstitution systems. A) Membrane protein reconstituted in amphipols (orange). The amphiphatic polymer wraps around the transmembrane domain (red) keeping the IMP soluble. B) A bicelle of long-chain lipids (orange) and short-chain lipids or detergents (purple). C) Nanodisc formed by lipids (orange) surrounded by two membrane scaffold proteins (MSPs, green) responsible for disc formation. D) Membrane protein reconstituted in a SMALP where the protein is able to stay in contact with its native lipids (orange) from the lipid bilayer after cutting out with a co-polymer (grey).

Amphipols are amphipathic polymers which wrap around the hydrophobic regions of IMPs (Figure 8). It has been shown that amphipols can solubilize IMPs and act as vehicles for releasing IMPs into the gas phase (83, 85, 86). They seem to work best however for native MS of monomeric IMPs, since complexes have been shown to (partially) dissociate (83). The most commonly used amphipol is the anionic A8-35, a polyacrylate polymer which has octyl (hydrophobic) and isopropyl (hydrophilic) side chains. A8 stands for the average molecular weight of 8 kDa, and the number 35 represents the percentage of underivatized carboxylate groups. The remaining groups carry octylamine chains (25%), or are derivatized with isopropylamine (40%) (87). Together these three units form the amphipathic amphipol that solubilizes the IMP via multiple contact points with the transmembrane domain. Proteins solubilized in amphipol are more resistant to gas-phase unfolding than their detergent-solubilized counterparts (85), and they are also more stable in solution, possibly because the amphipol contacts more sites on the IMP than detergents do (21, 88) (Note 11).

Lipid-based reconstitution systems, e.g. bicelles and nanodiscs, have been successfully used to study IMPs by many structural methods, and have also proven useful for native MS (57, 83, 89). Bicelles (Figure 8) can be made with diameters ranging from 20 to 40 nm (90), with the size tuned by adjusting the ratio of long-chain to short chain lipids (91). Nanodiscs (Figure 8) are produced using membrane scaffold proteins (MSPs), with different MSPs producing discs with diameters ranging from 9.8 (MSP1D1) to 17 nm (MSP1E3D1) (92, 93). Due to the high lipid content of nanodiscs, the ability to generate nanodiscs with heterogeneous lipid mixtures, and the realization that IMPs reconstituted in nanodiscs bound to their entire lipid annulus can be studied by native MS, the obtained mass spectra can be difficult to interpret. This has been aided by developments in spectral deconvolution software (94, 95).

Typically, nanodiscs and bicelles are prepared from IMPs that have been purified in detergent. Contact with detergent prior to reconstitution in a lipid environment may still be structurally perturbing as detergents are harsh surfactants and may stabilize non-native states (20). However, IMPs can also be produced by cell-free (CF) expression (96), and inserted into preformed empty nanodiscs. This approach is particularly elegant, not only because it avoids potentially critical detergent contacts, but also because it enables overexpression of IMPs without using traditional bacterial culture approaches. IMPs incorporated into nanodiscs by CF expression have been analyzed by LILBID-MS (97).

Another promising approach for the reconstitution of IMPs in native membranes is SMALP technology. SMALPs use a specific variant of nanodisc-forming amphipathic polymer that extracts pieces of membrane bilayer in a cookie-cutter-like fashion together, along with the incorporated membrane protein (98). Their size distribution is however more polydisperse than for typical nanodiscs, making them less suitable for native MS.

5. Case Studies

5.1. Using ion mobility to investigate conformational changes

Extending native MS with IM can provide additional information on the IMP's conformational state and its possible transitions (50, 99). In the case of the pentameric MscL protein, a mechanosensitive ion channel that opens in response to high cellular pressure (100), native IM-MS experiments were conducted to examine conformational changes upon channel opening. Cysteine mutations were implemented in MscL monomers, which enabled covalent binding of a positively charged drug (2-(trimethylammonium) ethyl methanethiosulfonate bromide, MTSET) inside the channel, resulting in Coulombic repulsion, that forces channel opening in a manner similar to external membrane pressure (101). Since the channel is pentameric it can carry up to five of these positively charged MTSET moieties inside the pore. As the number of charges inside the pore was increased, the channel opened (IM profile highlights various different opening states of the channel) which was visualized as a stepwise increase in CCS (Figure 9A). More than one MTSET was required to be present in the pore to initiate the Coulombic repulsion which opened the pore and resulted in an increased CCS. Molecular dynamics simulations were used to demonstrate that the increase in size of the pentamers correlated with gradual pore opening (Figure 9B) (50).

