Probing membrane protein-lipid interactions

Mark T. Agasid,[†] Carol V. Robinson[*,†]

[†] Department of Chemistry, University of Oxford, 12 Mansfield Road, Oxford OX1 3TA, U.K.
Corresponding author: Robinson, Carol V (carol.robinson@chem.ox.ac.uk)

Keywords

- mass spectrometry
- membrane proteins
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Highlights

- Mass spectrometry connects lipid-binding to changes in protein conformation and subunit stoichiometry
- State-of-the-art instrumentation identifies bound lipids
- Mass spectrometry defines lipid densities observed in X-ray and Cryo-EM structures

Abstract (100-120)

Structure determination of membrane proteins has highlighted the many roles played by lipids in influencing overall protein architecture. It is now widely accepted that lipids surrounding membrane proteins play crucial roles by modulating their conformational, structural, and functional properties. Capturing often transient lipid interactions and defining their chemical identity, however, remains challenging. Recent advances in mass spectrometry have resolved questions concerning lipid interactions by providing the molecular composition of intact complexes in association with lipids. Together with other biophysical tools, a picture is emerging of the dynamic nature of lipid-mediated interactions and their effects on conformation, interactions and signalling.

Introduction

Cellular membranes and their constituent integral and peripheral proteins perform crucial roles in biological systems. Specifically, membrane proteins initiate a multitude of physiological processes through intricate interaction networks involving other membrane-bound proteins, intracellular proteins, and lipids, thereby enabling cells to interact and respond to their extracellular environment. Unsurprisingly, membrane proteins serve as important centres by which we can influence cellular function to combat pathophysiological states.[1] Hence, while membrane proteins account for ~23% of the genome, they are disproportionately represented in drug discovery, constituting >60% of drug targets.[2]

Breakthroughs in membrane protein structure determination using X-ray crystallography and single-particle cryo-electron microscopy (cryo-EM) have brought atomistic detail to the interactions

governing ligand interactions and associated protein conformational changes. Notably, information about the surrounding lipid environment, which maintains the membrane protein-fold and function, is more difficult to ascertain. Sufficient resolution and characteristic lipid shapes aid in discerning lipid-like densities, but most critical lipids are difficult to distinguish from co-purifying lipids or detergent molecules.[3] While generally serving as a hydrophobic 'solvent' to support the fold of membrane proteins, lipids may also interact specifically to alter conformational status or interactions. Specific binding is usually assigned to lipids in tailored pockets, which are distinguished by terms such as 'structural' or 'non-annular' lipids. By contrast, non-specifically binding lipids augment the nanoscale bilayer properties (e.g. hydrophobic thickness, fluidity, and curvature) physically surrounding membrane proteins, and are known as annular lipids. Though predominately phospholipids, glycolipids, and sterols compose biological membranes, a host of additional lipid species exist,[4] and it is often the less abundant lipids that play significant roles in membrane protein lifetimes.[5] These multitudinous annular interactions in turn affect biologically important facets such as membrane protein trafficking, localization, as well as structural and functional properties.[6,7] Understanding how specific lipids affect the structural and functional features of individual membrane protein systems is the challenge that is the focus of this review.

Validating the mass spectrometry platform

Mass spectrometry (MS) has become a significant structural biology tool to interrogate protein-lipid interactions. By transferring intact protein complexes from solution to the gas phase it is possible to monitor directly binding of small-molecules and lipids to protein complexes.[8,9••] Additional information, provided using lipidomics analysis, details the different types of lipids co-purified with a complex that may be fit into membrane protein structures with unknown lipid-like densities.[10,11] When combined with hydrogen deuterium exchange (HDX-MS), the conformational plasticity of protein complexes in the presence of different membrane mimetics and lipid conditions, can also be evaluated.[12•] Moreover, native MS is finding increasing utility to evaluate preparations of protein complexes prior to cryo-EM analysis.[13,14] An overview of recent MS-based membrane protein-lipid examples is shown in Figure 1.

