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Mass spectrometry-enabled structural biology of membrane proteins

Antonio N. Calabrese *, Sheena E. Radford

Astbury Centre for Structural Molecular Biology, School of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK

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ABSTRACT

The last ~25 years has seen mass spectrometry (MS) emerge as an integral method in the structural biology toolkit. In particular, MS has enabled the structural characterization of proteins and protein assemblies that have been intractable by other methods, especially those that are large, heterogeneous or transient, providing experimental evidence for their structural organization in support of, and in advance of, high resolution methods. The most recent frontier conquered in the field of MS-based structural biology has been the application of established methods for studying water soluble proteins to the more challenging targets of integral membrane proteins. The power of MS in obtaining structural information has been enabled by advances in instrumentation and the development of hyphenated mass spectrometry-based methods, such as ion mobility spectrometry-MS, chemical crosslinking-MS and other chemical labelling/footprinting-MS methods. In this review we detail the insights garnered into the structural biology of membrane proteins by applying such techniques. Application and refinement of these methods has yielded unprecedented insights in many areas, including membrane protein conformation, dynamics, lipid/ligand binding, and conformational perturbations due to ligand binding, which can be challenging to study using other methods.

Contents

Abbreviations: ATD, arrival time distribution; CCS, collision cross section; CID, collision induced dissociation; CIU, collision induced unfolding; DDM, n-dodecyl-β-D-maltoside; ESI, electrospray ionization; FPOP, fast photochemical oxidation of proteins; HDX, hydrogen-deuterium exchange; HRFP, hydroxyl radical footprinting; IMS, ion mobility spectrometry; MS, mass spectrometry; MP, membrane protein; *m/z*, mass-to-charge ratio; OMP, outer membrane protein; SMALP, styrene maleic acid lipid particle; XL, chemical crosslinking.

* Corresponding author.
E-mail address: a.calabrese@leeds.ac.uk (A.N. Calabrese).

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1. Introduction

Membrane proteins (MPs) represent a disproportionately large number of therapeutic targets (~60%) [1–3] given that they comprise only 25–30% of the proteome [4,5]. MPs perform numerous essential cellular functions, including signaling, transport, cell adhesion and catalysis [6–10]. Despite their importance, structural information pertaining to this class of proteins is relatively sparse when compared with water soluble proteins. The so-called 'resolution revolution' [11,12] in cryo-electron microscopy (cryo-EM) has meant that structural information of MPs is now accessible for samples where X-ray crystallography has failed, or is unsuitable. Despite this increase in capability, both of these methods rely on trapping or enriching the conformational state of the protein of interest prior to study, whether this be by plunge freezing or crystallization. This makes it impossible to interrogate protein dynamics directly and to define the functional cycles of proteins, where conformational changes may be required and protein states are in dynamic equilibrium. Moreover, lipid/ligand binding, lowly-populated states, and co-populated conformations are difficult to discern by cryo-EM and crystallization, although class averaging in cryo-EM data analysis can be used to unpick structural ensembles [13–15]. Structural mass spectrometry (MS) methods do not require freezing or crystallization, the lack of ensemble-averaging in native MS enables ready detection of co-populated conformations [16,17] and ligand bound states [18–23], and the methods are not (often) restricted by protein size (especially methods which utilize bottom-up analyses) [24–26]. Real-time MS measurements also enable kinetic information to be determined regarding protein interactions with ligands or binding partners [27–30]. Consequently, MS-based methods are being increasingly applied in structural studies of both water soluble proteins and MPs.

The burgeoning field of structural MS has flourished over recent years, with instrumental and methodological advances spawning new approaches to interrogate both water soluble and MP structures and their interactions [31–35]. Broadly speaking, the structural MS approaches that have been developed can be separated into two classes, namely native (or non-covalent) MS and labelling-MS (Fig. 1). Native MS exploits the ability of electrospray ionization (ESI) to maintain non-covalent interactions upon ionization, thereby preserving tertiary and quaternary structure for interrogation *in vacuo* [34,36]. This approach allows protein subunit stoichiometry and architecture to be studied (the latter when coupled with ion mobility spectrometry, IMS) [33,37]. Labelling approaches include chemical crosslinking (XL) [26,38–41], hydrogen/deuterium exchange (HDX) [42,43], and surface labelling methods, including chemical labelling (CL) [44–46], and hydroxyl radical footprinting (HRFP) by methods such as synchrotron radiolysis [47] or fast photochemical oxidation of proteins (FPOP) [48,49]. In these methods, the protein is labelled in solution, and is subsequently proteolysed before the resultant peptides are analyzed by MS either qualitatively or quantitatively. Each of these MS-based approaches provides a different type of structural information and, in many cases, integrating data from several of these methods (perhaps supplemented by other data) can be informative [50]. Whilst these approaches provide low resolution structural information, the challenging nature of numerous biological systems (e.g. size, conformational dynamics, intrinsic disorder, low abundance, heterogeneity and transiency), including those for proteins embedded in membranes (which have the added challenge of protein solubility), often precludes the use of high resolution methods such as X-ray crystallography, cryo-EM or nuclear magnetic resonance (NMR) spectroscopy. In many instances, the approaches used to study MPs by structural MS have been developed from those

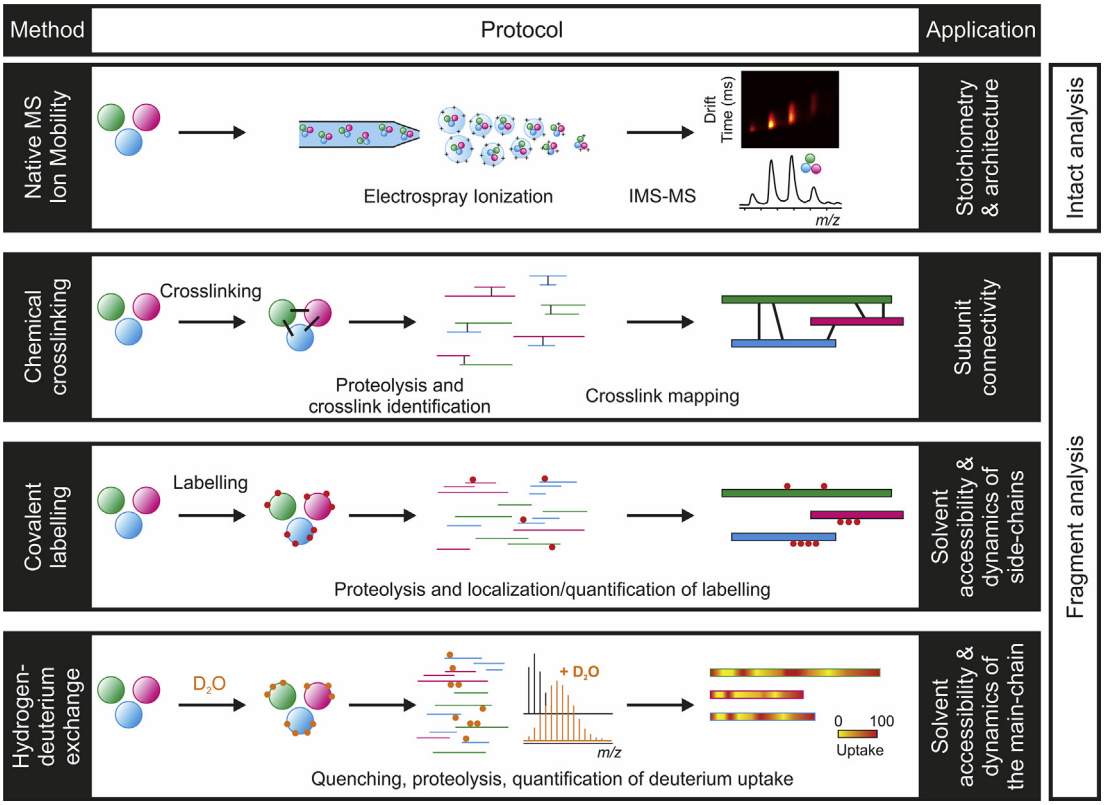

Fig. 1. Structural Mass Spectrometry. Summary of structural mass spectrometry methods, workflows and the information obtained from each experiment. Native MS and IMS-MS involves analyzing proteins intact, whilst maintaining non-covalent interactions. The other methods depicted typically involve analyzing peptide fragments and mapping the labelling sites onto the protein structure.

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utilized to study water soluble proteins, but the methods have had to be refined in order to overcome challenges unique to MPs, especially the requirement for solubilization by detergent micelles, membrane bilayers or other amphiphiles [51,52].

In this review, structural MS methods that have been utilized and/or developed to study MPs are described, demonstrating the vast array of applications where MS can provide unique insights into the biochemical processes mediated by MPs. MS studies have yielded insights into MP conformation, the stoichiometry of MP complexes, lipid/ligand binding, and conformational perturbations due to ligand binding, amongst others. The rapid advances made in the field of structural MS of MPs, especially the fast pace with which instrumentation is advancing, make MS uniquely placed to enable future discoveries, with the consequence that MS-based methods are bound to remain a pivotal component of the structural biology toolkit.

2. Native mass spectrometry to study membrane protein structure

Native (or non-covalent) mass spectrometry relies on the transfer of proteins into the gas-phase whilst retaining intermolecular interactions, and thus (some aspects of) tertiary and quaternary structure [36]. This has been enabled by the advent of soft ionization methods, especially electrospray ionization (ESI) [53]. ESI produces multiply charged analyte ions, with each protein species represented in a mass spectrum by a series of peaks, each of specific mass-to-charge ratio (m/z) termed a charge-state distribution, which usually resembles one or more Gaussian distributions [54]. Multiple Gaussian distributions for a single species indicates the presence of several conformations [55,56]. Compact states acquire fewer charges during ESI compared with expanded structures, as fewer ionizable sites are solvent exposed [57]. The fundamental principles of ESI have been extensively studied to date, and several mechanisms for ion formation have been proposed [53,58]. Typically, a miniaturised version of ESI, termed nano-ESI, is the ionization method of choice for native MS analyses, as it only requires sample flow rates of nL/min (compared with μL/min for conventional ESI), reducing sample consumption (~1 μL) (Figs. 1 and 2). Nano-ESI is also more sensitive and tolerant to buffer contaminants than conventional ESI [59], and since the droplets formed by nano-ESI are smaller, low source/desolvation temperatures can be used, enabling non-covalent interactions to be retained which may be destabilized as a result of heating [60]. Combined,