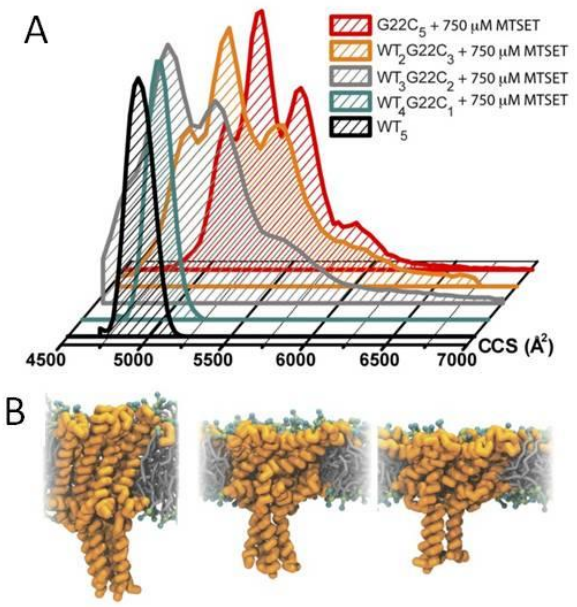

Figure 9: Stepwise channel opening of pentameric MscL highlighted by an increase in CCS and matched by a dynamic model. A) MscL with 0 (black), 1 (blue), 2 (gray), 3 (orange) or 5 (red) mutant subunits which enables the binding of increasing numbers of positively charged MTSET inside the channel causing its stepwise opening. Conformational changes were observed based on the increase in CCS which correlates with the number of charged MTSET molecules in the pentamer. B) Structural models of MscL under applied tension to the membrane. Figure adapted with permission from (50).

5.2. Top-down sequence information of membrane proteins

'Top-down' methods are becoming increasingly commonplace to gather sequence information for IMPs. IMPs can be introduced into the vacuum in a denatured (e.g. acid unfolded) or native (detergent solubilized) state. Here we will only consider the case of native IMPs characterized by top-down MS and the reader is referred elsewhere for a more complete discussion of top-down analysis of denatured IMPs (102).

This native top-down approach enables multimeric IMP complexes to be studied to determine how proteoforms (i.e. phosphorylation state, alternative splice variant, point mutant) may influence the stoichiometry of a complex, with the sequencing information used to confirm the identity of the exact proteoform implicated. In recent work, three IMPs, [the hepatitis C Virus (HCV) p7 viroporin, the mechano-sensitive ion channel of large conductance (MscL), and the Kirbac potassium channel 3.1] were studied by native top-down MS after liberation from detergent micelles (103) (Note 12). Mass selection of a precursor ion of each complex was performed followed by CID. Interestingly, predominantly b fragment ions were detected, along with several y fragment ions. Sequence coverage was low, 11 % and 18 % for MscL and Kirbac, respectively, mostly from fragment ions arising from the N-terminal portions of the proteins. Few fragments arose from the central or C-terminal portions of the proteins, as residual structure may remain which prevents fragmentation. Fragmentation of the smaller p7 viroporin, however, resulted in a sequence coverage of 73 %, consistent with a lack of residual structure. Interestingly, the fragment ions arising from CID of all three proteins were detected from regions of transmembrane helical secondary structure (as determined by high resolution structures or by prediction algorithms). Combined, in a single experiment native top-down MS of IMPs allows the oligomeric state, accurate subunit mass, and (partial) sequence information of the subunit(s) to be determined (103).

5.3 The role of lipids in membrane protein stability and functionality

In certain cases some lipids are so strongly bound to IMPs that even after purification with harsh detergents the lipid interactions remain unperturbed (104). There has recently been increased interest in the role of lipids in IMP structure and stabilization, resulting in the development of advanced lipid-based IMP reconstitution methods (see section 4.1). Here we will discuss examples where lipids have been observed to play a structural role in the case of detergent-purified IMPs.