Many important membrane protein-lipid observations made via MS have subsequently been validated. Phosphatidylglycerol (PG) lipids, identified by native and ion mobility MS to stabilize the *E. coli* ammonia transporter, AmtB, and induce distinct conformational changes,[15] have recently been found to be a necessary membrane component for the full translocation cycle.[16] Negatively charged lipids observed to bind differentially to the outer membrane porin, OmpF, by native MS were also found to affect OmpF channel opening/closure times.[17,18] Cardiolipin (CDL) was observed to stabilize and facilitate assembly of the NhaA Na^+/H^+ antiporter.[19,20] In addition, pairing MS observations with functional assays has shown that PG binding increases Erwinia ligand-gated ion channel (ELIC) function,[21••] PIP_2 enhances coupling of Class A G protein-coupled receptors with heterotrimeric G_s proteins,[22] and PA and PE selectively bind the two-pore domain potassium channel, K2P4.1.[23] In this review, we consider further advances in MS methods and applications, highlighting its interplay with cryo-EM, and describe further challenges and new prospects as both tools advance.

Figure 1. Recent membrane proteins studied by mass spectrometry. Membrane protein examples include: *transporters/translocases* – eukaryotic purine/H+ symporter (UapA, PDB 5IUC), boron transporter (Bor1P, PDB 5SV9), lactose permease (LacY, PDB 6C9W), xylose transporter (XylE, PDB 4QIQ), Glycerol-3-phosphate (GlpT, PDB 1PW4), Mycobacterial membrane protein Large 3 (MmpL3, PDB 6OR2), Phospho-N-acetylmuramoyl-pentapeptide translocase (MraY, PDB 5JNQ), bacterial Leucine Transporter (LeuT, PDB 5JAE), Prensenilin homologue from *M. masisnigr*i (PSH, PDB 4Y6K); E Coli lipid flippase (MurJ, PDB 6CC4), Translocator protein (TSPO, 4UCI), Ammonia channel (AmtB, PDB 6B21), and multidrug transporter from L. lactis (LmrP, PDB 6T1Z), Sodium/hydrogen exchanger 9 (SLC9A9, Nhe9, PDB 6Z3Y); *membrane-bound enzymes* – Human V-ATPase (PDB 6WM4), Mammalian V-ATPase (PDB 6VQ6); *ion channels* - Erwinia ligand-gated ion channel (ELIC, PDB 6V03), G protein-coupled inwardly-rectifying K^+-channel (Kir3.2, PDB 6XIT), two pore domain K^+-channel (K2P, PDB 3UM7); *porins* – outer membrane protein F (OmpF, PDB 3POX); to *receptors* - neurotensin receptor 1 (NSTR1, PDB 4XEE), adenosine 2A receptor (A2aR, PDB 6GDG), and β-1 adrenergic receptor (B1AR, PDB 7JJO). Notable experiments performed in different membrane mimetics (orange), mass spectrometry methods (red), or mass spectrometry milestones (blue) have additional descriptions found near protein structures. Inside the vesicle shows the major mass spectrometry approaches used to interrogate different complexes including native mass spectrometry and native top-down (nativeomics), lipidomics, and hydrogen-deuterium exchange coupled to mass spectrometry (HDX-MS). Unless otherwise noted, membrane proteins were studied in detergent micelles.

Lipid-induced changes in protein stoichiometry

Membrane lipids can exert several effects on membrane protein complexes. In addition to serving as non-classical ligands for modulating protein function, lipids can also stabilize the interfaces of membrane proteins to facilitate oligomerisation,[24,25] allosterically affect the binding of other lipid partners,[26,27] alter coupling with other proteins,[22] and stabilize specific protein conformations [28]. Native MS is particularly adept at following lipid-induced changes in stoichiometry because these changes result in shifts of apparent protein mass, highlighted in the examples below.

