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Review

Mass spectrometry—A versatile tool for characterising the lipid environment of membrane protein assemblies

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ABSTRACT

Biological membranes are selectively permeable barriers important for cell organization and compartmentalization. Their organisation strongly depends on the lipids that constitute the lipid bilayer as well as the proteins that reside in the membrane. Unravelling the organisation of biological membranes is therefore of great importance to understand cellular function driven by integral and peripheral membrane proteins. Recent developments in mass spectrometry made it a powerful tool contributing to our present-day understanding of membrane composition and organisation. The two main deliverables of mass spectrometry are (i) the identification and quantification of the membrane components, and (ii) the analysis of their structural arrangements. In this review article, we first briefly discuss the aspects of membrane organization that are accessible through mass spectrometry. We then provide detailed insights into the various mass spectrometric strategies which help identifying lipids from membranes or membrane protein assemblies, unravelling the lipid binding modes in membrane proteins and uncovering their structural roles. We further discuss the growing interest in membrane mimetics providing membrane proteins with a native-like lipid environment for structural and functional studies and the possibilities of mass spectrometry to contribute in these experiments.

1. Introduction

1.1. Key components of biological membranes

Biological membranes separate the aqueous interior of cells or cellular compartments from their, in most cases also aqueous, outer environment. Their organization is influenced by the various types of lipids and proteins that are the components of biological membranes as well as the abundancies they display. In most biological membranes, the protein content is ≥50% while the lipids contribute most of the remainder. Depending on the cell type and the compartmental membrane, sugars constitute up to 10% of the membrane content (Guidotti, 1972). The lipid bilayer of most biological membranes comprises three major lipid classes including glycerophospholipids, sterols and ceramide-based sphingolipids (Harayama and Riezman, 2018). In mammals, the most abundant sterol is cholesterol and its derivatives, while in plants and fungi others sterols are present. Of these, phospholipids are most abundant. They are composed of a glycerol base carrying two fatty acyl chains as well as one phosphate group. The latter can be modified with a variety of alcohols. As a result, phospholipids are manifold and usually classified by their polar head groups (Fahy et al., 2009, 2005). One of the characteristics of phospholipids is their ability to spontaneously form lipid bilayers in aqueous solutions (Watson, 2015). In these lipid bilayers, the hydrophilic head groups face the aqueous surrounding and the hydrophobic fatty acyl chains form the hydrophobic core of the membrane (Fig. 1**A**).

In addition to the lipids, proteins and sugars are also components of biological membranes (Watson, 2015). Membrane proteins carry out important cellular processes such as transport of ions and other compounds across the membrane, catalysis of chemical reactions and mediation between the cell and its environment. There are three types of proteins that associate with lipid membranes: peripheral (extrinsic), integral (intrinsic) and lipid-anchored proteins (Fig. 1**A**). Peripheral proteins are directly attached to the lipid membrane or to integral membrane proteins and consequently do not interact with the hydrophobic core of the phospholipid bilayer. Their association with the membrane is mediated through electrostatic or other non-covalent interactions with the phospholipid head groups. Integral membrane proteins, on the other hand, contain one or more domains that are embedded in the phospholipid bilayer. They usually contain

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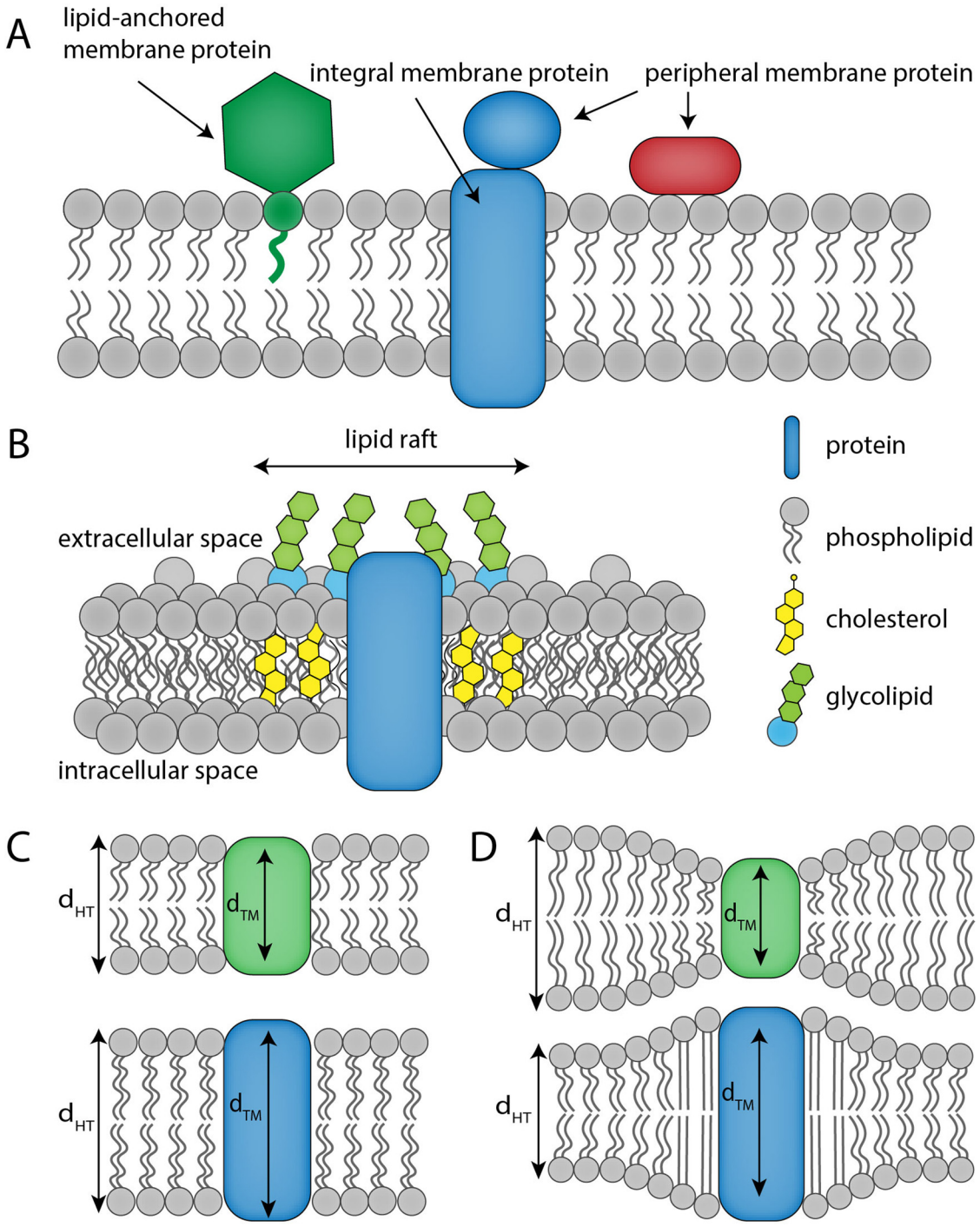

Fig. 1. Organization of biological membranes. (A) Classification of membrane proteins. Lipid-anchored proteins (green) are covalently linked with the membrane through lipids. Integral membrane proteins (blue) penetrate the phospholipid bilayer. Peripheral membrane proteins (blue and red) are associated with the membrane (red) or with integral membrane proteins (blue). **(B)** Domain formation in biological membranes. Lipid rafts are enriched in cholesterol, glycosphingolipids and proteins. **(C)** The lipid composition determines membrane thickness. Integral membrane proteins are perfectly embedded in the membrane when membrane thickness (d_{HT}) and length of the transmembrane domain of integral membrane proteins (d_{TM}) match. **(D)** Hydrophobic mismatch occurs when the length of the transmembrane domain (d_{TM}) and the hydrophobic thickness of the bilayer (d_{HT}) do not match. Negative (top panel) and positive (bottom panel) mismatch are shown.

hydrophobic amino acids, which interact with the fatty acyl chains of phospholipids thereby embedding the protein in the lipid bilayer. While peripheral membrane proteins can be removed from the lipid membrane through environmental changes, for instance changes in pH, integral membrane proteins are not easily released from the membrane (Lee, 2004). Other membrane-associated proteins are not embedded in the membrane, but are linked to the membrane through a covalently bound lipid anchor. These proteins can be located on either side of the cell membrane. The different lipid groups which link the proteins to the membrane may play a central role in protein interaction and function (Hentschel et al., 2016).