The Robinson group investigated the dimerization of membrane transporters in the presence or absence of specific lipids. They found that some IMP complexes require structural lipids for oligomer stabilization when the protein-protein interactions alone are insufficient (67). For example, the bacterial leucine transporter LeuT, purified in OG, was detected as a dimer by native MS, but the measured mass was consistent with lipids remaining bound (Figure 10). After delipidation, by exchanging the detergent to neopentyl glycol (NG), only monomeric LeuT was observed. Even when the protein was returned to OG, the delipidated LeuT was still not able to dimerize. Addition of *E. coli* polar lipid extract to delipidated LeuT again caused the protein to form a lipid-bound dimer, meaning that specific lipids function to stabilize the dimer interface (Note 13). The addition of dilysocardiolipin did not result in a dimerization of the protein again (Figure 10), providing evidence that specific lipid binding is required to mediate dimerization. Specific lipids were also required for the dimerization of the *E.coli* Na^+/H^+ antiporter NhaA (67).

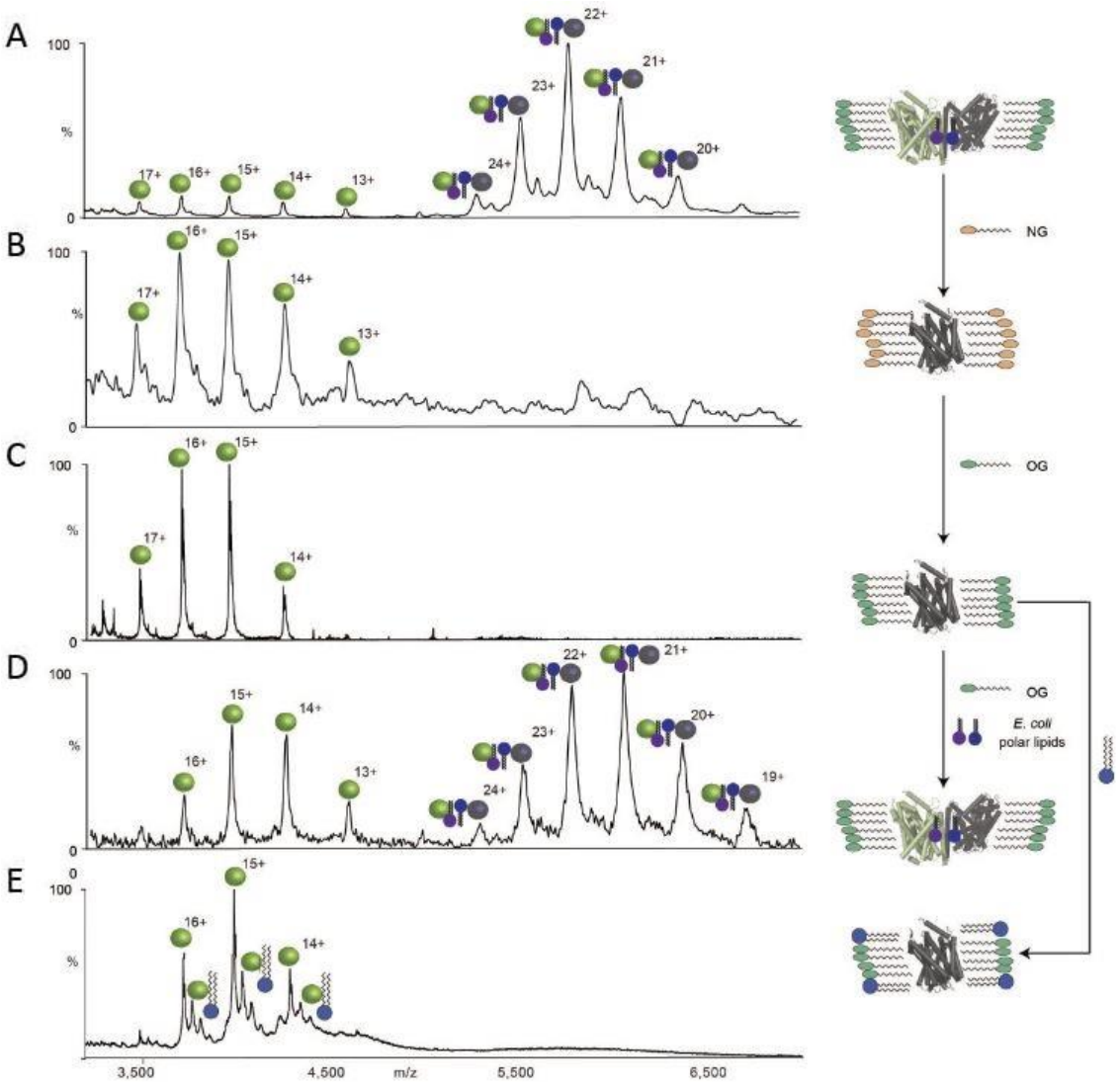

Figure 10: The role of lipids in membrane protein oligomerization. A) Mass spectrum of LeuT liberated from OG micelles shows predominantly lipid-bound dimers, with some delipidated monomer. After detergent exchange to neopentyl glycol (NG) the spectrum exhibits only delipidated monomer (B) and exchange back to OG micelles did not lead to dimer reassembly (C). LeuT dimerization was promoted by adding *E. coli* polar lipids to the delipidated monomeric LeuT (D). The mass spectrum (E) recorded after delipidation and in the presence of dilysocardiolipin (blue head group) shows no dimerization of LeuT. Reproduced with permission from (67).