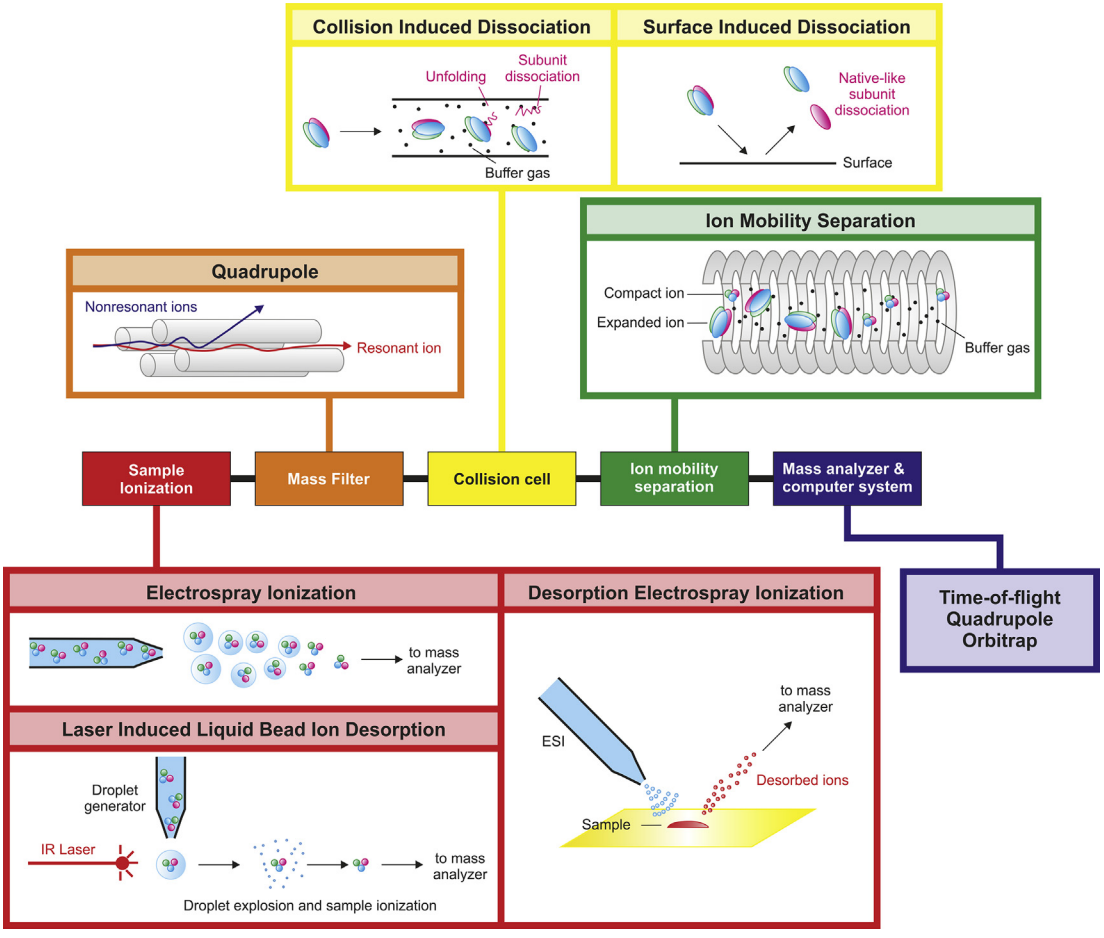

Fig. 2. Components of a typical mass spectrometer. Mass spectrometry analyses begin with sample ionization (red box). Typically, ESI is used, whereby an electric potential is applied to a fine tapered capillary containing the sample. This results in the formation of charged droplets containing the analyte, which liberates ions into the gas-phase for mass analysis. Alternative ionization methods have been used for analysis of MPs by native MS, including LILBID and DESI (see text). A quadrupole (orange box) can be used as a mass filter to select ions of particular m/z. Ions can be collisionally activated by a number of means (yellow box), including CID (where ions are accelerated in the presence of an inert buffer gas, which leads to protein unfolding, ejection of subunits from complexes and/or peptide bond cleavage), or SID (where ions are accelerated into a surface, resulting in disassembly of complexes). Ion mobility (green box) can be used as an additional gas-phase separation technique. It separates ions based on their mobility through a buffer gas filled cell under the influence of an electric potential. A number of ion mobility devices are available including travelling wave IMS [85,86], conventional IMS [87], and trapped IMS [88]. Instruments may also be configured with IMS devices before the quadrupole mass filter. Finally, ions must be mass-analyzed (blue box) by, for example, time-of-flight, quadrupole or Orbitrap devices. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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these unique properties of nano-ESI mean that is the method of choice for native MS analyses. Buffers typically used for *in vitro* studies of proteins (e.g. Tris-HCl, phosphate, HEPES, MOPS, MES etc.) are non-volatile and, therefore, generally not compatible with ESI-MS as they lead to adduct formation and/or ion suppression [60]. Consequently, MS compatible solutions, such as aqueous ammonium acetate, ammonium bicarbonate or ammonium formate, are commonly used for native MS applications, and buffer exchange must be stringent [60]. Ammonium acetate is typically utilized for native MS applications, as ammonium bicarbonate has been shown to generate high protein charge states (relatively unfolded ions) when analyzed at pH 7, but this can be regulated by changing the capillary voltage and source temperature [61–63]. It is important to note that at pH 7, ammonium acetate does not function as a buffer at all, but is effective around pH 4.75 and 9.25 (pKa values of the CH_3COOH/CH_3COO^- and NH_4^+/NH_3 acid/base pairs, respectively) [64].

The requirement to use specific non-volatile buffers for native MS studies can be problematic for the analysis of proteins/complexes that are stabilized by specific or non-specific ion adduction, or in instances where high (or low) ionic strength conditions promote complex formation or dissociation. Moreover, ionic strength, salt composition and pH are carefully tuned *in vivo*, and many biochemical studies use buffers that attempt to mimic such conditions. Aqueous ammonium acetate solutions are often insufficient for this purpose. Several strategies have been developed that enable the presence of mM concentrations of non-volatile salts in nano-ESI analyses. These include adding supercharging reagents [65], additives [66–68], or high ammonium acetate concentrations to prevent salt-induced ion suppression/adduction [60,69,70]. More recently it has been shown that using submicrometer nano-ESI capillaries (<0.5 μm compared with conventional emitter sizes of 1–10 μm) enable the detection of well-resolved charge state distributions for native proteins and protein complexes analyzed from high-salt biochemical buffers (e.g. 25 mM Tris-HCl, 150 mM NaCl and phosphate buffered saline) [71,72]. This technology has also been demonstrated to be applicable for the analysis of detergent solubilized MPs by nano-ESI (in 150 mM NaCl, 25 mM Tris-HCl, 1.1% (w/v) n-octyl-β-D-glucoside) [73]. Further developments in this area will enhance the range of proteins/assemblies that can be studied by nano-ESI-MS and ensure that native MS becomes more synergistic with other biochemical analyses.

There is much experimental evidence to demonstrate that protein conformations *in vacuo* are not significantly affected by the ionization process and the loss of the solvent [74,75], although some data suggest that a degree of reorganization does occur [76]. Indeed, there is evidence that structural compaction occurs in unstructured loop regions [77,78], in the case of intrinsically disordered proteins [79,80], and where large cavities may be structural features of proteins/assemblies [81]. Moreover, the lack of the hydrophobic effect in the gas-phase, which stabilizes protein structural states and protein complexes, means that some assemblies

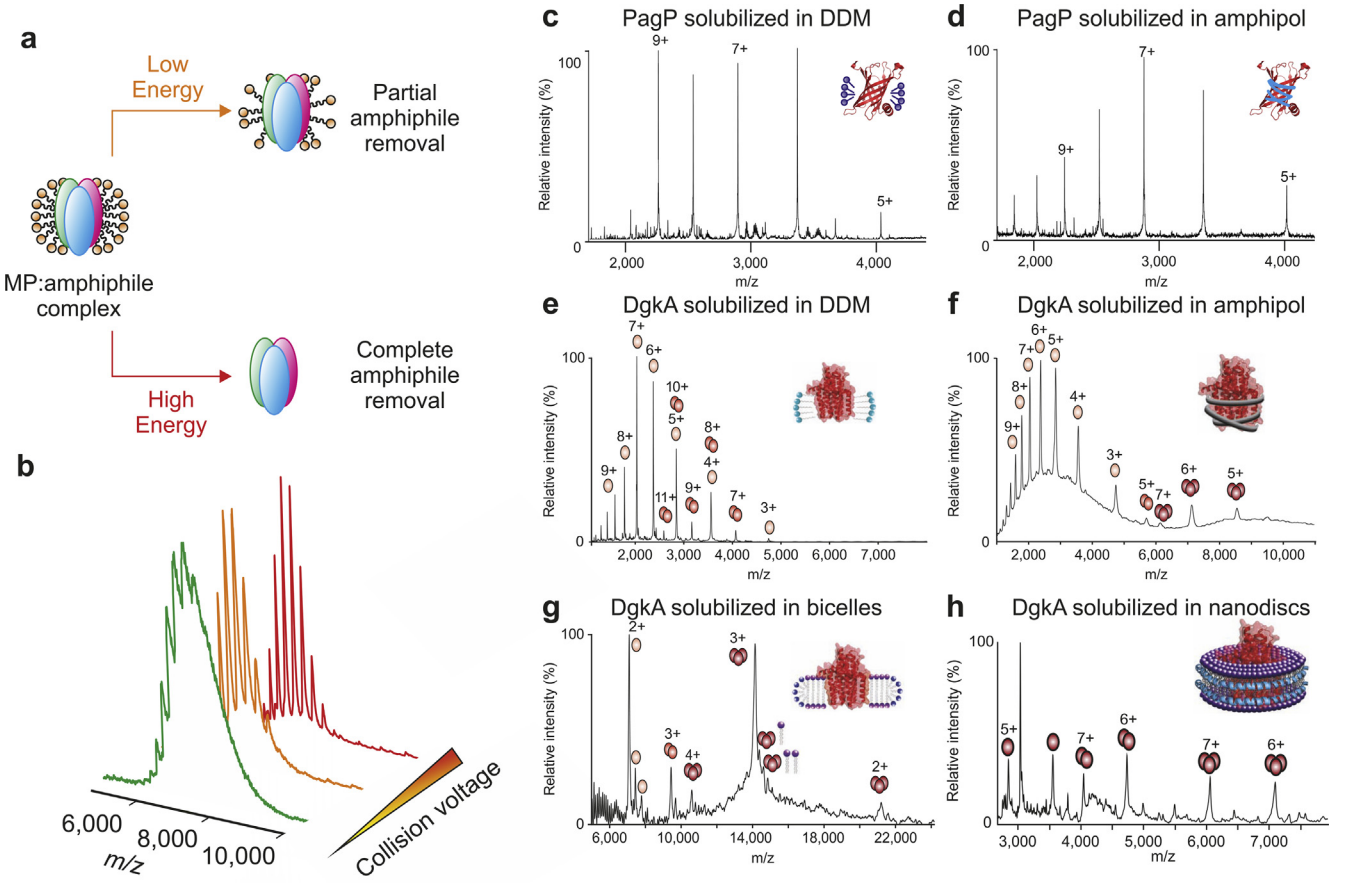

Fig. 3. Native mass spectrometry of membrane proteins. (a) In order to observe ions corresponding to a MP, the solubilizing amphiphile must be removed by energetic collisions after transfer into the gas-phase. Applying insufficient energy results in only partial amphiphile removal and a poorly resolved spectrum (see b). (b) Increasing the collision voltage in the mass spectrometer results in increased peak resolution as a result of detergent removal. (c,d) nano-ESI mass spectra of PagP solubilized in (c) DDM and (d) amphipol A8-35 [89]. In c the charge state distribution comprises two Gaussian distributions, suggesting two conformations are present. Spectra were acquired at a collision energy of 100 V. (e-h) nano-ESI mass spectra of DgkA solubilized in (e) DDM, (f) amphipol A8-35, (g) bicelles and (h) nanodiscs [90]. Part b is adapted from Ref. [84] with permission. Parts c and d are reproduced from Ref. [89]. Parts e-h are reproduced from Ref. [90] with permission.