Studies of the Na^+/H^+ antiporter, NHE9, showed diverging results regarding lipid residency at the homodimer interface.[29] Molecular dynamics simulations suggested a compact dimer interface that precluded lipid diffusion, while cryo-EM structures highlighted a slight movement of transmembrane (TM) 3 that could accommodate a lipid between protomers. As phosphatidylinositolphosphate (PIP) lipids were known to affect protein function, native MS was employed to probe the oligomerization potential following doping of a brain lipid mixture. Without brain lipids, NHE9 existed in a monomer-dimer equilibrium. However only dimeric NHE9 was observed with ~1 kDa lipid adducts. Thermal shift assays using a GFP-fusion construct showed PIP_2/PIP_3 lipids were responsible for NHE9 thermal stabilization and likely contributed to protein oligomerisation. Lysine-to-glutamine triple mutants located in the TM2-TM3 loop (K58Q, K105Q, K107Q), abrogating PIP lipid interaction and show only delipidated monomers (Figure 2B), demonstrating the importance of PIP_2 or PIP_3 for formation of functional dimers. Thus, native MS was able to clarify discrepancies in results between the two other methods.

Sometimes more than one lipid class is required to stabilize the interface of membrane proteins. The protein UapA is a case in point. UapA, a member of the nucleobase ascorbate transporter family, is responsible for the H^+-dependent uptake of purines and is functionally a homodimer. Native MS analysis of a thermostabilized, conformationally locked, inward-facing mutant (UapAG411V$_{\Delta 1\text{-}11}$) confirmed that co-purified lipids were important for facilitating UapA dimerisation, with delipidated samples displaying a predominately monomeric stoichiometry.[30] Lipidomics analysis identified three major co-purified phospholipids, PC, PE, and PI, with PE and PI lipids rescuing the functional UapA homodimer. Surprisingly, supplementing delipidated UapA with both PE and PI further increased the dimer ratio beyond that of the individual lipids, suggesting the existence of alternative lipid binding sites.

Most native MS studies are performed in detergent micelles.[31] Although detergents efficiently extract proteins from membranes, the micellar structure may be a poor surrogate to the native bilayer, causing altered function and fold of extracted membrane proteins.[32] Researchers are moving toward other membrane mimetics, such as nanodiscs, styrene maleic acid copolymers, and membrane-derived vesicles, which have been recently been reviewed.[33,34]

Defining unassigned lipid densities in structural maps

Single-particle cryo-EM has accelerated the acquisition of high-resolution membrane protein structures, bringing detail into the intricate structural and conformational landscapes of membrane protein complexes.[3] Except in exceptional cases,[35••,36] distinguishing and assigning critical lipids bound to proteins from detergent or background lipids remains challenging from maps alone as multiple lipids may fit into the density. A combination of native MS and lipidomics has found utility in defining these lipid moieties.

Structures of the multidrug porter LmrP, bound to its ligands had additional lipid-like electron density cohabitating the ligand binding pocket.[37] Lipid density was insufficient to differentiate the phospholipid headgroup. Since *L. lactis* membranes are predominately composed of PE, PG, and CDL lipids, and EM density ruled out the tetradentate acyl tail of CDL, native MS investigations using PG-containing nanodiscs were performed to evaluate lipid binding. Interestingly, several PG lipids bound to LmrP. A N116Y mutation created a steric clash within the ligand binding cleft that inhibited PG binding according to native MS and impaired transport properties for a host of molecules in functional studies. This study highlighted how PG occupation within the binding pocket enabled LmrP to accommodate a diverse range of hydrophobic substrates.

In a recent cryo-EM structure of the complete human V-ATPase from kidney multiple lipids were observed binding within the c-ring.[38] Lipidomics analysis showed PC, PE, PS, and cholesterol were present in the complex (Figure 2A), with PC, PE, and PS lipid molecules associated with subunits a, e, and RnaseK. Additionally, glycolipids, dolichol phosphate-linked glycan (Dol-pp-G) and monosialoganglioside (GM1) were also observed through lipidomics (Figure 2B) and placed within EM density maps. We are now able to consider the roles of sterols, phospholipids, and glycolipids on the mechanisms of V-ATPase assemblies.