Both, proteins and lipids, can be covalently modified by sugars, which are usually found on the hydrophilic sides of the phospholipid bilayer. Due to the diversity of sugar chains, this modification can be

manifold. As a result, glycolipids serve as annular lipids, receptors and markers at the cell surface or as membrane matrix lipids (Yamakawa and Nagai, 1978). Similarly, glycosylation of proteins has diverse functional and structural roles, for instance facilitating proper protein folding or cell-cell adhesion (Dwek, 1996; Varki, 1993).

1.2. Organization of biological membranes

The organization of biological membranes is usually described on the basis of the fluid mosaic model. This model, postulated by Singer and Nicolson in 1972, proposes that membranes are fluid phospholipid bilayers in which proteins and lipids are randomly distributed and freely move. It is further assumed that the proteins penetrate either entire or half membranes (Singer and Nicolson, 1972). The fluid mosaic model has been refined over the last decades. It is nowadays generally accepted that biological membranes contain additional lipid classes (such as sterols or sphingolipids) and that proteins penetrate the lipid bilayer depending on their hydrophobicity. In addition, proteins form multi-subunit complexes containing membrane-embedded and peripheral subunits. Both, lipids and proteins, interact specifically and exist in "microdomains" whose functional role is increasingly being appreciated.

The existence of specific domains is an important aspect of membrane organization: The proteins that reside in the membranes are important for cellular function and, in order to coordinate their function, lipid bilayers often segregate into platforms of action - so-called lipid rafts (Fig. 1**B**). Lipid rafts were initially defined as detergent-resistant glycolipid-enriched (DRG) membrane fractions with a high content of cholesterol, glycosphingolipids, sphingolipids and glycosylphosphatidylinositol (GPI)-anchored proteins (Brown and Rose, 1992). The lipid raft hypothesis proposes that sphingolipids, sterols and specific proteins such as GPI-anchored or acylated proteins as well as specific transmembrane proteins preferentially associate, thereby inducing segregation in the lipid membrane and preventing homogeneous lateral distribution (Lingwood and Simons, 2010). The lipid raft concept further proposes that lipid rafts are highly dynamic ceding them a significant lateral mobility.

Another important aspect in respect to membrane organization is membrane thickness which affects both, the structure and function of membrane proteins (Lee, 2011). Membrane thickness depends on length and saturation of the fatty acyl chains as well as membrane packing and composition (Klose et al., 2013). When thickness of the hydrophobic membrane core and length of the membrane-spanning domain of an integral membrane protein match, a protein is perfectly embedded in the lipid bilayer (Fig. 1**C**). However, if the thickness of the membrane is not consistent with the transmembrane-spanning domain of the protein, hydrophobic mismatch occurs (Fig. 1**D**). More precisely, when the transmembrane-spanning domain of the protein is too long or too short to match the hydrophobic core of the membrane, positive or negative hydrophobic mismatch is observed.

Depending on the type of mismatch, the response of membrane lipids and proteins may vary (Jensen and Mouritsen, 2004). For instance, lipids modulate membrane thickness to overcome the mismatch by stretching (positive mismatch) or disordering (negative mismatch) their acyl chains (Killian, 1998; Lee, 2004). Proteins, on the other hand, may adopt a different conformation by bending their transmembrane helices thus reducing their hydrophobic length and thereby overcoming the mismatch (Killian, 1998; Lee, 2003). In other cases, helices simply adjust their hydrophobic length by changing their orientation (Killian, 1998). Protein clustering was also found to be a result of hydrophobic mismatch minimizing the tension caused at the mismatching protein-membrane interface (Milovanovic et al., 2015).

Membrane thickness can also be varied by the amount of cholesterol which increases membrane thickness substantially (Kučerka et al., 2008; Rukmini et al., 2001). Consequently, hydrophobic mismatch can be caused by domains enriched in cholesterol (Milovanovic et al., 2015). In a nutshell, membrane thickness and, as a result, hydrophobic mismatch affect protein folding, conformation, oligomerisation and activity (Dumas et al., 1999; Jensen and Mouritsen, 2004). The knowledge of the exact lipid bilayer composition including the fatty acyl chain composition of the lipids is therefore of utmost importance to understand membrane organisation.

1.3. Protein-lipid interactions in biological membranes

Another important aspect for membrane organisation is the knowledge on the interactions formed between proteins and lipids. In 1984, E. Sackmann postulated that lipids have a significant effect on the activity of proteins (Sackmann, 1984). However, the analysis of protein-lipid interactions in biological membranes proved challenging and is still in progress. For a long time, it has therefore been the goal to unravel the structures of integral membrane proteins including their interactions with lipids. First insights have emerged from high-resolution structures of membrane proteins containing lipid molecules. Some examples are the structures of bacteriorhodopsin (Grigorieff et al., 1996; Mitsuoka et al., 1999), cytochrome c oxidase (Shinzawa-Itoh et al., 2007; Tsukihara et al., 1996) or the bc1 complex (Hunte et al., 2003; Lange et al., 2001) which localized multiple lipids in the cavity between the subunits or in the periphery of the complexes.

Based on these insights and considering the membrane lipid environment, protein-lipid interactions are classified into three groups: annular lipids, non-annular lipids (also called co-factor or structural lipids) and bulk lipids (Lee, 2011; Palsdottir and Hunte, 2004) (Fig. 2). The lipid shell surrounding intrinsic membrane proteins is usually called the lipid annulus (Lee, 1977). Annular lipids mediate between the protein and the lipid environment and are therefore important for

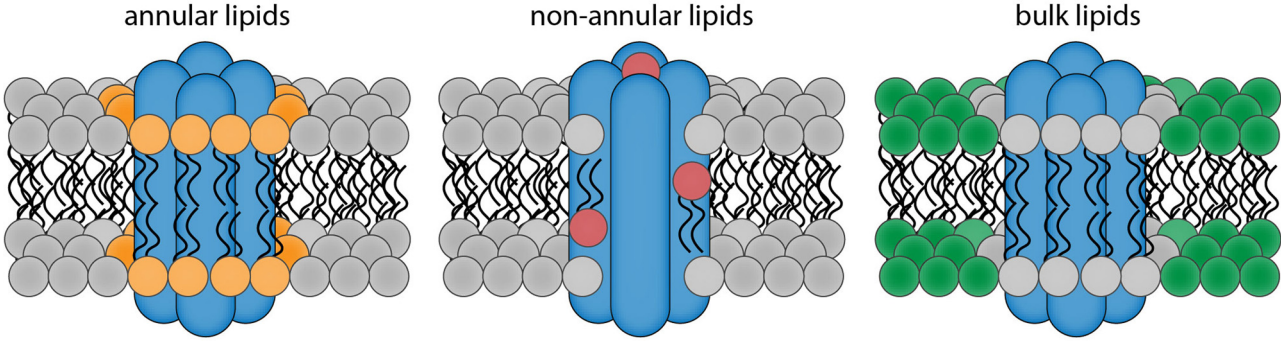

Fig. 2. Types of lipids in biological membranes. Annular lipids (orange) mediate between the protein and the lipid environment and are therefore important for vertical positioning of the protein in the lipid bilayer. Non-annular lipids (red) are bound in cavities of the protein surface, between transmembrane helices of a protein or at the protein-protein interfaces of multi-subunit protein complexes. Bulk lipids (green) usually do not make contact with the membrane-embedded protein and rather represent the bulk lipid composition of the protein's membrane environment.

vertical positioning of the protein in the lipid bilayer (Palsdottir and Hunte, 2004; Lee, 2004). Annular lipids exchange fast with their lipid environment and their interactions with membrane proteins are difficult to capture (East et al., 1985). Nonetheless, electron paramagnetic resonance spin labelling experiments provided information on lipid stoichiometry, selectivity and exchange dynamics (for review see (Marsh and Horváth, 1998)). In some cases, interactions with annular lipids could also be maintained in protein preparations for high resolution structures (Grigorieff et al., 1996; Shinzawa-Itoh et al., 2007; Tsukihara et al., 1996).