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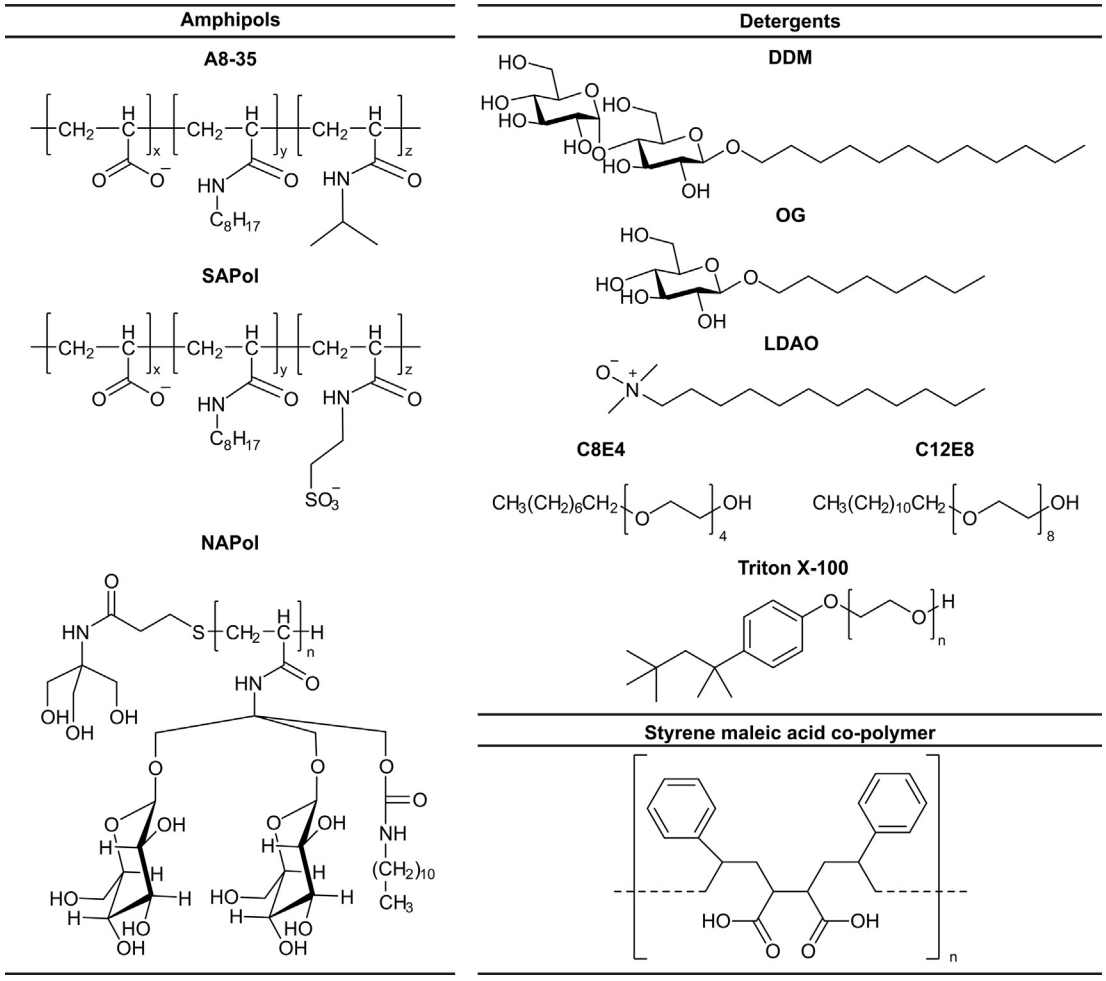

Fig. 4. Example structures of polymers and detergents used to solubilize MPs. Several structures of amphipols, detergents, and the styrene maleic acid co-polymer are shown. A8-35 is the most well characterized amphipol and it comprises free acids for solubility (x), whilst hydrophobicity is provided by octyl (y) and isopropyl (z) grafting [91,92]. Taurine (z) groups are used in SAPol to confer solubility at low pH [91,92]. NAPol uses glucose-based moieties to confer solubility. In the case of C8E4 and C12E8, "C8" and "C12" denotes an aliphatic chain length of 8 and 12, respectively, whilst "E4" and E8" refer to the number of repeats of the ethylene glycol unit. The styrene maleic acid co-polymer is generated by hydrolysis of a styrene maleic anhydride co-polymer [93].

may be too labile, and will not retain their native fold during nESI, although this is not always the case [82,83]. In the analysis of water soluble proteins using native MS, gentle ionization and instrument conditions are typically used to preserve non-covalent interactions [60]. However, for MPs, mass analysis requires the dissociation of the MP from the solubilizing amphiphile, which must occur after transfer of the MP-amphiphile complex into the gas-phase (Fig. 3a,b). This requires the optimization of a number of instrument parameters to ensure the transmission, detection and characterization of MPs by nESI-MS [84].

2.1. Detergent-based reconstitution methods for native MS

Most structural methods used to study MPs exploit the ability of detergents to retain the structural and functional integrity of MPs in solution, and much work has been conducted to find detergents compatible with native MS analyses. MS-compatible detergents must be able to solubilize the protein efficiently, maintaining a functional, stable MP in solution, but additionally must be able to be removed from the MP after transfer into the gas-phase without significantly impacting the structure/stoichiometry of the MP [84]. Initial studies were conducted using n-dodecyl-β-D-maltoside (DDM) as the detergent (Fig. 4) [94], but in many instances this detergent has proved unsuitable as the activation energies required to dissociate the detergent micelle and liberate the 'naked' MP result in MP destabilization and unfolding. Typically, the sampling cone, collision energy, collision gas pressure and source pressure must be optimized, and in many cases increased from values typically utilized to analyze soluble proteins by MS, to permit detergent removal whilst retaining native protein structure (Fig. 3a,b) [84]. For water soluble proteins, the cone voltage and collision energy are typically set to ~10–50 V, whilst for MPs the optimal voltage/energy is often up to 200 V. A published protocol describes in detail the optimization procedure to be undertaken in order to study MPs by native MS [84]. Recently there has been an upsurge in the use of other non-ionic detergents for native MS analysis of MPs, for example, Triton X-100, tetraethylene glycol monooctyl ether (C8E4), octaethylene glycol monododecyl ether (C12E8), lauryldimethylamine N-oxide (LDAO), and n-octyl-β-D-glucoside (β-OG) (Fig. 4) as it has been shown that these detergent micelles dissociate at much lower activation energies, often resulting in MP ions that are more native-like [95–97]. These detergent micelles may be dissociated more easily than those formed by DDM as their non-ionic nature means that the only stabilizing forces are hydrophobic interactions which are weakened in the gas-phase, whilst DDM and β-OG micelles are also stabilized by hydrogen-bonding [95]. It has also been proposed that the relative ease of detergent removal may relate to the stability of the protein within

the detergent micelle, i.e. harsher detergents require lower activation energies [97]. This means that different proteins solubilized in the same detergent may require different energies to be liberated into the gas phase. It has been shown that the detergent used can influence significantly the observed charge states of a MP, and it has been posited that detergents may act as charge-carriers that result in nano-ESI droplets and MP ions with reduced charge (so the MP ions observed are more native-like) [97]. It should be noted, however, that it still remains the case that screening must be performed to determine empirically the optimal detergent to study a given MP by native MS [84]. This can be a significant bottleneck in native MS workflows [84].

2.2. Detergent-free methods for native MS

It is becoming more and more apparent (from EM, X-ray crystallography and MS studies [22,96,98–100]) that there is an intimate relationship between MPs and the surrounding lipid environment, whereby the binding of key lipids is important for maintaining MP structure and function [98]. Detergents are a poor mimic of the native bilayer, may not permit the retention of MP-lipid interactions [101], and in many cases disturb the correct, functional oligomeric state of MP assemblies [90,95,96]. Consequently, a variety of detergent-free methods have been developed to solubilize MPs for biophysical analyses, namely nanodiscs [102], bicelles [103], liposomes [104], styrene maleic acid lipid particles (SMALPs) (also called lipodisq) [93] and amphipols (Fig. 4) [91,92]. Each of these methods has advantages and disadvantages, and only amphipols and the membrane 'discs' of nanodiscs and bicelles have been utilized as vehicles to deliver native MPs into the gas-phase to date [105].

2.2.1. Amphipols

Amphipols are amphipathic polymers that wrap around the hydrophobic regions of MPs, binding in a quasi-irreversible fashion [91,92]. A range of amphipols has been developed, but the most well characterized, and the only one commercially available, is A8-35, which is synthesized by grafting isopropylamine and octylamine groups onto a polyacrylic acid polymer precursor (Fig. 4) [91,92]. The solubility of MP-amphipol complexes is conferred by the remaining free acids, whilst a hydrophobic environment for MP stabilization is formed by the isopropyl and octyl moieties. MPs solubilized in amphipol have improved thermal, chemical and kinetic stability when compared with those solubilized in detergent micelles [106,107]. The synthetic route to A8-35 means it is highly heterogeneous in mass and the degree of grafting, but the polymer has been shown to form relatively homogeneous particles of ~40 kDa [91,92,107]. The name A8-35 is derived from the chemical composition of the polymer: it was initially presumed to have a mass of ~8 kDa (however further evidence has shown it to have a mass of ~4.5 kDa), and ~35% of free acid groups remain ungrafted [92,108,109]. A variety of other amphipols has been developed that differ in the average mass of the polymer, the proportion of acid groups left unmodified and also the chemistry of the polymer itself [91,92,107]. For example, a sulfonated amphipol (SAPol) has been developed in which taurine is grafted in place of the isopropyl amine (Fig. 4), and a structurally dissimilar non-ionic APol (NAPol) (Fig. 4) has been synthesized that uses glucose-based moieties to maintain solubility.

The first reported examples of successful native mass spectra of MPs solubilized in a non-detergent medium were the β-barrel outer membrane proteins (OMPs) OmpT and PagP (Fig. 3 c,d) that were successfully refolded directly into A8-35 [110]. Further studies revealed that the monomeric α-helical inner membrane proteins Mhp1 and GalP could also be liberated from amphipol A8-35 [89], and whilst the trimeric *E.coli* diacylglycerol kinase (DgkA) could be liberated from amphipol A8-35, the dominant species was the monomer (Fig. 3f) [90]. This suggested that amphipols may not be the optimal medium for promoting the retention of oligomeric MPs in native mass spectrometry. However, a direct comparison with DDM suggested that monomeric MPs (inner and outer MPs) solubilized in A8-35 are more resistant to gas-phase unfolding than their detergent-solubilized counterparts, possibly because the amphipol-MP complex dissociates more slowly than a detergent-MP assembly (so the naked protein is not subjected to the harsh MS conditions for as long) [89]. Further systematic study of a collection of amphipols revealed no obvious trends when altering the charge or size of the amphipols used [111], suggesting that, like detergents, no one amphipol could prove the 'magic bullet' solution for MS studies of MPs, and that screening of amphipols is required to optimize native MS conditions on a sample-by-sample basis.