Lipids have also been shown to have allosteric effects on IMPs. In one case, the role of lipids on the interaction between the trimeric ammonia channel AmtB and the regulatory protein GlnK was studied (105). The affinity of the AmtB-GlnK interaction was determined by MS in the presence of various lipid types (Figure 11) (Note 14). Up to three lipids bound to AmtB (one per subunit), with lipid binding modulating the affinity (K_D) of the AmtB-GlnK interaction. For example, binding of the lipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine [POPC] increased the affinity. This is likely because lipid binding triggers allosteric modulation of the conformational dynamics of AmtB. In some cases, binding of more than one lipid resulted in a further increase/decrease in binding affinity, suggesting that the binding of multiple lipids cooperatively influences the binding affinity (105)(Figure 11). Combined, the data demonstrated that the allosteric influence of lipid binding on the AmtB-GlnK interaction is modulated by both the identity of the lipid head group and the length of the hydrophobic tail (105).

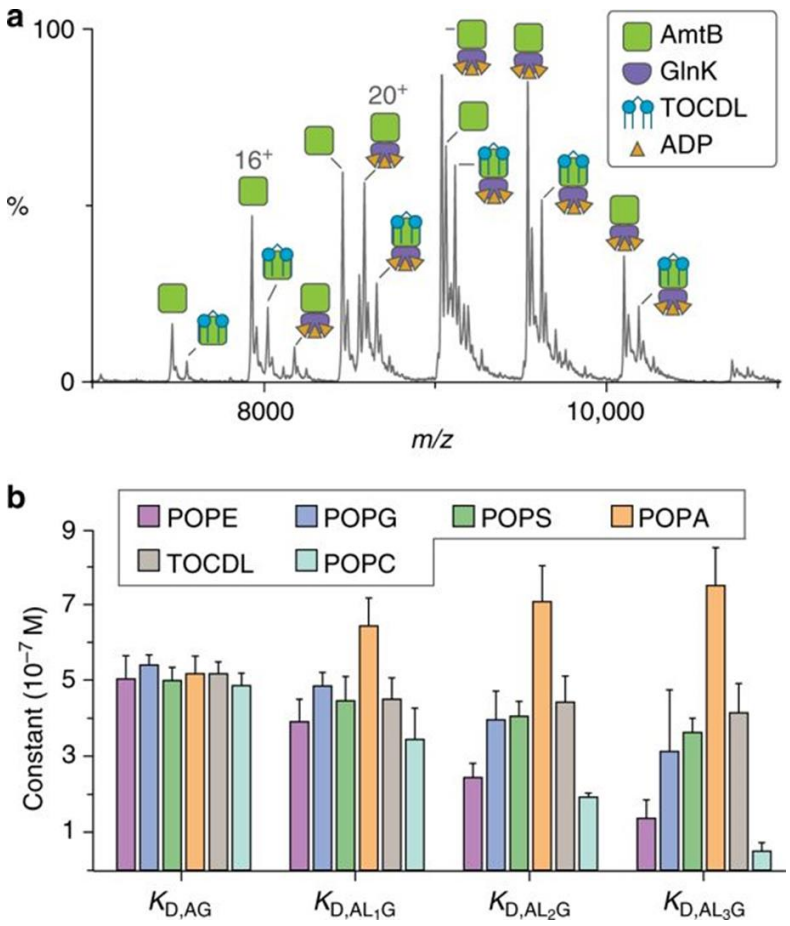

Figure 11: Native mass spectrometry used to observe the binding of lipids with different head groups and their allosteric effect on the complex formed by GlnK and AmtB. The mass spectrum in (a) demonstrates the complex formation between GlnK and AmtB in combination with ADP and the lipid 1,1',2,2'-tetraoleoyl-cardiolipin (TOCDL) which binds to the complex. (b) The K_D value of the binding between GlnK and either apo AmtB or AmtB bound to phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), and phosphatidic acid (PA) containing 1-palmitoyl-2-oleoyl (PO, 16:0-18:1) tails, and TOCDL. As can be seen, the different head groups of the lipids have an influence on the K_D value of the complex and thus indirectly affect the molecular interactions between AmtB and GlnK. Here POPC is the lipid with the best stabilization effect on the complex while POPA is the least. This effect was shown to be further influenced by the number of lipids bound. Reproduced with permission from (105).