Non-annular lipids are found in cavities of the protein surface, between transmembrane helices of a protein or at the protein-protein interfaces of multi-subunit protein complexes. Due to the harsh purification procedures utilising detergents, most lipids resolved in high-resolution studies of membrane proteins are non-annular lipids. Enhanced delipidation losing non-annular lipids is often linked with a decrease in the protein's activity (Lee, 2004; Palsdottir and Hunte, 2004). Non-annular lipids are therefore also termed structural or co-factor lipids.

Interactions between integral membrane proteins and annular or non-annular lipids are consequently mediated through two interaction modes; first, hydrophobic interactions between the side chains of the protein's membrane domain and the fatty acyl chains of the lipids or, second, hydrophilic interactions between polar amino acid chains and the polar head groups of the lipids (Lee, 2011; Palsdottir and Hunte, 2004). Bulk lipids, on the contrary, usually do not interact with the membrane-embedded protein and rather represent the general lipid composition of the protein's membrane environment.

1.4. Motivation

Recent developments in mass spectrometry (MS) made it a powerful tool for the analysis of protein-lipid complexes (Barrera and Robinson, 2011; Landreh and Robinson, 2015). In so-called 'native' MS, non-covalent interactions are maintained providing insights into specific lipid binding to individual membrane proteins or their complexes (Barrera et al., 2013, 2009). Importantly, MS further allows the identification of lipids, defining the lipid class as well as the fatty acyl chain composition (Cajka and Fiehn, 2014; Han and Gross, 2005; Shevchenko and Simons, 2010). Combining both, the identification of lipids and the targeted analysis of protein-lipid complexes by native MS provides new avenues for structural and functional analysis of membrane associated protein complexes.

In the following sections, we provide an overview on the various MS strategies employed to unravel the lipid environment of membrane proteins. Specifically, we discuss MS-based lipid identification in membrane protein assemblies with emphasis on the interactions formed between proteins and lipids. We also introduce the application of membrane mimetics for MS analyses, offering the possibility to study membrane proteins in a native-like lipid environment.

2. Identification of lipids in membranes

As discussed, the lipid composition of biological membranes defines their organisation. More precisely, depending on the lipid classes present in the membrane, domain formation is favoured or disfavoured and the biophysical properties of the membrane are manipulated. The differences in fatty acyl chain composition also shape the membrane, for instance by modifying membrane thickness. The knowledge on the exact lipid composition is therefore essential when studying membrane protein structure and function. There are two key technologies used to analyse complex lipid mixtures: traditional thin layer chromatography (TLC) and state-of-the-art MS.

2.1. Lipid extraction

For identification of membrane lipids, extraction protocols are usually applied. During these procedures, proteins and minerals are removed facilitating lipid analysis.

2.1.1. Lipid extraction after Folch

Folch's method is the most popular procedure for extracting lipids from biological samples (Folch et al., 1957). During the general workflow, tissue samples are first homogenized in a mixture of chloroform/methanol (ratio 2:1). Note that including methanol as a polar component in the extraction mixture is important, as it improves the solubilisation of lipids from cell membranes. When 0.2 equivalents of water or aqueous buffer are added to the chloroform/methanol mixture, an aqueous and an organic phase form. Hydrophilic components and salts are enriched in the upper phase which mostly contains water and methanol. The lower, organic phase contains the lipids. In a final step, the lipid-containing phase is washed with water to minimize the amount of salts, non-lipid and hydrophilic components. Folch's method is very effective for isolating a broad range of phospholipids and neutral lipids from various biological samples.

2.1.2. Lipid extraction after Bligh-Dyer

The Bligh-Dyer method (Bligh and Dyer, 1959) is based on Folch, however this lipid extraction procedure utilises lower amounts of chloroform and methanol. In the first step, homogenized tissue is mixed with a chloroform/methanol solution (ratio 1:2). After mixing, additional chloroform is added to the homogenate (ratio 2:2). Water or aqueous buffer are then added to induce phase separation. After separation of the two phases, the upper, aqueous phase is removed. When compared with Folch's method, the additional extraction step using chloroform provides better extraction of non-polar lipids (Sündermann et al., 2016).

2.1.3. Lipid extraction using MTBE

MS is the major tool to analyse entire lipidomes, however, it is particularly sensitive towards the quality of the lipid extracts. Co-extracted components of biological samples include various salts which affect MS analyses. Previous studies showed that lipids of all classes can be extracted using methanol/chloroform (Folch and Bligh-Dyer, see above). In these procedures, the lipids are mostly enriched in the lower chloroform-containing phase. However, this lower fraction usually also contains insoluble precipitated particles, which might clog the electrospray ion source or the liquid chromatography (LC) system.

A recently introduced lipid extraction method therefore employs methyl-tert-butyl ether (MTBE)/methanol for extraction of complex lipidomes. Applying this protocol, lipids are recovered into the MTBE phase, which, because of its low density, constitutes the upper phase of the two phase system. This facilitates sample handling and avoids contamination of the lipid extract with insoluble particles (Matyash et al., 2008).

2.2. Thin layer chromatography

TLC is a traditional method for separating complex lipid mixtures. It is based on polarity differences between the various lipid classes, including phospholipids, sphingolipids and sterols (Fuchs et al., 2011). Usually, the stationary phase is composed of a polar silica gel and the mobile phase contains significant amounts of apolar solvents such as hexane or chloroform. TLC separation is then caused by the relative affinity of the lipids towards the stationary and the mobile phase. Lipids dissolved in the mobile phase, migrate over the stationary phase. During this movement, lipids with higher affinity to the stationary phase travel slower than those with lower affinity. Following this principle, separation of lipids is achieved.