2.2.2. Disc technologies for native MS

A variety of methods for generating membrane discs for MP reconstitution has been developed, each of which has found applications in EM, X-ray crystallography, NMR and MS [93,102,112,113]. All of these membrane discs comprise a lipid bilayer (either native or synthetic) that is solubilized by some means. Thus, membrane discs are a more favorable mimic of the native environment experienced by MPs *in vivo* (compared with detergents). Bicelles comprise a planar lipid bilayer surrounded by a rim of detergent or short-chain lipid. The ratio of long-chain lipid to detergent/short-chain lipid can be tuned to control the size of the discoidal particle [114]. By contrast, the bilayers of nanodiscs are solubilized by two copies of amphipathic membrane scaffold proteins (MSPs), that have been specifically engineered to form a disc of a defined size (several MSPs have been described to date) [102].

Seminal work described the utility of bicelles and nanodiscs as vehicles for the delivery of MPs into the gas-phase by ESI [90]. In this work, bicelles and nanodiscs (along with amphipols) were used to solubilize the trimeric MP DgkA, which could be observed intact after high energy collisional activation of the assemblies (although some dissociation to the monomer and dimer was observed) (Fig. 3 e-h). Monomeric LacY and sensory rhodopsin II (pSRII) could also be liberated from nanodiscs and bicelles. In the case of DgkA, the detergents studied (DDM and n-decyl-β-D-maltoside, DM) did not stabilize the native trimer (Fig. 3e), suggesting that either the trimer dissociated in solution in these detergents or the energies required to liberate the protein from the micelle destabilize the complex. Overall, these results thus highlight the importance of the lipid environment in stabilizing some MP assemblies for study in the gas-phase.

This earlier work focused on the use of high collision energies to liberate intact MP assemblies devoid of adducts, however a more recent study, enabled by the development of high-resolution instrumentation, has focused on using lower collision energies to study MP-lipid interactions in nanodiscs [115]. This study revealed that collision induced dissociation (CID) of MP-reconstituted nanodiscs (containing the MPs AmtB and AqpZ) occurs in a stepwise, sequential manner, with the MSP and bulk lipid being the first entities removed from the complex, leaving the annular lipid shell, for the most part, attached to the MP. Higher energies can further dissociate lipids from the MP, with ionic lipids removed last. These spectra are complex, due to the heterogeneity of the MP-lipid complexes observed (MPs with 0 to 120 bound lipids were co-populated), and require deconvolution algorithms to assign the complex series of overlapping peaks [116]. Such analyses could be leveraged to further understand how MPs interface with their surrounding lipid environment. Empty nanodiscs have also been

studied by native MS allowing, for example, the precise lipid composition to be determined [117–120].

2.3. Alternative ionization methods

Whilst most native MS studies of MPs have employed standard nano-ESI as the ionization method, other studies have explored the use of alternative methods with promising results. Two methods have emerged as most promising, laser induced liquid bead ion desorption (LILBID) [121,122] and desorption electrospray ionisation (DESI) [123,124] (Fig. 2).

In LILBID, microdroplets are generated which are then irradiated with a mid-infrared laser. This causes the droplet to explode and release ions into the gas-phase, which can then be analyzed by MS (Fig. 2). Unlike ESI, ions in LILBID spectra are more lowly charged (often singly charged, negative ions are observed) and molecules typically present over fewer charge states. The charge a protein/complex acquires upon LILBID has been shown to correlate qualitatively with its net solution charge (as determined from its amino acid sequence) [121], suggesting that the ions generated may be more native-like than those from ESI methods, which produce highly and non-natively charged ions. The current generation of LILBID instruments has relatively poor spectral resolution, making ligand identification in binding studies challenging [125]. Like ESI, the method was first attempted utilizing detergent solubilized MPs, and a range of MP assemblies has been studied [121,126]. Recently, it was demonstrated that MP complexes reconstituted in nanodiscs could be analyzed by LILBID [125]. Interestingly, complexes of the MPs studied in nanodiscs together with the scaffold protein were observed in the spectrum, which the authors posit is due to membrane destabilization during droplet explosion. By comparison, in ESI mass spectra of nanodisc reconstituted MPs, it was demonstrated that at low collision energies, the scaffold proteins and bulk lipids are ejected from the complex, with the MP retaining lipids of its surrounding annular belt [115]. Increasing the collision energy results in dissociation of these bound lipids.

In DESI, a buffer solution is electrosprayed and a sheath gas is used to direct the charged droplets to a surface at which the analyte has been previously deposited. The analyte is dissolved when the droplets impact with the surface. Droplets are subsequently ejected from the surface, desolvation occurs and the ions are drawn into the mass spectrometer (Fig. 2) [123,127]. Using DESI, MPs prepared in detergents and deposited onto glass surfaces could be desorbed by electrospraying a detergent-containing solution onto the surface [128]. Analysis by high resolution MS revealed that the charge state distributions observed depend on the detergent used in the desorption plume, suggesting that on-surface detergent exchange was occurring. Moreover, specific binding to MPs could be observed by adding ligands to the desorption spray [128]. Excitingly, this opens up the possibility for the study of membrane-embedded MPs on surfaces directly by DESI-MS.

2.4. Stoichiometry, ligand and lipid binding and thermodynamics revealed by native MS

The earliest applications of the use of MS to analyze MPs was in elucidating/confirming subunit stoichiometry of MP complexes. It is important to note, however, that the subunit stoichiometry observed by MS has been shown to be influenced by the detergent used to solubilize the MP assembly [95,96]. Native MS methods can also be used to detect lipid, drug, or other molecules binding to MPs. Intriguingly, MS has been used to show that delipidation of certain MP complexes results in their destabilization and dissociation into their constituent components [22]. This is related to the strength of the oligomerization interface, with complexes comprising interfaces with low buried surface areas and few salt bridges requiring lipid binding for stabilization [22]. Additionally, the binding of lipids that have been carried through purification protocols has been observed in native mass spectra for several MPs [20,21,89,129]. For example, in the case of the F_0F_1 ATPase from *Enterococcus hirae*, the membrane embedded ring was identified as a decamer with ten cardiolipin molecules bound. In comparison, the homologous ATPase from *Thermus thermophilus* has a 12-subunit membrane embedded ring, to which six phosphatidylethanolamine lipids were bound, consistent with a 6-fold symmetry and suggestive of a structure comprising a hexamer of dimers with each dimer containing a lipid binding site [130]. Lipid interactions could be important for understanding how the inner dimensions of the ring are controlled and how the ring interacts with other subunits of the rotary ATPase. More generally, specific lipid binding events carried through the purification pipeline despite stringent MP purification/delipidation protocols could hint at a regulatory role [20,23]. Studies of the ABC transporter P-gp [27] revealed that this protein preferentially binds to negatively charged lipids and cardiolipins (over zwitterionic lipids), and that binding to an inhibitor enhances cardiolipin binding, demonstrating that native MS could be used to probe synergistic binding events. Native MS has also been used to capture off-target drug interactions with MPs [131] and, excitingly, to monitor ligand binding to G-protein-coupled receptors (GPCRs), enabling native MS methods to be applied to GPCR drug discovery [132].

In the past, resolution has prevented MS from being applied to studies of small molecule binding to MPs and MP assemblies, especially for the study of multiple lipid binding and lipid and drug co-binding events. However, the advent of Orbitrap instrumentation with higher sensitivity and resolving power has enabled applications to be developed that would be impossible with older generations of instruments. For example, the Orbitrap has enabled the study of multiple concomitant binding events to MPs for lipids that differ in mass by as little as 12 Da [133], allowing the relative binding affinities of different lipids/ligands to be quantified, the study of lipids co-purified with a MP, and drug binding in the presence of lipids [131,133]. Indeed, the resolving power of the Orbitrap instrument may also assist with the identification of proteoforms, glycoforms and post-translational modifications. It is also possible to analyze more complex samples, for example, nanodisc-embedded membrane proteins (see above) [115], thus permitting the identification of lipids which comprise the annular belt which would be difficult on lower resolution instruments (given the peak overlap in the spectra).

Temperature controlled ESI sources have also been developed that enable the thermodynamics of individual ligand binding events to proteins to be determined [134]. This has allowed the thermodynamics of individual lipid binding events to the ammonia channel (AmtB) to be studied, revealing that the thermodynamic signature (ΔH and $-T\Delta S$ values) of binding varies for different lipid types. This is complementary to the more typical experiment to determine binding affinity, in which titrations are used to determine binding affinities. For example, in the case of trimeric porin, OmpF, a peptide was shown to bind to each subunit by threading through the pores, and the affinity measured by a MS titration was in agreement with that determined by isothermal titration calorimetry (ITC) [135]. Such an approach has also been used to study the relative affinities of lipids and drugs for MPs, and the synergistic effects of binding on affinity [27]. These ligand binding analyses can be augmented with collision induced unfolding (CIU) analyses, which exploit the power of IMS-MS to resolve conformational changes upon collisional activation, that provide a measure of the structural stabilization imparted by binding (see following sections) [98,136].

2.5. Ion mobility spectrometry-mass spectrometry

IMS separates ions by their mobility through a cell filled with a buffer gas (He or N_2) under the influence of an electric field (Fig. 2) [33,37,57,137]. The drift time of an ion under these conditions is dependent on its mass, charge and size/shape. Larger, more extended ions experience more collisions with the buffer gas and therefore take longer to traverse the drift cell. The converse is true for smaller ions. Also, more highly charged ions are accelerated to a greater extent through the drift cell, and so have a higher mobility (lower drift time). For each ion species an arrival time distribution (ATD) is recorded, which separates, for example co-populated conformations. Importantly, the drift time of an ion can be converted to its collision cross-section (CCS), either directly or by calibration (when using a travelling wave IMS device) [74,138,139]. It should be noted, however, that care should be taken when calibrating travelling wave IMS data for membrane proteins, as CCS values can be underestimated [52,144]. For globular proteins, ions of the lowest charge state (highest m/z) typically represent the most compact native-like conformation, whereas higher charge state species are more expanded due to Coulombic repulsion [74]. Experimentally determined CCS values can be compared with those of structural models or high-resolution structures to provide insights into protein architecture. Several software packages, implementing different methodologies, have been developed to determine CCS values *in silico*. The most reliable CCS calculation method is the trajectory method, which is implemented in the software MOBCAL and other packages [141,142]. Recent work has demonstrated that alternative, less computationally intensive approaches can provide data of similar quality (e.g. the corrected projection approximation implemented in IMPACT [140], or the projection superposition approximation [143]).