5.4. Native MS can assist with high-resolution IMP structures

Crystallization of IMPs remain challenging, as demonstrated by the relatively few high-resolution structures available in the Protein Data Bank (PDB). However, low-resolution structural data and modeling approaches have been able to inform on the structures of IMPs in the absence of higher resolution data. Native MS has the potential to support the validation and/or refinement of model structures. For example, homology models of IMP can be tested by native IM-MS, by comparing the expected CCS of a homology model with the measured value, and native MS can be used to unequivocally determine the oligomeric state of a protein (104). Structural information can also be provided for complex, (hetero-)oligomeric assemblies, as native MS enables the study of subunit arrangements. For such studies, chaotropic solvents can be used to (partially) disrupt assemblies in solution prior to MS, and collision-induced dissociation can be employed to eject peripheral subunits in the gas phase, enabling the generation of topological models of protein assemblies (106). In case of homo-oligomeric protein complexes, e.g. multimeric ion channels, dissociation experiments will not generate much topological information, since the subunits are indistinguishable. In such cases, ion mobility can be used to investigate the arrangement of subunits. The CCS for both the intact complex as well as its subunits under mild solution disruption conditions can be matched to CCSs of models from proposed geometric subunit arrangements to experimentally determine the overall topology of the complex (107).

6. Future perspectives

In this chapter we have described how native MS can be used to characterize IMPs, which requires the IMP solubilized in detergent or some other hydrophobic environment to be volatilized and the protein liberated into the gas phase for analysis. Native MS approaches for IMP characterization are relatively new, dating back to 2008 when the detergent solubilized heterotetramer $BtuC_2D_2$ was studied (76). Since then many other IMPs have been characterized by native MS, enabling their subunit stoichiometry and lipid interactions to be studied, and their structure interrogated by IM. A crucial factor for the success of all of these studies is undoubtedly the hydrophobic environment used for solubilizing the IMP. The reconstitution system has a major impact on both structure and function, and even the presence of single lipids may play a regulatory or stabilizing role. Therefore, it is important to use a solubilization system which mimics the native lipid bilayer as best as possible, which is the major bottleneck of IMP characterization in general. SMALP technology, where a co-polymer is used to excise the IMP directly from a lipid bilayer, is an elegant way to avoid the loss of native lipids. Cell-free expression of membrane proteins and their direct incorporation into empty nanodiscs is also another way to generate samples for native MS without using harsh detergents that may be structurally destabilizing. Importantly for native MS, the secondary interactions between the protein and the reconstitution system must not be stronger than interactions between single subunits and/or lipids, so as to enable gentle release of the membrane protein complex without perturbing the protein's structure. To date, this process has not been successful for SMALP embedded IMPs, most likely because of strong interactions with the amphipathic polymer and the polydispersity of the particles generated. It may be necessary to fine-tune the polymer structure to improve the homogeneity of SMALP particles and allow for easier IMP release. This would enable IMP characterization in the native lipid environment and circumvent the need for testing of different hydrophobic environments, elevating IMP characterization by native MS to the next level.

7. Acknowledgements

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8. Notes

Note 1

Automated nanoESI sample inlet systems are commercially available, such as the Nanomate (Advion, Ithaca/NY, USA). More commonly, nanoESI is performed using glass capillaries with a thin metal coating (applied with a Sputter coater) or an inserted metal wire. These capillaries can be purchased or produced in-house with a capillary puller, e.g. Sutter Instrument P-97 (Novato/CA, USA). Analyte ions are generated by applying a voltage (1.2-1.6 kV) to the capillary containing the sample solution.

Note 2

Applying a gas pressure from behind the sample (0-2 bar) (nanoflow gas) may be required to initiate a spray, but spectra without this nanoflow gas are preffered. The capillary voltage should set to the lowest possible value that gives a stable spray (ca. 1.2-1.4 kV).