Once separated, lipids are visualized as spots on the TLC plate using

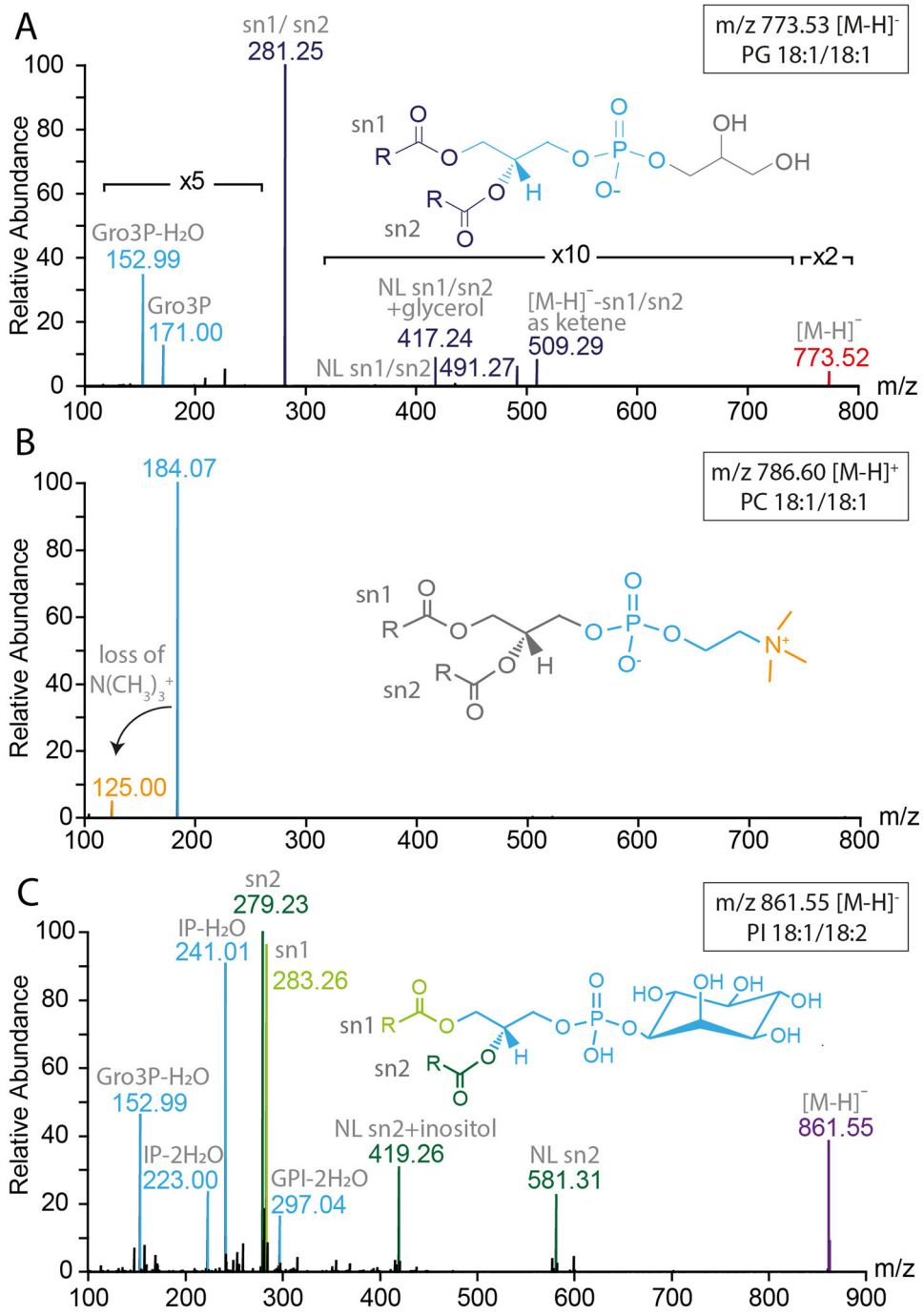

Fig. 3. Fragment spectra of different phospholipids. Characteristic fragment ions define the lipid class and fatty acyl chain composition. Fragment ions: NL, neutral loss; Gro3P, glycerol-3-phosphate; inositol phosphate; GPI, glycerol phosphatidyl inositol. **(A)** PG 18:1/18:1. Negative ion mode. **(B)** PC 18:1/18:1. Positive ion mode. **(C)** PI 18:1/18:2. Negative ion mode.

various staining solutions containing, for instance, iodine (Vioque and Holman, 1962), sulphuric acid (Klein et al., 1998) or silver nitrate (Martínez-Lorenzo et al., 1994). A characteristic of the lipid classes is the retention factor (Rf value) which is defined as the distance covered by the lipid divided by the distance covered by the solvent. The Rf value therefore provides information on the identity of a lipid in a complex mixture. Comparison with standard lipids allows the identification of lipid classes in the mixture.

2.3. Mass spectrometry-based lipid analysis

The identification of lipids by MS relies on accurately determined masses of intact lipid molecules and the subsequent detection of specific fragment ions in tandem MS experiments. When studying lipids from biological membranes, electrospray ionisation is usually the method of choice. As the ionization efficiency of lipids depends on the lipid class (i.e. the lipid head group), the lipids are usually analysed in either negative or positive ion modes (or both) (Schuhmann et al., 2012). Fig. 3 shows example mass spectra of different lipid classes. However, for some lipid classes a full structural characterisation is hard to achieve by tandem MS. For instance, PC readily ionises in positive ion mode, however, only head group fragment ions are observed making the identification of the exact lipid species difficult (Fig. 3**b**). In negative ion mode, PC usually ionises with formate or chloride yielding

information on the fatty acid composition (Godzien et al., 2015).

Even though the lipid class and the lipid species can be identified by tandem MS, this approach fails when the sn-position of fatty acyl chains or the position of double bonds are of interest. In these cases, additional experiments or specialised fragmentation techniques are required. The double bond positions, for instance, can be identified following Paterno-Büchi reactions (Ma and Xia, 2014) or ozonolysis (Thomas et al., 2006). Recently, ultraviolet photodissociation (UVPD) was introduced allowing the determination of both, the sn- and double bond positions (Williams et al., 2017).

A typical MS-based lipidomics workflow includes sample preparation, MS analysis and data processing (Yang and Han, 2016). For MS analysis, lipids are either separated by LC (LC–MS) (Cajka and Fiehn, 2014; Danne-Rasche et al., 2018) or directly introduced into the mass spectrometer (so called 'shotgun' experiments) (Han and Gross, 2005). The development of highly sensitive and mass accurate instrumentation nowadays allows the analysis of complex lipid mixtures in single shotgun experiments (Schwudke et al., 2011). The development of dedicated software further facilitates data analysis (Herzog et al., 2013, 2012, 2011; Husen et al., 2013; Ni et al., 2017)

For lipid quantification, the sample of interest is usually spiked with standard lipids which show a higher or lower mass when compared with the analytes. This mass difference is caused by deuteration or by employing lipid species with longer/shorter fatty acyl chains (Brügger et al., 1997). Quantification is then usually performed by comparing the peak intensities of the standard lipid and the analyte.

3. Identification of associated lipids in protein assemblies

Integral membrane proteins contain one or more segments which are embedded in the phospholipid bilayer of biological membranes. As mentioned above, they are therefore often co-purified with associated lipids. For solubilisation and extraction of membrane proteins from biological membranes various detergents are used. Similar to phospholipids, detergents are amphiphilic and spontaneously form micelles in an aqueous environment when their concentration exceeds the critical micelle concentration (CMC). When using detergents at concentrations above the CMC during the extraction from natural membranes, membrane proteins incorporate into the detergent micelles and mixed detergent-protein-lipid complexes are formed (Garavito and Ferguson-Miller, 2001).

3.1. Native mass spectrometry reveals lipid binding to membrane protein complexes

MS of intact protein complexes (so-called 'native' MS) has emerged as a key technology in structural MS. It delivers information on the stoichiometry, topology, subunit interactions and ligand binding (Schmidt and Robinson, 2014). Importantly, native MS is well-suited to study membrane proteins and helps defining the lipid binding partners and accessing their effect on protein stability (Fig. 4**A**) (Barrera et al., 2013; Bechara and Robinson, 2015; Landreh et al., 2016). However, to achieve this goal, non-covalent protein and protein-ligand interactions have to be preserved in the gas phase of the mass spectrometer – a venture which is not possible under conventional conditions. Therefore, instrument parameters have to be adjusted for transmission of high mass complexes (Sobott et al., 2002), and volatile, aqueous buffers are employed to maintain protein-ligand interactions in solution and during electrospray ionisation (Hernández and Robinson, 2007).