Comparison of the measured CCS with that of a high-resolution (or model) structure determined *in silico* can then be performed

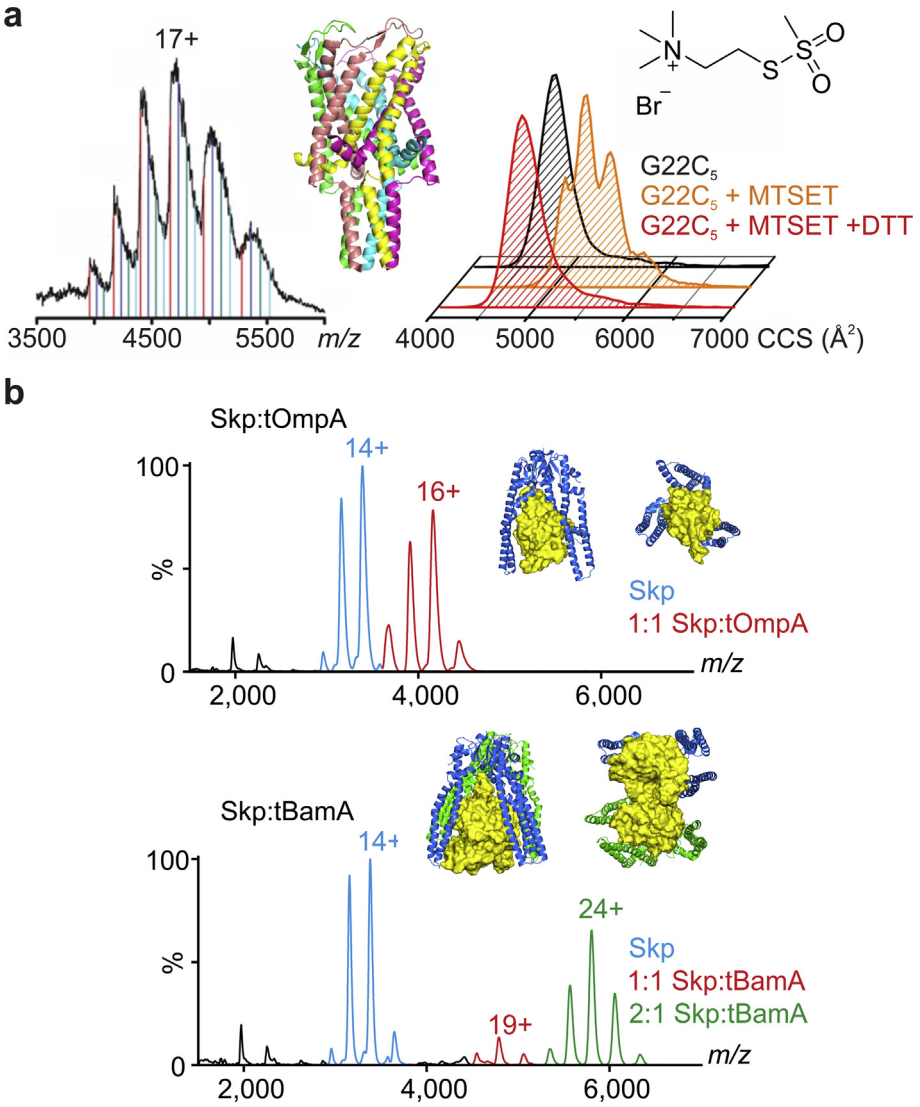

Fig. 5. Ion mobility spectrometry-mass spectrometry to study protein structure. (a) Native mass spectrum of the MscL pentamer solubilized in Triton X-100 (left panel). The colored lines indicate the expected m/z values for the pentameric complex (red), and the complex with detergent molecules attached (other colors), indicating that complete detergent removal was not achieved. The structure of the pentameric MscL channel (PDB: 2OAR) [148] (middle). The CCS distribution for the 16+ ions of pentameric MscL (right panel). Upon binding MTSET (inset) the CCS increases indicating channel opening, addition of DTT causes the CCS to revert to that of the closed state [95]. (b) Native nano-ESI mass spectra of Skp in the presence of tOmpA (upper panel) and in the presence of tBamA (lower panel), showing the complexes formed. Skp forms 2:1 assemblies with the larger tBamA, but only 1:1 assemblies with the smaller tOmpA. Inset are the model structures that agree favorably with the IMS data (tOmpA and tBamA are shown in yellow, and Skp is shown in green and blue) [81]. Part a is adapted from Ref [95], with permission. Part b is adapted from Ref [81], with permission. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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[140–143], to provide insights into protein architecture. Combining IMS with low resolution modelling [145], conventional, or steered molecular dynamics (MD) simulations [81,129] is also gaining traction as a method for obtaining structural insights into proteins/assemblies. IMS also enables structural perturbations, for example, as a result of ligand binding, to be studied (see below).

As IMS-MS reports on the ensemble of structures adopted, it is a promising tool to study the dynamics and conformational heterogeneity of both water soluble and MPs and their assemblies. For example, study of the V-type ATPase from *T. thermophiles* revealed that the membrane-embedded V_0 subcomplex explored more conformations than the water soluble V_1 assembly and that ATP binding dampened the motions of V_0 [146]. IMS can also be used to investigate the conformational changes/transitions of MPs. For example, the homopentameric mechanosensitive channel of large conductance (MscL) from *E. coli* (Fig. 5a) opens in response to increases in membrane tension as a result of hypoosmotic shock. Despite the functional importance of such channels, it was impossible to monitor the global structural changes that occurred during channel gating. Nano-ESI-IMS-MS, combined with molecular dynamics simulations, provided the ideal solution to study this phenomenon. MscL was solubilized in Triton X-100 and analyzed by native MS [95]. Cys-containing monomers of MscL were then incorporated step-wise into the pentamer. The introduced Cys residue reacts with the molecule [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET) (Fig. 5a, inset), allowing the incorporation of up to 5 positively charged MTSET moieties in the MscL pore, which results in pore opening due to Coulombic repulsion [147]. Incorporation of MTSET in the pore led to stepwise opening of the pore, which could be detected by IMS, with up to four co-populated states observed (Fig. 5a). The assemblies of unfolded outer membrane proteins (OMPs) with the periplasmic chaperone Skp have also been studied by nano-ESI-IMS-MS, to understand how the chaperone accommodates its many client proteins of a range of sizes in its hydrophobic cavity [81]. Once chaperone bound, these complexes are water soluble, so no detergents are required for their characterization by IMS-MS. The data demonstrated that larger OMPs bind additional Skp molecules to sequester them successfully and prevent their aggregation. IMS data were compared with models generated *in silico* by MD, to understand the architecture of these assemblies, revealing that the chaperone functions by both expanding its central cavity and by multivalent chaperone binding to accommodate larger substrates (Fig. 5b).

2.6. Collision induced dissociation and collision induced unfolding

Collisional activation of MP complex:detergent assemblies often leads to dissociation (Fig. 2). However, this can result in useful information concerning the arrangement of the assembly. For example, ESI-MS of the *E. coli* β-barrel assembly machinery (BAM), a heteropentameric complex that comprises subunits A, B, C, D and E, revealed dissociation into subcomplexes, AB and ACDE [149]. This is consistent with the known architecture of BAM – it comprises two subcomplexes, AB and CDE, with A being the central subunit [150,151].

Pairing collisional activation by CID (Fig. 2) with IMS has led to the development of collision induced unfolding (CIU) [152,153], which has given an extra dimension to the information that can be obtained from ligand binding experiments. In CIU, a native-like ion is activated by CID prior to ion mobility separation, resulting in unfolding of the ion which can be monitored by its CCS derived from IMS. CIU is repeated at increasing collision voltages and the CCS of the ion plotted under each condition (Fig. 6a). CIU has found several applications. For example, it can be used to infer the domain structure of proteins [154], or for studying the effect of ligand binding on protein stability [136]. Software packages have been developed for the quantitative comparison of CIU trajectories for this purpose [136,155], and to assist in data collection and

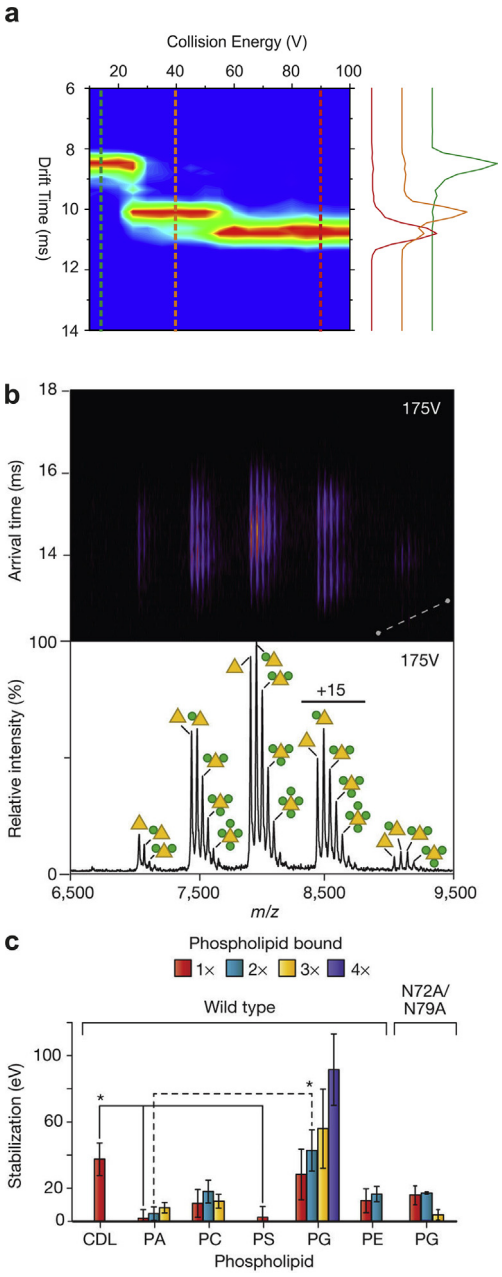

Fig. 6. Collision Induced Unfolding. (a) Example of a CIU experiment. An ion is selected in the quadrupole and IMS data are acquired after applying increasing energies. This results in gas-phase unfolding. At each collision energy an ATD is recorded, with the data typically plotted as a heatmap. On the right hand side are three ATDs at three different collision energies (indicated by the dashed lines of corresponding color in the heatmap), demonstrating that in this case an intermediate species is populated upon unfolding. (b) IMS-MS analysis of AmtB in the presence of phosphatidylglycerol (PG). Up to four lipid binding events are observed in the spectrum. Peaks labelled with a yellow triangle correspond to AmtB, each green circle represents a bound lipid. (c) Quantified stabilization of AmtB bound to different lipids (CDL: cardiolipin, PA: phosphatidic acid, PC: phosphatidylcholine, PS: phosphatidylserine, PG: phosphatidylglycerol, PE: phosphatidylethanolamine). The most significant stabilizing effects are observed for CDL and PG. The N72A/N79A mutant disrupts the PG binding site, which also removes the stabilizing effect of PG [98]. Parts (b) and (c) are reproduced from Ref. [98] with permission. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

analysis [156]. Recently, Laganowsky *et al.* used a CIU approach to quantify the stabilization imparted on MPs by bound lipids, and found that lipids important for function enhance the resistance of MPs to CIU (e.g. phosphatidylinositol phosphate imparts the highest stabilisation on MscL, consistent with its proposed role in mechanosensation, and cardiolipin which modulates the function of AqpZ also performs a stabilizing role). Additionally, crystallization of AmtB in the presence of the lipid that imparted the highest CIU-measured stabilization in the MS screen (Fig. 6b,c) yielded a new conformation of AmtB, suggesting a role for lipid binding in modulating both the structure and function of MPs [98].