Figure 2. Mass spectrometry defines lipid densities. (a) MS combined with cryo-EM identifies a number of lipids bound to the c-ring of human V-ATPase including phospholipids (phosphatidylglycerol (PG), phosphoethanolamine (PE), and phosphotidylcholine (PC)), cholesterol (CLR), gangliosides (GM1), and a glycolipid, Dol-19-PP-$GlcNAc_2Man_5$ (Dol-pp-G). Representative mass spectra showing detection of **(b)** Dol-19-PP-$GlcNAc_2Man_5$. **(c)** Structure of TSPO showing the location of unassigned lipid density on the homodimer structure. **(d)** Fitting of lipids identified by the **(e)** native top-down approach into the unassigned electron density of dimeric TSPO (PDB 4UC1) shows better agreement of a PE lipid headgroup than PG.

Differentiating annular from non-annular lipids

Extracting lipids from protein complexes severs their intimate connection, making it difficult to distinguish annular versus non-annular lipid contacts important for protein structure and function. Previously, delipidation protocols for native MS have been developed to differentiate these two lipid binding patterns.[39,40] Long incubation periods with excess detergent gradually delipidate membrane protein complexes, preferentially removing bulk lipids while retaining tightly associated lipids. This approach revealed that co-purified lipids were important for maintaining dimeric forms of the bacterial leucine transporter, LeuT,[19] and an inner membrane lipid flippase, MsbA [39]. Recently, an *in situ* delipidation method using stepwise increases of delipidating detergent supplemented into electrospray buffers was developed. This approach enabled distinction of specifically bound lipids from non-specific, annular interactions for three different membrane proteins, the prensenilin homologue PSH from *Methanoculleus marisnigri*, LeuT, and the *E. coli* lipid flippase, MurJ.[41]

Maintaining lipid-binding to membrane protein complexes and then subsequently identifying the individual bound lipids following dissociation is the ideal approach. This native-top down experiment is now possible on new MS platforms that enable transmission of large protein complexes and have sufficient MS stages to obtain diagnostic fragment patterns for individual lipids.[9••] With the instrument, the lipid composition of the full annular belt of *E. coli* water channel, aquaporin Z, was explored, showing a preference for shorter chain unsaturated lipids than previous reports,[42] and a lipid associated with unidentified electron density (Figure 2C) in the mitochondrial outer membrane transporter (TSPO) was also identified. The homodimeric *Rhodobacter sphaerodies* homologue (RsTSPO) co-purified with PG and PE lipids (C32:1 to 36:2, Figure 2E) associated with the complex. Fitting the most abundant PE lipid identified (16:0/18:1) into the crystal structure of a TSPO single mutant, A139T, showed good correlation with electron density that could not be assigned in the original structure (Figure 2D).[43] Thus with new MS instruments it is now feasible to concomitantly interrogate protein structure and define associated lipids.

Probing lipid-induced changes in protein conformation

Protein function is often linked to relatively minor changes in conformation which requires additional MS approaches, like hydrogen-deuterium exchange coupled to mass spectrometry (HDX-

MS), to capture these changes in membrane proteins.[12•] Briefly, in a HDX-MS experiment, protein is incubated in a deuterated solvent to facilitate exchange of solvent-accessible labile hydrogens along the amide backbone of the protein (Figure 3A). Comparison of deuterium uptake between two conditions can be mapped onto known protein structures or topological maps to inform on protein conformation, dynamics, ligand binding sites, and allosteric effects.[44] With recent developments enabling analysis across a broad array of membrane and membrane-like environments, including detergent micelles, nanodiscs, SMALPs, bicelles, and vesicles [12], new insight into how lipid composition modulates membrane protein conformational dynamics can be extracted.