During native MS of membrane proteins, detergent micelles, in which the proteins are solubilized, are removed in the gas phase through collisional activation with inert gas molecules releasing the 'free' protein (Fig. 4**A**) (Barrera et al., 2009, 2008). For the identification of associated lipids, conditions are carefully adjusted to preserve the oligomeric state of membrane proteins and at the same time maintain associated lipids. Applied in this way, lipid binding to numerous membrane proteins, for instance UapA (Pyle et al., 2018), P-gp (Marcoux et al., 2013), MacB and LmrCD (Barrera et al., 2009), has been uncovered. A recent development in native MS employs a high-mass, high-resolution orbitrap mass spectrometer resolving a cohort of bound lipids with only minimal mass differences (Gault et al., 2016).

Several recent studies not only revealed lipid binding *per se* but also provided insights into the mode of binding or the structural and functional role of associated lipids. As an example, a significant amount of annular lipids was identified for the heterodimeric ABC transporter TmrAB. Despite harsh purification using detergent, the complex showed only unresolved signals in the mass spectra. Following a delipidation protocol, charge state series could be resolved and negatively charged phosphatidylglycerol species were found to be tightly associated with the dimeric complex (Bechara et al., 2015).

In a similar way, the lipid plugs of ATPases (Schmidt et al., 2013; Zhou et al., 2011) and cytochrome c oxidase (Liko et al., 2016) (Fig. 5**A**) were defined. For this, mass differences of subcomplexes containing or omitting the bound lipids provided insights into the binding stoichiometries. Combined with computational approaches the arrangements of proteins and lipids could be visualised.

However, it becomes apparent that the information on the masses and stoichiometries of the bound lipids alone is not sufficient. The lipid class and the fatty acyl chain composition including the number/position of double bonds remain to be elucidated to unambiguously identify and characterize the associated lipid species. Some of the discussed studies therefore combined native MS with (quantitative) lipidomics experiments (Fig. 5). The following paragraphs discuss the identification of lipids bound to membrane proteins.

3.2. Identification of lipids after their extraction from membrane protein assemblies

The most obvious way to identify the lipids bound to membrane proteins is their extraction from the purified protein sample (Fig. 4**Bi**). For this, the various extraction protocols that are commonly used to extract lipids from membranes or tissue (see above) can be employed. The lipid extract can then be dried and reconstituted in MS compatible buffers. LC–MS or shotgun experiments are subsequently used to identify the extracted lipids.

An early study following this strategy identified phospholipids bound to the transporter associated with antigen processing (TAP). For this, extracted lipids were separated by liquid chromatography and PE, PC and PI lipids were identified from high-resolution mass spectra (Schölz et al., 2011). Even though the exact information on fatty acyl chain composition was missing, these lipids were found to play key roles in activity of the TAP complex (Eggensperger et al., 2014; Schölz et al., 2011).

In another recent study specific lipid binding to the purine transporter UapA was reported (Pyle et al., 2018). Extraction and LC–MS/MS analysis revealed binding of PC, PE and PI phospholipids. Of these, PE and PI were found to stabilize the UapA dimer. Molecular dynamics simulations and mutational analyses revealed lipid binding at the dimer interface, consequently suggesting a role in stabilising the functional dimer (Pyle et al., 2018) (Fig. 5**B**).

3.3. Identification of lipids after protein hydrolysis

Another strategy to characterize the identity of lipids bound to membrane proteins is their analysis after proteolytic hydrolysis of the proteins. Using for instance a protease such as Trypsin, proteins are hydrolysed into peptides and bound lipids are released. In principle any protease can be employed for these experiments. Peptides and lipids are then separated by LC according to their different elution profiles (see Fig. 4**Bii** for details). However, detergent molecules originating from protein purification procedures are also contained and often result in abundant signals. Nonetheless, direct elution of the lipids into a high-

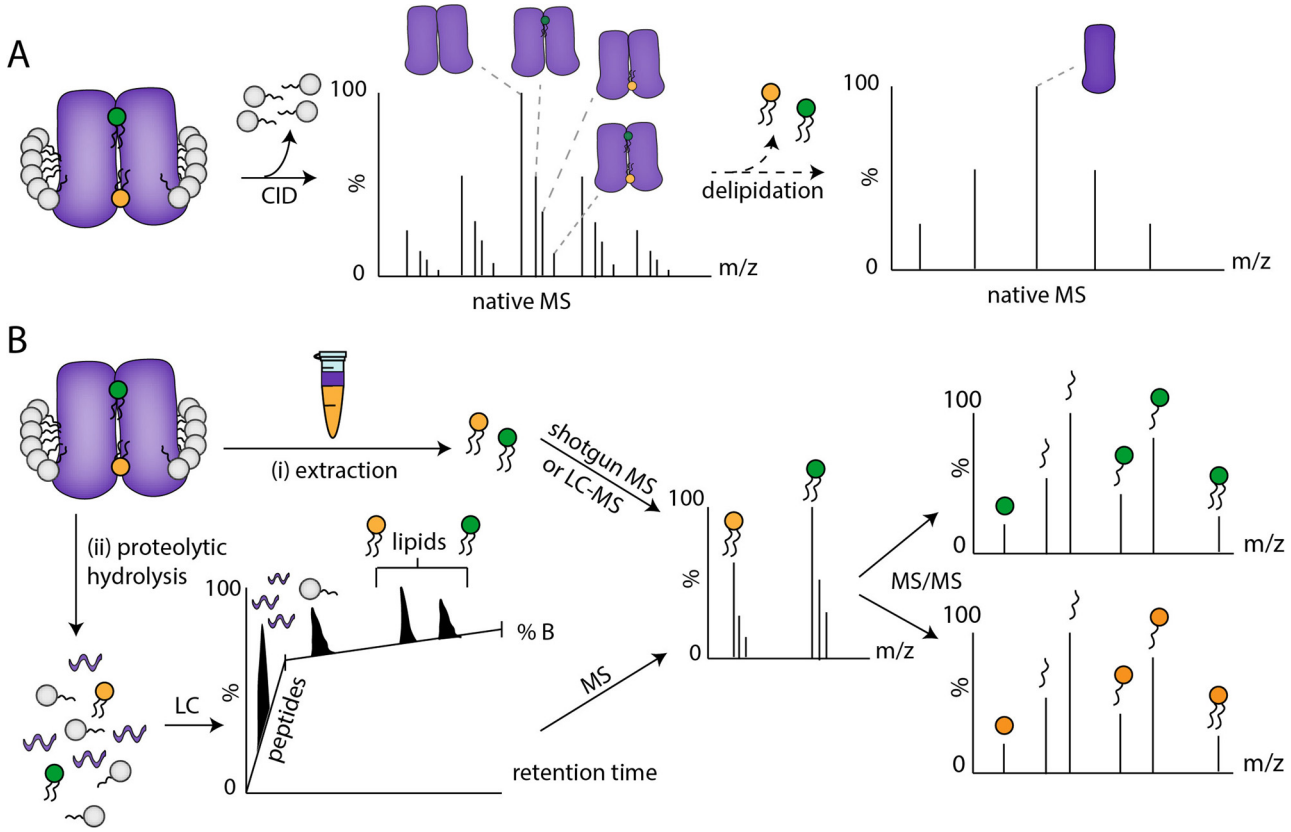

Fig. 4. Identification of membrane protein associated lipids. (A) After dissociation of the detergent micelle, native MS reveals binding of co-purified lipids to membrane proteins. Delipidation often causes dissociation of membrane protein complexes. **(B)** Lipids are extracted and analysed by shotgun-MS or LC–MS (i) or proteins are hydrolysed and the lipid-peptide mixture is separated by LC (ii). Lipid masses are determined by MS and lipid species are identified from MS/MS spectra.

resolution mass spectrometer allows unambiguous identification of phospholipids.