Complementary to CID, surface induced dissociation (SID) has also been used to dissociate protein assemblies (Fig. 2) [157], and has also shown promise as a tool to study MPs [158]. SID involves directing ions to a surface, where each ion experiences a single, fast, energetic collision, unlike CID where multiple energetic collisions with a buffer gas occur [157]. Typically, subcomplexes formed by SID are more native-like (when compared with those formed by CID) and IMS data for the trimeric MP complexes AmtB and AqpZ suggest that the monomer and dimer formed upon SID of the intact trimers populate native-like CCSs [158]. IR laser activation has also shown promise as an alternative method to liberate MPs from detergent micelles [159].

2.7. Discussion

Native MS-based structural methods have been instrumental in the characterization of numerous biochemical processes. Whilst initial experiments focused on the use of native MS as a tool to discern protein complex stoichiometry [60], the advent of IMS-MS has significantly enhanced the structural information accessible [37,74,138,145,153]. Despite the relatively low structural resolution afforded from IMS measurements, knowledge of a protein's collision cross section can provide critical information that can be used to refine structural models and to map conformational changes [16,33,79,80,145,160–162]. This is especially useful in the study of protein dynamics and structural interconversions, as co-populated species with different m/z and/or CCS can be interrogated individually without ensemble averaging. It is important to note, however, that the range of complexes which can be studied by native MS methods is restricted to those that survive intact in the gas phase which, in turn, is determined by the nature of the non-covalent interactions involved in complex formation. For example, since hydrophobic interactions are weakened in the gas phase, macromolecular complexes that rely on hydrophobic contacts for assembly may not survive intact during the transfer to the gas phase [82,83]. In addition, the loss of a solvation shell may result in structural reorganization, as evidenced for water soluble proteins, including intrinsically disordered proteins or complexes with 'open' topologies as found in extended polyproteins, such as IgGs or ring-like subunit assemblies [76,81,163]. In these cases it is advisable to perform molecular dynamics simulations to model the behavior of molecules in the gas phase.

In the case of MPs, CCS values obtained by calibration of travelling wave IMS data must be analyzed with caution. Whilst changes in structure monitored by travelling wave IMS may be interpretable, the energies required to liberate the MP from the detergent micelle/nanodisc, and the lower net charge acquired by MPs upon ESI (relative to water soluble proteins) mean that calibration of MP drift time data with CCSs of water soluble proteins can be erroneous [52,144]. In such cases care must be taken to choose suitable soluble protein calibrants, or it may be necessary to measure CCSs directly from drift tube (conventional) IMS devices, or to perform a calibration using CCSs of MPs measured previously on a drift tube (conventional) IMS device [144]. However, a lack of commercially available MPs and the absence of a suitable CCS database that can be utilized for calibration makes such a task challenging, at least at the current time. In cases where native MS may not be suitable, or may provide insufficient resolution, structural studies using solution-based labelling methods such as FPOP-MS, HDX-MS, or other approaches, followed by MS detection (and quantification) can be used to provide powerful information about structures, protein complexes and conformational changes in solution, including for MPs in different amphiphiles, as discussed in the sections below.

3. Labelling methods to study membrane protein structure

Covalent labels have been used in structural MS studies for many years, with many of these methods having been applied to the study of MPs, as detailed below. All of these approaches share a common generic workflow; (1) a labelling experiment is performed in solution, (2) the proteins are digested with a protease, and (3) the sites of modification are identified by MS. These sites of modification can then be used to infer binding interfaces, solvent accessible regions, and/or conformational dynamics of a protein/complex.

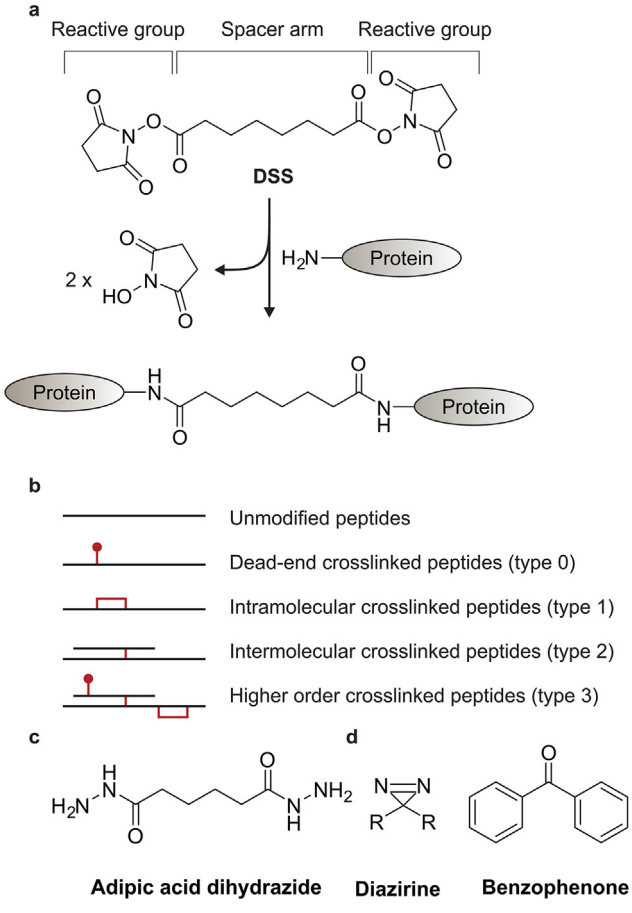

Fig. 7. Chemical crosslinking. (a) Structure of the common crosslinking reagent DSS. Its water soluble counterpart BS3 comprises sulfonated NHS-ester reactive groups. The reaction of the NHS-esters with amines on a protein is shown. (b) Common crosslinked peptides that can be identified after proteolysis. Typically it is the intermolecular crosslinked peptides (type 2) that provide the most informative structural information, especially when the two peptides involved originate from different proteins of an assembly. (c) A crosslinker containing hydrazide reactive groups reacts specifically with activated carboxylic acids. (d) Photoactivatable crosslinking groups that can be incorporated into crosslinker designs.

3.1. Chemical crosslinking

Chemical crosslinking (XL) has been employed for decades to study protein interactions, but the advent of high resolution MS and robust proteomics workflows has enabled this methodology to be reborn as a technique that provides residue-level structural information [26,38–41]. After reaction with a suitable XL reagent, a protein/complex is proteolysed and the crosslinked peptides identified by MS. There are many commercially available XL reagents, but they all involve the same basic structure of two reactive groups separated by a spacer arm of defined length (Fig. 7a) [26,38–41]. Additional functionalities may be incorporated in the spacer arm, such as functional groups that are cleavable (either chemically or by CID) [164] or groups that can be used for crosslink enrichment (e.g. biotin or alkynes) [165,166]. Crosslinked peptides can also be enriched by size exclusion or strong cation exchange chromatography [167,168]. Enrichment is often necessary to enable efficient detection of crosslinked peptides amongst their more highly abundant non-crosslinked counterparts. Intermolecular crosslinks, which are the most structurally informative, comprise two peptides covalently joined by the XL reagent (Fig. 7b). MS/MS methods can be used to identify the peptides involved, and the residues that are covalently joined. The spacer arm imposes a distance restraint which can then be used for structural modeling, model validation, or comparison with high-resolution structural data [26,38–41]. Analytical challenges have meant that significant effort has been spent on refining XL-MS methodologies, especially to enrich for XL peptides (they are of inherently low abundance), and to develop automated (or semi-automated) methods of spectral assignment and XL identification [168–172].

A variety of reactive group chemistries can be incorporated into crosslinker designs, but by far the most commonly encountered ones are the N-hydroxysuccimide (NHS)-esters, which react primarily with Lys side-chains, but also Ser, Thr and Tyr (Fig. 7a) [173,174]. Bis(sulfosuccinimidyl)suberate (BS3), or its membrane permeable counterpart disuccinimidyl suberate (DSS) (Fig. 7a), are probably the most frequently used XL reagents. Both comprise two NHS-ester reactive groups (in BS3 the NHS-esters are also derivatized with sulfonyl groups to confer water solubility) separated by a 11.4 Å spacer arm. The utility of hydrazides as reactive groups that target carboxylic acids (i.e. side chains of Asp and Glu) has also been demonstrated; however, their low reactivity means that they must be activated with a coupling reagent [175]. The reagents adipic acid dihydrazide (11.4 Å spacer arm) (Fig. 7c) and pimelic acid dihydrazide (12.3 Å spacer arm), combined with the coupling reagent 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMTMM), have been used recently to study the TRiC/CCT chaperonin and 26S proteasome complexes [175]. Photoreactive groups (e.g. diazirine, benzophenone) (Fig. 7 d,e) can also be incorporated into crosslinker designs, which have an (essentially) non-specific side-chain reactivity (but do have some preferences) [176]. Cell permeable reagents can also be used to define the interfaces of protein assemblies in a cellular context [165,166]. Additionally, photoactivatable groups (benzophenones or diazirines) (Fig. 7 d,e) can be incorporated into proteins *in vivo* by the cellular machinery (by using an aminoacyl-tRNA synthetase/tRNA pair that incorporates unnatural amino acids at an amber stop codon), and used for interaction/interactome analysis [177,178].

Application of XL-MS to study the architecture of water soluble protein assemblies is now well-established, with many impressive studies of proteasomes, ribosomes, polymerases, the mediator complex, and transcription factors, amongst others [179]. Reports of XL-MS of MPs are few and, generally speaking, limited to large assemblies with extra-membrane regions of significant size. XL has been applied in integrative studies to investigate signaling complexes of GPCRs. In one example, the β_2 adrenergic receptor and β-arrestin 1 complex was characterized by combining XL-MS, HDX-MS and negative stain EM [180]. The structure of the complex between the β_2 adrenergic receptor and a GPCR kinase was also studied by XL-MS, again using an integrated approach, combined with HDX-MS, negative stain EM, mutagenesis, molecular dynamics and docking, allowing a dynamic model of the complex to be derived [181]. XL-MS has also been used to study the architecture of complexes involved in photosynthesis [182–184], demonstrating the organization of the individual components of the photosynthetic apparatus when they form a megacomplex in a cyanobacterium [182].

Comparative XL has also been developed, whereby isotopically encoded crosslinkers are used to crosslink a protein in different states, for example in the absence or presence of different additives/components/post translational modifications. The relative intensities of the crosslinked peptides can be then quantified and used to determine structural alterations. For example, the F_0F_1 ATPase has been probed by comparative XL, revealing that phosphorylation is involved in regulating nucleotide binding [40,41].

3.2. Hydrogen/deuterium exchange

Hydrogen/deuterium exchange (HDX)-MS can be used to study protein conformation, dynamics, ligand binding sites and allosteric effects [42,43]. Typically, in HDX-MS experiments, the protein solution is diluted in deuterated buffer, resulting in the labile hydrogen atoms being exchanged for deuterium (only main chain amide hydrogens are observable as side chain amines and carboxylic acids exchange too rapidly to measure). Exchange is allowed to take place for defined, measured, time periods and the reaction then quenched by reducing the pH to ~2.5 (where the intrinsic rate of HDX is minimized) [43]. Samples are digested with a protease that is active at low pH (typically pepsin, but other enzymes, e.g. nepenthesin [185,186] can be used), and the resultant peptides are then separated by LC (at low temperature and pH, again to prevent back-exchange), analyzed by MS, and the extent of deuterium incorporation determined [43]. Interest in HDX-MS has been enhanced recently by the commercialization of integrated HDX-MS platforms, which can be interfaced with liquid handling robotics to automate the labelling, quenching and injection steps [187,188]. As with all structural MS techniques, working with MPs has its own unique challenges. For example, the hydrophobic transmembrane segments of MPs can be inherently difficult to digest and observe by LC-MS, although this is often protein and digestion condition dependent [189], and does not always preclude structural analyses. Recent studies on detergent and lipid solubilized proteins have demonstrated that both are compatible with HDX-MS [181,190–195].