For example, lipid headgroup chemistry can have significant effects on protein structure and dynamics. A comparative study using PC-PG-CDL vs PE-PG-CDL found PE lipids stabilized the inward facing conformation of the *E. coli* xylose transporter, XylE.[45••] Extracellular residues were protected against deuterium uptake in PE nanodiscs, whereas intracellular residues showed increased uptake (Figure 3B&C). Incubating XylE with the substrate xylose, reversed the deuterium uptake trend, showing XylE was functional and that the conformational equilibrium could be shifted toward the outer-facing conformation. Similar results in PE vs PC containing nanodiscs were found with the lactose permease, LacY, suggesting a general effect for PE stabilizing the inward conformation for these two transporters. Moreover, as PC is a non-native *E. coli* membrane lipid, this study illustrates the importance of membrane composition in shaping the conformational equilibrium of membrane transporters. In other cases, more subtle membrane composition variations, like lipid acyl tail composition (tail-length and degree of unsaturation), can affect protein conformations. [46]

Figure 3. Hydrogen-deuterium exchange mass spectrometry details lipid-induced conformational changes in membrane proteins. **(a)** An HDX experimental protocol for membrane proteins in lipid environments (adapted from Martens et al).[45••] Protein encapsulated in a membrane mimetic is first labelled at specific time points (1), the labelling reaction quenched and nanodisc assembly disassembled (2), endogenous lipids removed (3), and deuterium uptake quantified following enzymatic digestion of the protein (4) and peptide identification by mass spectrometry (5). Representative deuterium uptake for the xylose transporter, XylE, comparing the effects phosphatidylcholine-phosphatidylglycerol-cardiolipin (PC-PG-CL) nanodiscs versus phosphatidylethanolamine-phosphatidylglycerol-cardiolipin nanodiscs (PE-PG-CL). Differential deuterium uptake patterns mapped onto the **(b)** 3D and **(c)** topological structure of XylE show increased deuterium uptake on the intracellular face and decreased uptake on the extracellular face in PE nanodiscs.

Future Perspectives and Concluding remarks

Many of the case studies highlighted in this review have emphasized phospholipid binding, largely as a result of their high abundance in cellular membranes. However, increased EM resolution is highlighting new players in the lipid bilayer, for example, the dolichol lipid in the kidney V-ATPase.[38] The combination of increased resolution both in MS and cryo-EM will provide more information about other less abundant lipids including sphingolipids, glycerolipids, and ceramides.

Low affinity interactions are however more difficult to capture by many techniques, not just MS. Cholesterol, for instance, though comprising up to 50% of membrane lipid mass, and implicated in protein function and membrane localisation, is difficult to capture. An emerging approach seeks to create photocrosslinkable analogs of various lipid types that covalently link themselves onto transmembrane side chains upon UV-irradiation.[47] Potentially these lipid analogs can inform of discrete pockets that open due to conformational changes in membrane proteins.

Recently, MS studies have begun to move away from proteins solubilized in detergent micelles. Reports utilizing other membrane mimetics that better preserve protein structure and function, such as nanoscale disc assemblies (see review by Overduin *et al*, this issue) and cellular membranes are increasing.[33,48] Similarly, structural determination directly from cell membranes is feasible with cryo-EM.[49] Potentially the use of a single membrane medium for EM and MS will offer comparable information regarding protein stoichiometry and subunit composition. Such comparators are important for proteins that are difficult to isolate from membranes or those whose structures and interactions are lost once removed from the lipid bilayer environment.

With emerging multistage mass spectrometers that can now couple native MS with '–omics' workflows, proteoform-specific information can be gleaned from heterogenous protein complexes, as well as information about co-purified endogeneous ligands.[9••,50] With these advanced MS tools, together with the atomic resolution now possible with cryo-EM, deciphering the enigmatic interactions and effects of lipid binding to membrane proteins will become increasingly feasible.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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