This strategy was successfully pursued to identify the lipids that associate with ATPases (Schmidt et al., 2013; Zhou et al., 2011), cytochrome c oxidase (Liko et al., 2016) or TmrAB (Bechara et al., 2015). In some cases, the lipids were also quantified by employing lipid standards, for instance to determine protein-lipid stoichiometries (Zhou et al., 2011) or to monitor delipidation of annular lipids (Bechara and Robinson, 2015). The advantage of the strategy described here is that lipids cannot be lost during sample preparation and are in any case included in the MS measurements. However, this approach makes a distinction of annular or non-annular from bulk lipids nearly impossible.

3.4. Combining native mass spectrometry and lipid profiling for determination of lipid identities and binding modes

A recent protocol combines native MS with solution-phase lipid profiling to monitor the delipidation process of membrane proteins and, at the same time, obtain information on the mode of lipid binding (Gupta et al., 2018). This protocol allows distinguishing bulk and annular lipids which are likely co-purified during detergent-based extraction from structural lipids which associate strongly with membrane proteins. The protocol includes: (i) the acquisition of native mass spectra of the detergent-purified membrane protein complex at various delipidation steps and, (ii) the extraction of associated lipids at each step followed by LC–MS/MS analysis for lipid identification. At early delipidation steps, i.e. immediately after detergent-purification and prior to additional washing steps, identified lipids comprise bulk, annular and structural lipids. At later delipidation steps, i.e. following additional washing steps, only structural lipids remain associated. By comparing the lipid content identified at the various delipidation steps bulk lipids and annular lipids can be distinguished from tightly bound structural lipids. In addition to lipid identification, complex stability and the oligomeric state of the membrane protein is monitored by native MS and can be correlated to the presence of lipids. Of note, bulk lipids and annular lipids are hard to distinguish by this procedure.

In a second set of experiments, tightly bound lipids are targeted. For this, the protein-lipid complex observed after delipidation is selected for tandem MS and lipids are assigned through neutral loss from the intact complex or through detection of charged lipids with low mass-to-charge ratio in the tandem mass spectra (Gupta et al., 2018). However, employing this strategy requires a specialised instrument set-up allowing multi-step dissociation experiments releasing the protein-lipid complex from the detergent micelle and selecting and dissociating the membrane protein complex in tandem MS.

The described protocol has been exemplified using three membrane proteins. Their full cohort of associated lipids was identified and structural lipids were differentiated from annular and bulk lipids (Gupta et al., 2018). Nonetheless, when working with detergent-purified membrane proteins, one has to keep in mind that the various available detergents differ in their delipidation properties, i.e. some detergents are rather harsh and remove the lipid shell around integral membrane proteins while others maintain associated lipids to a certain degree (Helenius et al., 1979).

4. Membrane mimetics

Over the last decade, there has been an ongoing effort to study the structure and function of membrane proteins in an active, native-like lipid environment. Unfortunately, detergents, which are usually used to solubilize membrane proteins, often have adverse effects on protein

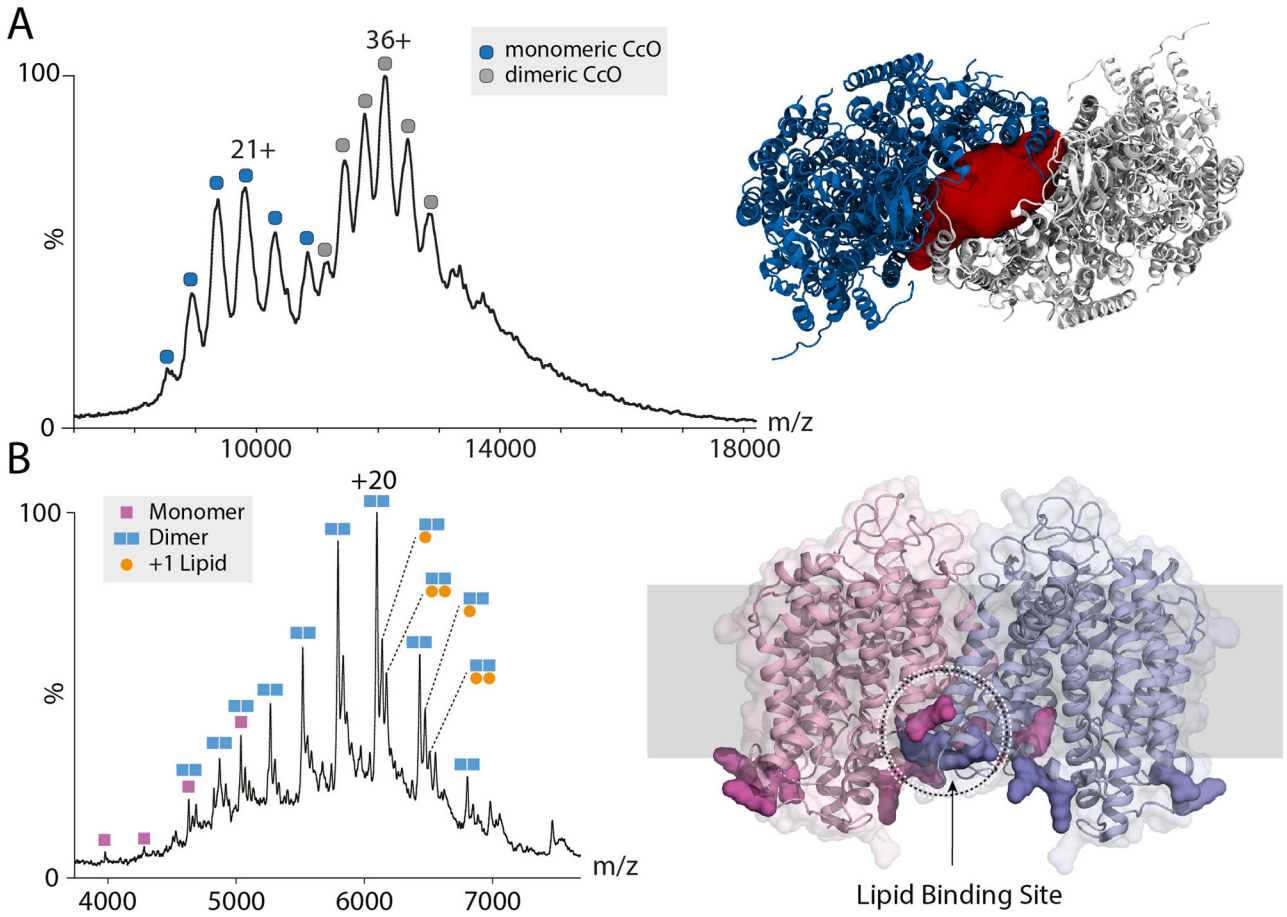

Fig. 5. Localisation of lipids in membrane protein assemblies. (A) Cytochrome c oxidase. Associated lipids were identified by LC–MS/MS after protein digestion. Monomeric and dimeric cytochrome c oxidase were observed in the native mass spectrum. The mass differences between the two subcomplexes revealed the mass of the lipids bound in the cavity. The volume of the 'lipid plug' was then obtained from the crystal structure and the number of bound lipids could be calculated. Figure adapted from (Liko et al., 2016). **(B)** Membrane transporter UapA. Associated lipids were identified after extraction by LC–MS/MS. The native mass spectrum shows binding of two lipid species to dimeric UapA. Lipid binding sites were determined by molecular dynamics simulations. Figure adapted from (Pyle et al., 2018).