Note 3

It is expected that the smallest surface-exposed subunit of the complex, which is the easiest to unfold and detach from the complex, will dissociate preferentially. If further collisional activation is performed, the peptide backbone of the ejected subunit can also fragment (as for peptides), yielding sequence information (so-called top-down MS/MS fragmentation).

Note 4

Calibration of travelling wave-IM is performed using protein ions whose CCS have been determined by conventional (drift tube). Denatured and native proteins can be used. No IMP calibrants are available, and so native water soluble protein calibrants should be used. The calibrants should be carefully chosen (108) to avoid erroneous results due to the overall lower charge of IMPs relative to water soluble proteins and the energy required to liberate the IMP from detergent/lipid.

Note 5

Software packages have been developed to visualize and quantitatively compare CIU trajectories (54, 109).

Note 6

The fine-tuning between collisional activation and gas pressures must be carried out with great care so that the native structure is not significantly perturbed and that ion transmission is not lost. Incomplete

collisional activation can result in broad peaks in the spectrum due to insufficient ion declustering, whereas excess collisional activation can cause protein unfolding (detectable with IM), dissociation of complexes and/or fragmentation. Residual detergent or lipid remaining attached to the IMP may protect the released protein against unfolding or dissociation, as has been observed for residual water or buffer molecules bound to native, water soluble proteins and complexes (80, 110, 111).

Note 7

On the Synapt, the optimal source pressure should be determined for each sample, by adjusting the isolation valve (Speedivalve) of the roughing pump. Source pressures ca. 3-5 mBar are typical, but pressures up to ~8 mBar can be achieved on the instrument. Care should be taken when adjusting the valve to ensure the instrument does not vent. Increased cone voltages can improve desolvation, and transmission of high *m/z* ions. This increased energy can aid the liberation of IMPs, but also result in gas phase unfolding/charge stripping of the protein (as determined by CCS analysis). Cone voltages > 100 V are typically required to observe IMPs. The Trap DC bias voltage, which accelerates ions into the IMS sector of the instrument, is important to optimise, with voltages of 80-100 V often required to observe IMPs. IMS parameters must be optimised to ensure ions separate, and minimise artefacts (e.g. sample rollover, where an ion packet is injected into the mobility cell before the previous one has exited the cell). Wave heights ca. 5- 25 V and wave velocities ca. 200-400 m/s are typical, and the gas pressure in the IMS cell is typically 0.5 mBar.

Note 8

Ion transfer and ion optics parameters must be optimized for each sample, to ensure efficient ion transmission. IMPs can be released using in source trapping, with typical desolvation times of 5 ms, and energies from 50-200 V. Alternatively, IMPs can released in the HCD cell using a HCD time of 5 ms, and energies from 50-200 V (71).

Note 9

Buffer exchange can be performed by dialysis, size exclusion chromatography or using centrifugal concentrators (e.g. Vivaspin, Sartorius) or desalting columns (e.g. Zeba columns, Thermo Fisher). It may be necessary to perform multiple buffer exchange steps to minimize salt adduction.

Note 10

Detergent screening can be conducted easily if the IMP has an affinity tag. The IMP can be immobilized on a resin (e.g. Ni-NTA for a His_6 tagged protein), washed with a new detergent and then eluted (61). The IMP can then be desalted using SEC or some other method.

Note 11

Amphipol removal requires very high energy regimes. In many cases it may be easier to start optimization from high voltage conditions which causes the IMP to be liberated from the amphipol, but also results in protein unfolding (as determined by IM). MS conditions can then be made gentler, so that that unfolding no longer occurs, but release does.

Note 12

For top-down analysis the IMP must be released from the detergent/lipid/polymer in the source region of the mass spectrometer to enable subsequent mass selection in the quadrupole. This can be achieved using in source trapping on the Orbitrap or using elevated cone voltages on Q-ToF instruments.

Note 13

Lipids should be prepared in detergent containing buffers using established protocols to ensure that adducts are removed (61). An excess of lipids may have disadvantageous effects on the spectrum. Therefore, it is recommended to wash the sample a few times with detergents using centrifugal concentrators (e.g. Vivaspin, Sartorius).

Note 14

Binding affinities can be determined by performing titrations with subsequent detection and quantification by MS. Sample conditions, however, must be carefully controlled for reliable K_D determination. Software for automated spectral deconvolution and K_D estimation can be used to aid analysis (95).

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