As with most analytical methods used to study MPs, MPs solubilized in detergent have been the primary targets for HDX-MS analyses. A variety of systems has been studied revealing many structural insights. For example, BmrA, an ABC transporter that adopts open and closed conformations in its transport cycle, was solubilized in DDM and HDX-MS was used to study the protein's conformational transitions (Fig. 8) [190]. The conformational changes in green cone opsin upon light activation have also been studied [196], as well as the alternating access mechanism of the Na^+/H^+ antiporter NhaA [191].

A particular interest in the field of HDX-MS has been the study of protein complexes involved in signaling cascades. In this area of work, the interaction of the β_2 adrenergic receptor with the trimeric G protein Gs was investigated by HDX-MS, revealing that the Gα subunit is the principle binding site (and not Gβ or Gγ) [197]. The binding of β-arrestin to the β_2 adrenergic receptor has also been studied by HDX, combined with chemical crosslinking

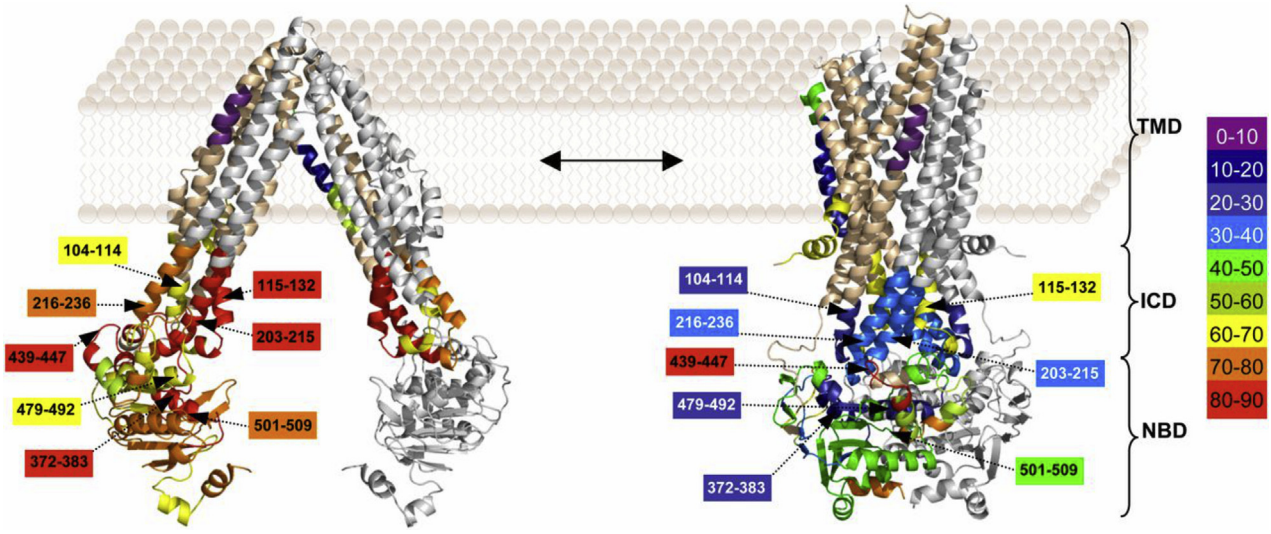

Fig. 8. HDX-MS of BmrA. The BmrA structure was switched from the open (left) to the closed (right) conformation by the mutation E504 [190]. The structures shown are 3D models of dimeric BmrA, one subunit is shown in white the other in cream. Regions are colored according to the percentage of deuterium exchange after 1 hr (see key on right). Upon closing, regions of BmrA are significantly protected from exchange. TMD = transmembrane domain, ICD = intracellular domain, NBD = nucleotide binding domain. Figure is reproduced from Ref. [190].

and low resolution EM, allowing a model of the complex to be constructed [180].

If HDX of MPs is performed in the presence of lipids (e.g. nanodiscs, bicelles or liposomes), the lipids are often removed prior to MS analyses to prevent them interfering with ionization and LC separation [43,198], though more recent work suggests that this is not always necessary [181,192–195,199]. HDX-MS in nanodiscs has been used to study propeptide binding to γ glutamyl carboxylase [192], and to study the conformational dynamics underlying the alternating access mechanism of LeuT [193]. Bicelles have been used to study the complex of β_2-adrenergic receptor with a GPCR kinase, combined with data from other methods, including chemical crosslinking [181]. Interestingly, GPCRs solubilized in bicelles yielded better sequence coverage in HDX-MS workflows than those solubilized in detergent [199]. The conformational dynamics of the F_0F_1 ATP synthase during catalysis have also been studied by HDX-MS using protein solubilized in inside-out membrane vesicles, revealing that the rotor shaft is destabilized when pumping protons against a transmembrane gradient [194]. More recently, the SMALP platform was implemented to study the rhomboid protease GlpG in different lipid conditions, to identify regions of the protein sensitive to the lipid environment [195].

The rate of HDX depends both on the chemical step of breaking and making NH and ND bonds, respectively, as well as the probability than an NH will be solvent exposed. Whilst the intrinsic rate of HDX will depend on the dielectric constant of the environment, for MPs the dominant determinant of the rate of HDX will be solvent exposure, meaning that HDX-MS is well-suited to study MP folding, and also motions even in complex lipid environments [42,43,200]. One of the areas in which HDX has provided significant insights has been the field of protein folding [200–203]. Application of HDX-MS to the study of MP folding has been limited to date. However, recent work has used HDX-MS complemented with HDX-NMR experiments to study the folding of *E. coli* OmpX into detergent [204]. However, deuterium incorporation measured by MS was at the intact protein level, while residue-specific information was obtained by NMR. Nevertheless, this study demonstrates the feasibility of using HDX-MS as a method of probing MP folding.

3.3. Hydroxyl radical footprinting

Generation of hydroxyl radicals (·OH) for hydroxyl radical footprinting (HRFP) of proteins can be achieved by a number of means, including electrochemical methods, corona discharge methods and Fenton chemistry [205,206]. However, present day experiments are typically performed using synchrotron radiolysis or laser irradiation, as they permit the greatest temporal control of the radical dose. Synchrotron radiolysis of water can be utilized to generate ·OH for reaction with the solvated protein in standard buffered solutions without additives [207,208]. The length of the synchrotron pulse can be used to control the time that the sample is exposed to ·OH, so that dose–response curves can be generated over μs-ms ranges [47]. Alternatively, in fast photochemical oxidation of proteins (FPOP), a protein solution is prepared in the presence of low concentrations of H_2O_2. Irradiation at 248 nm with a pulsed laser leads to the generation of ·OH that can react with solvent accessible side-chains [209]. The presence of scavengers (glutamine or histidine) tunes the lifetime of the ·OH such that a majority of the labelling reactions occur on timescales faster than protein folding/unfolding (~1 μs) [209–211], although recent evidence suggests that radicals may be longer-lived [212]. Alternatively, the scavenger dose can be tuned to extend/reduce the labelling pulse, allowing dose-response experiments to be performed [213].

Characteristic modifications of +16 Da are typically detected following FPOP or radiolysis experiments (other, less common modifications are possible [214], e.g. +14 Da modifications as a result of aldehyde/ketone formation). These modifications can be quantified by LC-MS at either the peptide or residue level. Whilst solvent accessibility is the primary driver of modification, different side-chains also exhibit different reactivities, with the most reactive being sulphur-containing (Cys and Met) and aromatic (Trp, Tyr, Phe and His) residues (the reaction rates of the side-chains with hydroxyl radicals span four orders of magnitude) [47,214]. Given that the modifications in HRFP experiments are covalent, rigorous sample workups (e.g. protein precipitation, detergent removal and addition of denaturants), and varied protease digestion protocols (alteration of protein:protease ratios and digestion

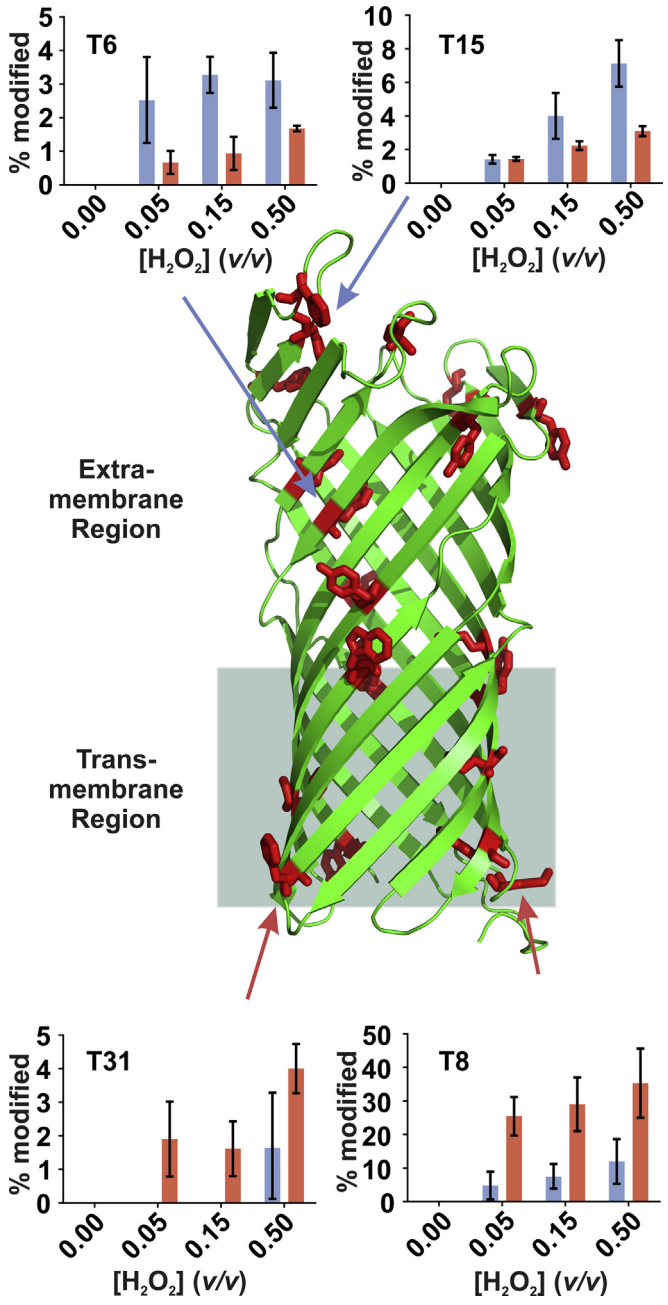

Fig. 9. FPOP-MS analysis of OmpT solubilized in either DDM detergent micelles or amphipol A8-35. Graphs show the % modified of four peptides in DDM (blue) or amphipol (orange). Arrows show the residues modified in each case. Residues in the extra-membrane region are more readily labelled in DDM, whilst residues at the lower boundary of the trasmembrane region are more readily labeled in amphipol. Figure is reproduced from Ref. [218]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

times) can be utilized to maximize sequence coverage and thus the information obtainable (unlike HDX where the labile nature of the label makes this more challenging). This is particularly pertinent in the study of MPs, which can be difficult to digest for LC-MS analyses [189].