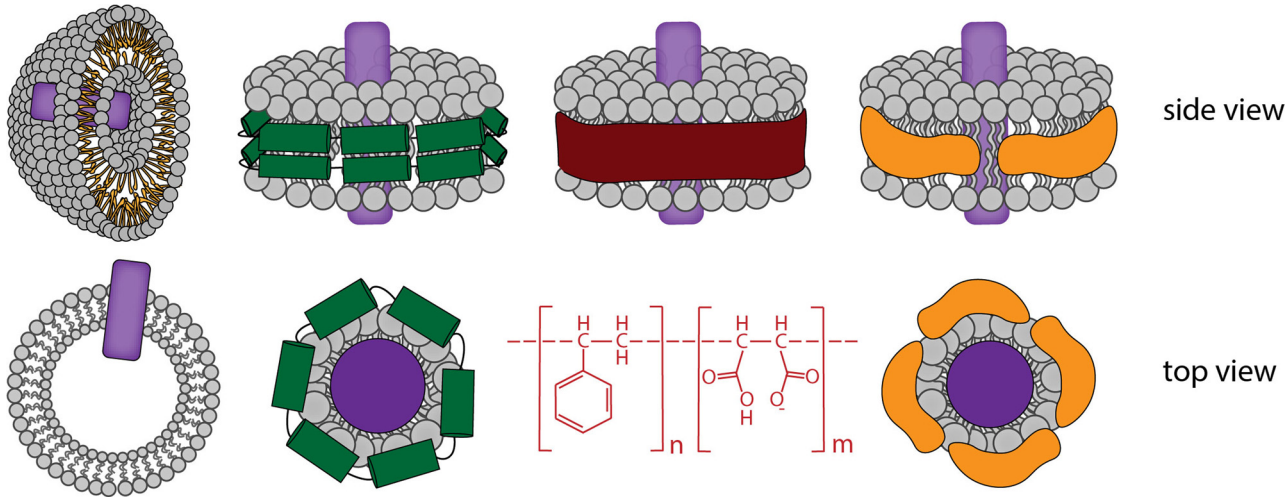

Fig. 6. Membrane mimetics. Top and side view of liposomes, nanodiscs, SMALPs and SapNPs. The SMA-copolymer unit is shown for SMALPs.

activity and stability. Therefore, various membrane mimetics (e.g. liposomes, nanodiscs and others) have been developed to overcome these difficulties (Fig. 6). Following the success in studying membrane protein structure, MS also emerged as a key method for analysing membrane proteins assembled in membrane mimetics (Hellwig et al., 2018; Hopper et al., 2013; Li et al., 2016; Marty et al., 2016b).

4.1. Liposomes

Liposomes might be considered the simplest membrane mimetic (Fig. 6). They have therefore often been employed in biophysics, chemistry, biochemistry and biology. Liposomes are spherical vesicles that consist of one or more phospholipid bilayers surrounding an

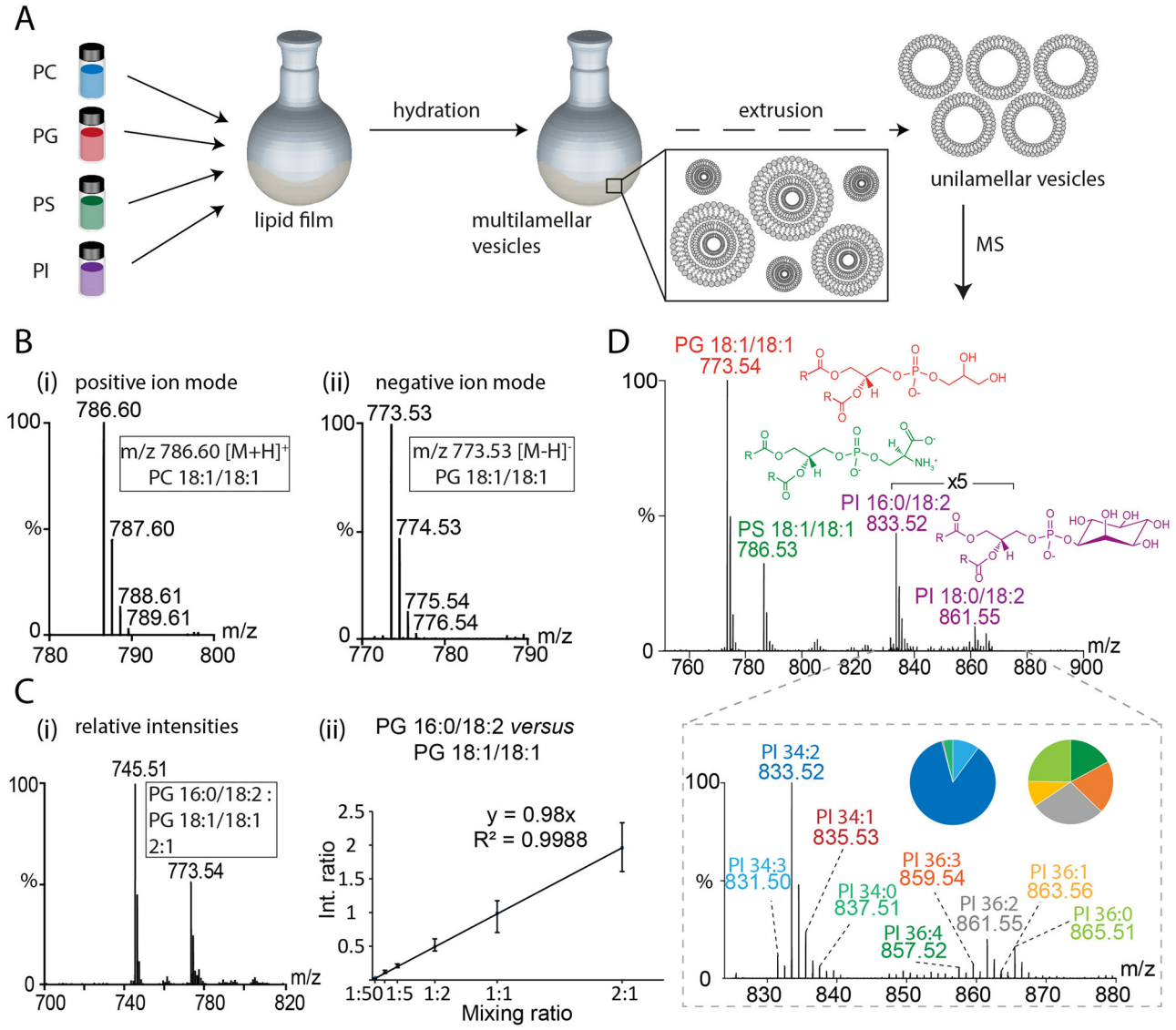

Fig. 7. Identification of lipids from liposomes. (A) Workflow for preparation of liposomes. A lipid film is prepared from a lipid mixture. During hydration of the lipid film MLVs form. ULVs are prepared from MLVs by extrusion. **(B)** Mass spectra of different lipids obtained from ULVs. (i) PC 18:1/18:1 was analysed in positive ion mode and (ii) PG 18:1/18:1 was analysed in negative ion mode. **(C)** Two PG species were mixed in varying ratios. (i) The peak intensities observed in the mass spectrum reflect the mixing intensities. (ii) Mixing ratio and observed intensity ratios from 1:50 up to 2:1. **(D)** MLVs were prepared from PG 18:1/18:1, PS 18:1/18:1 and soy PI extract in 1:1:0.3 ratios. Several PI species were observed (purple and inset). The various PI species were quantified by calculating their theoretical isotope pattern. Panels (B), (C) and (D) were adapted from (Frick et al., 2018).

aqueous interior. Similar to biological membranes, the polar head groups of the phospholipids are oriented towards the aqueous surrounding while nonpolar fatty acyl chains are oriented towards the fatty acyl chains of the opposite lipid monolayer. Depending on the number of lipid bilayers, liposomes are classified as multilamellar vesicles (MLVs) or unilamellar vesicles (ULVs). Accordingly, MLVs have an onion-like structure containing several concentric lipid bilayers while ULVs are composed of a single phospholipid bilayer (Sharma and Sharma, 1997). Depending on their size, ULVs are further divided into small, large and giant ULVs (also called SUVs, LUVs and GUVs, respectively).

Liposomes can be prepared at varying sizes from a variety of lipids including cholesterol, natural and non-natural phospholipids (see Fig. 7**A** for workflow). Their variability in size, composition and amphipathic character makes them amenable for diverse applications. Initially, they were used to trap hydrophilic or hydrophobic compounds and release the enclosed molecules at designated target sites. Liposomes are therefore ideal drug-carriers (Gregoriadis, 1976a, 1976b; Gregoriadis and Ryman, 1971). Later, proteins were incorporated into the liposome membrane allowing functional and structural studies in a native-like environment. However, a problem that usually occurs when using so-called proteo-liposomes is that the correct orientation of the proteins in the liposome membrane is difficult to achieve and usually two populations (inside-out and outside-out) are obtained (see (Luis G. Cuello et al., 1998) for an example). In addition, liposome membranes are curved and the protein structure or orientation might be affected.

Even though liposomes are well-suited membrane mimetics, a sophisticated application for MS is still missing. In early studies, liposomes were employed for hydrogen/deuterium exchange studies of membrane peptides (Demmers et al., 2001, 2000), however, due to their size and heterogeneity liposomes were generally not considered further as suitable for MS studies (Marty et al., 2016b). Nonetheless, a recent study identified and quantified lipids directly from the lipid bilayer of liposomes (Fig. 7) (Frick et al., 2018). Importantly, the intensities of different lipid species corresponding to the same lipid class reflect the correct mixing intensities, promising an analysis directly

from natural bilayers in future studies (Fig. 7**C** and **D**).

4.2. Nanodiscs

Nanodiscs are disc-shaped lipid bilayers encircled by two belts of an engineered, amphipathic membrane scaffolding protein (MSP) (Bayburt et al., 2002) (Fig. 6). The diameter of the disc can be varied by the length of the MSPs and the lipid composition is defined by the lipids employed during assembly. The length of their fatty acyl chains can be adjusted to the size of the proteins under investigation (Denisov and Sligar, 2017). In comparison with detergent micelles, nanodiscs are well-defined and represent native-like lipid bilayers. Consequently, they have successfully been employed in a growing number of functional and structural studies (summarized in Denisov and Sligar, 2016).

Due to their favourable characteristics keeping membrane proteins in an active form, their application for MS was also explored. Starting with 'empty' nanodiscs, they were first characterised as well-defined and potentially monodisperse with only minimal variation in the number of incorporated lipids (Marty et al., 2012). Deconvolution algorithms allowed the interpretation of the rather complex mass spectra with overlapping charge states (Marty et al., 2015, 2014). Next, the release of protein oligomers from the lipid bilayer of nanodiscs through collisional dissociation in the gas phase was explored (Henrich et al., 2017; Hopper et al., 2013). It is worth mentioning that the application of nanodiscs revealed the native oligomeric states of the proteins while this was not always achieved when using detergents. The advanced application of nanodiscs therefore delivered insights into membrane proteins and their lipid interactions. Accordingly, the annular lipid belt of integral membrane proteins (Marty et al., 2016a) as well as lipid-dependent oligomerisation were uncovered (Henrich et al., 2017).

Even though nanodiscs are promising for analysing membrane proteins in their native states and environment there are some drawbacks. MSP production and purification is time-consuming and finding the appropriate lipid composition to establish a fully active lipid environment is challenging and requires additional experiments. Another drawback is the relatively high collisional energy required to release the membrane proteins from the disc in the gas phase of the mass spectrometer as well as the additional signals from the bulk lipids and MSP. Recent improvements therefore include the development of special MSPs for native MS which facilitate interpretation of complex spectra (Reid et al., 2017) and the addition of chemical reagents allowing ejection of membrane protein oligomers with few lipids bound or even the intact membrane protein nanodisc complex (Keener et al., 2018).

4.3. Stryene maleic acid lipid particles

A recent innovation allows studying membrane proteins in their natural lipid environment. Using amphiphilic styrene-maleic acid (SMA)-copolymers, incorporates the membrane proteins together with their surrounding lipid environment from the native membrane thereby forming so-called SMALPs (Jamshad et al., 2011; Knowles et al., 2009) (Fig. 6). This is particularly beneficial as the lipids surrounding a protein in a membrane are often important for their structure and function. Another advantage of SMALPs is that membrane proteins are comparatively stable in these membrane mimetics (Simon et al., 2018). However, when compared with nanodiscs, which employ a scaffold protein of defined mass, stoichiometry and diameter, the number of SMA polymers is not defined giving SMALPs a certain heterogeneity.

As SMALPs are very recent development, they have not yet been applied in many MS experiments. Nonetheless, SMALPs were explored recently by LILBID-MS (Laser-induced liquid bead ion desorption MS) (Hellwig et al., 2018). Using a low activation energy, masses of intact SMALPs including protein oligomers and their lipids were revealed. Due to heterogeneity of SMALP populations, peak broadening was observed in these mass spectra. At higher activation energies, the incorporated proteins were released from the lipid disc in their monomeric states. Even though the oligomeric states of the membrane proteins could not be preserved in these experiments, SMALPs are promising tools for future studies combining functional and MS analyses.

4.4. Saposin-derived lipid nanoparticles

Another recent, innovative approach is based on the ability of saposin proteins A, B, C or D to form disc-shape constructs in the presence of lipids - so-called saposin-derived lipid nanoparticles (SapNPs) (Fig. 6) (Frauenfeld et al., 2016; Lyons et al., 2017). Crystal structures further confirmed that saposines are found in soluble or lipid-bound states (Popovic et al., 2012). For membrane protein analysis, saposines are used as scaffolding proteins, solubilizing membrane proteins in their lipid environment without detergents (Frauenfeld et al., 2016). First studies used negative stain electron microscopy to structurally characterize P-type ATPases in SapNPs in the absence of detergents (Lyons et al., 2017).

The advantage of saposines is their ability to adjust to proteins of different sizes (Frauenfeld et al., 2016) and thereby reducing the time-consuming search for scaffolding proteins and complex reconstitution (Flayhan et al., 2018). As this approach is fairly new, there is still the need for investigating its combination with analytical techniques such as MS.

5. Summary and conclusions

The organisation of biological membranes is affected by the protein and lipid components as well as their interplay. Besides the traditional techniques, MS is gaining importance in characterising the lipid environment of membrane-embedded proteins as it directly delivers insights into the interactions formed between proteins and lipids. Most importantly, MS allows the identification of lipids in biological membranes or associated with membrane proteins. Using native MS, a technique which preserves non-covalent interactions in the gas phase, direct lipid binding as well as binding stoichiometries can be explored. Combining both approaches, MS considerably contributes to our understanding of membrane organisation including formation of protein and lipid microdomains.

Recently, the application of membrane mimetics became increasingly popular allowing the study of membrane proteins in a native-like lipid environment. An advantage of MS is its compatibility with these membrane mimetics making their application in future studies promising. Since the extent of information achievable by MS is growing, the combination with computational workflows is required. In that way, models of protein-lipid assemblies as well as microdomains in the lipid bilayer can be obtained.

Conflict of interest

None.

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