Typically, FPOP experiments involve flowing the sample of interest through a capillary which is irradiated with a laser to induce ·OH formation. Coupling this fluidics setup with rapid online mixing has allowed the folding mechanisms of proteins to be studied (by initiating folding on-line and irradiating at defined time-points thereafter). In the case of MPs, this has allowed the kinetic folding mechanism of bacteriorhodopsin to be studied [215], and topology mapping/validation of MPs [216,217]. A comparative study also investigated the difference in the solvent accessibility of the outer membrane protein OmpT in amphipol A8-35 compared with the detergent DDM [218]. This study revealed additional intermolecular contacts with the amphipol (Fig. 9), consistent with the known abilities of amphipols to stabilize MPs compared with detergents [219,220].

Ligand binding [221], conformational dynamics [222] and conformational changes [223,224] of MPs can also be studied using synchrotron radiolysis HRFP. This method also has the advantage of being able to study ordered water molecules in protein structures [225,226]. Such water molecules are particularly important for MPs, as tightly bound water molecules in the membrane-spanning regions of the protein will be activated upon irradiation and thus label the protein in these regions (which would likely not be accessible in FPOP experiments, as these require H_2O_2 to diffuse to the labelling site).

More recently, in cell FPOP has emerged as a possibility [227,228], with a modification of this approach being used to study the conformation of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) [229]. Excitingly, this opens up new possibilities to obtain structural information on MPs in their native environment by FPOP-MS.

3.4. Other chemical labelling methods

Other chemical labels with relatively promiscuous reactivities have been developed to study the architecture of MPs. Recent work has revealed that a small hydrophobic, trifluoromethyl aryldiazirine probe could be used to footprint the membrane spanning regions of trimeric *E. coli* OmpF, with the trimerisation interface remaining relatively unlabeled when compared with the remainder of OMP [230,231]. A complementary approach to label proteins with trifluoromethyl radicals ($\cdot CF_3$) has also been described recently [232]. Utilizing an FPOP platform, addition of $NaCF_3SO_2$ in addition to H_2O_2 prior to laser irradiation triggers a series of radical reactions that generate $\cdot CF_3$, which reacts with amino acid side-chains. This approach is complementary to FPOP, as $\cdot CF_3$ labels side-chains that are relatively unreactive with ·OH. Application to MPs again resulted in the observed modifications being localized to the extra-membrane regions [232].

Other probes of solvent accessibility with increased residue specificity, when compared with radical based labelling methods, are also well-established [44]. Cys specific labelling has long been employed in the study of MP structure [233]. In particular, detection and quantification of labelling of solvent-exposed Cys residues by the Cys-specific reagent N-ethylmaleimide (NEM) using MS is a widely used strategy to inform on the structural state of a MP [234–238]. In one recent example, the conformational transitions of the secondary active transporter Mhp1 was studied using NEM-labelling detected by MS [239]. Mhp1 utilizes an alternating access mechanism in its transport cycle, whereby the protein adopts both inward- and outward- facing conformations, and has a Cys residue in a defined position that is only exposed in the inward-facing form [239]. Substrates were shown to switch the conformational equilibrium of the protein, and point mutations were studied in the ligand binding site, revealing that whilst some abrogate binding others cause structural destabilization [239].

The solvent accessibility of Lys residues can be interrogated by using succinic anhydride, which has been used to study the ligand induced conformations of the β_2 adrenergic receptor [237], or sulfo-succinimidyl acetate, which has been used to study the interaction of rhodopsin with the G protein transducin [240]. NHS-esters have also been used to study the solvent accessibility of Lys residues (e.g. so-called 'dead-end' (Fig. 7b) crosslinks formed during XL-MS experiments) [40,41]. The reagent butane-2,3-dione can be used

to probe Arg accessibility, and has been used to study the conformational transitions in LacY, in combination with diisopropyl carbodiimide labeling of carboxyl groups [241]. Diethylpyrocarbonate (DEPC) is also a commonly encountered modifying reagent, and whilst it primarily targets His it also has the potential to react with Lys, Arg, Tyr, Thr and Cys residues [242,243]. DEPC, along with all other covalent labels, is able to provide restraints for integrative modeling workflows, as exemplified in recent work on the F_0F_1 ATPase from spinach chloroplasts (in combination with XL-MS data) [243]. In this work, the data were used as restraints in molecular dynamics simulations to determine regions of the protein with intrinsic flexibility and to study the conformational dynamics of the peripheral stalk.

3.5. Discussion

When using labelling strategies to characterize MP structure and conformational changes utilizing structural MS studies, the labelling reagent(s) used must be carefully chosen. For example, water soluble reagents can readily probe, and hence identify, regions of a protein complex that are exposed to solvent, and more lipophilic reagents can be used to modify residues buried within the membrane [231]. Quantitative comparison of residue accessibility in such cases can be misleading, however, if the reactivity of the probe in the different environments is not already known and hence calibrated. More generally, the timescales required for many of the chemical reactions to reach a suitable yield are often incompatible with motions on a biologically-relevant timescale. In the case of XL and, indeed, most covalent labelling methods, samples are often incubated with the reagents for up to 1 h [26,38,41,174]. Given that many global motions of proteins occur on the μs – ms timescale this significantly limits the conformational dynamics that can be investigated by such techniques, and the information obtained often comprises distance restraints that may be consistent with multiple conformations. Presently, this remains a challenge for interpretation using computational methods, and it can be difficult, or impossible, to unpick conformational families consistent with the data, the relative population of those conformational families and the rates of their interconversion. Here again, however, data from other methods, such as single molecule Förster Resonance Energy Transfer (FRET) [244,245], small angle X-ray scattering (SAXS) [246,247], and double electron–electron resonance (DEER) electron paramagnetic resonance (EPR) [248], can provide independent information that complements MS data so that the two approaches can be used in synergy (so-called 'integrated structural biology' [249]) to create molecular models. This issue is not unique to MS footprinting methods, with structural techniques such as NMR and cryo-EM also potentially being resolution limited by dynamic averaging [13–15,250,251]. Powerful NMR methods, such as relaxation dispersion, however, can provide structural, kinetic and thermodynamic information about rare species in dynamic equilibrium, in favorable cases even for large (MDa) protein complexes [250,251]. In the same vein, particle classification methods in cryo-EM can be used to tease out different protein structures within a dynamic ensemble provided that each conformer is significantly represented so that medium to high resolution data can be obtained [13–15].

In the case of FPOP labelling, the lifetime of the hydroxyl radicals is in the order of μs [209–211]. Thus, online mixing can allow conformational dynamics in response to ligand binding or another stimulus to be probed on stopped-flow (ms) timescales [252,253]. Such methods have enabled MP folding to be monitored [215]. These fast reacting functional groups have recently been extended further to include diazirines which are also activated by laser irradiation [230,231]. This approach has the advantage that the diazirine probes used are membrane permeable, as shown by a recent study in which the majority of modifications were shown to be located in the transmembrane region of the MP, OmpF [231]. Hence, this diazirine is unique amongst the reagents shown to be suitable for footprinting of MPs.

In the case of HDX, the timescales accessible are limited by back exchange, in which the deuterium labels introduced are exchanged with protons from the bulk solvent during sample workup (an issue that is not encountered using the covalent labelling methods discussed above). Moreover, the time required for mixing with deuterated solvents means that labelling times lower than a second are unachievable using manual (and most automated) methods. Stopped-flow and microchip devices can be used to shorten the labelling pulse, significantly reducing the timescales accessible to HDX-MS, however, such devices remain non-routine [203,254].

Finally, it should be borne in mind that methods which require covalent modification of a protein of interest may trap artificial, non-native, protein conformations, for example if modification of an amino acid induces a conformational change and formation of a conformer not formed by the unmodified sequence. While there is evidence that XL of proteins does not globally impact their structure, some local changes have been reported [255]. It is important that this be kept in mind when analyzing any data, but given the resolution of the distance constraints often obtained from XL-MS (Cα-Cα distances typically in the order of 30 Å, depending on the XL reagent used) [256], it is not clear that this is a significant concern. Moreover, the population of modified (relative to unmodified) residues is often low, especially in FPOP, meaning that any structural perturbations are likely to be difficult to detect. In any case, for FPOP given that the labelling pulse (μs) is over before any major structural changes can occur this potential concern is probably immaterial [209–211]. However, if conformational changes upon modification are suspected (e.g. in the case of dynamic systems), these could be assessed in control experiments, for example by IMS-MS or HDX analyses of the covalently modified proteins, or by NMR, as previously conducted for the water soluble proteins carbonic anhydrase and alcohol dehydrogenase 1 [255]. What is clear is that covalent labeling of proteins monitored quantitatively by MS is now an essential part of a structural biologists toolkit, able to provide important information in residue-specific detail on the nature of protein structures, their dynamics, binding and folding/unfolding transitions. Further development of reagents with enhanced chemical properties (e.g. faster reaction rates) will expand the repertoire of conformational changes of water soluble proteins and MPs that can be studied by footprinting MS methods and the range of biological questions which can be addressed.

4. Conclusions and outlook

Significant advances have been made in the study of MPs by structural MS methods. In particular, work to determine the optimal amphiphiles to be used, and the suitability of membrane discs for MS analyses has been essential. Despite these successes, integrating the range of data obtained by MS into a structural model can be complex, especially for dynamic systems, wherein model building guided by computer simulations is an essential component of the MS toolkit. Nevertheless, advancements in these areas have been many-fold over recent years, paralleled by impressive advancements in instrument design. Moreover, the integration of MS data with those from other structural methods, including cryo-EM and NMR, presents an opportunity to study increasingly complex systems using an integrative structural biology approach.

The future potential of in-cell MS methods is also an exciting and growing area of MS capability. The use of XL-MS to investigate the interaction networks of proteins, and the structure of assemblies in cells, along with FPOP to study conformation and confor-

mational changes, remain areas wherein significant advances are likely to be made in the months and years ahead. Such analyses will enable MPs to be studied directly in cellular membranes, rather than *in vitro* and in reconstituted systems. Given that the native membrane surrounding a MP *in vivo* is highly complex, and much more so than in reconstituted systems, this represents a significant advantage for MS over other approaches, especially given that the composition, lateral structure and thickness of each membrane (cellular and organellar) differs. To this end, further exploitation of MPs solubilized in SMALPs (that have been directly excised from the membrane along with endogenous lipids) provides an exciting opportunity to study MPs by MS in a near-native lipid environment.

Structural biology is increasingly becoming integrative [249], in that information from a range of complementary techniques is combined to not only determine the structure of a protein/assembly, but also to interrogate conformational dynamics and study other motions important for function. Rapid developments in instrumentation and methodologies to date, together with promises for future advances, guarantee that MS will remain an integral component of the structural biology toolkit, that is sure to reveal the secrets of how MPs perform the vast array of functions and interactions essential for life.

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