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Advances and challenges in preparing membrane proteins for native mass spectrometry

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ABSTRACT

Native mass spectrometry (nMS) is becoming a crucial tool for analyzing membrane proteins (MPs), yet challenges remain in solubilizing and stabilizing their native conformations while resolving and characterizing the heterogeneity introduced by post-translational modifications and ligand binding. This review highlights recent advancements and persistent challenges in preparing MPs for nMS. Optimizing detergents and additives can significantly reduce sample heterogeneity and surface charge, enhancing MP signal quality and structural preservation in nMS. A strategic workflow incorporating affinity capture, stabilization agents, and size-exclusion chromatography to remove unfolded species demonstrates success in improving nMS characterization. Continued development of customized detergents and reagents tailored for specific MPs may further minimize heterogeneity and boost signals. Instrumental advances are also needed to elucidate more dynamically complex and labile MPs. Effective sample preparation workflows may provide insights into MP structures, dynamics, and interactions underpinning membrane biology. With ongoing methodological innovation, nMS shows promise to complement biophysical studies and facilitate drug discovery targeting this clinically important yet technically demanding protein class.

1. Introduction

Membrane proteins perform vital cellular functions including material transport, signal transduction, adhesion, enzymatic catalysis, structure, and communication (Levental and Lyman, 2023; Tosaka and Kamiya, 2023) (Fig. 1). G-protein-coupled receptors mediate responses to hormones and neurotransmitters via signal transduction (Gurevich and Gurevich, 2019; Maeda and Schertler, 2013). Voltage-gated and ligand-gated ion channels regulate nerve and muscle activity by controlling ion flow. (Cuello et al., 2017; Levental and Lyman, 2023; Liu et al., 2019). Transporters facilitate nutrient uptake, such as the glucose transporter GLUT4 and lactose permease (Kimanius et al., 2019; Yuan et al., 2022). Multi-drug resistance pumps enable bacterial expulsion of toxins and antibiotics, conferring survival and drug resistance (Kaur et al., 2021). Integrins ensure tissue integrity and processes like wound healing via cell-cell and cell-matrix interactions (Doyle et al., 2022). Major histocompatibility complex proteins present antigens and initiate immune responses (Migalska et al., 2019). Chemotaxis receptors allow bacteria to sense and respond to chemical gradients, guiding movement toward favorable conditions (Karmakar, 2021). Given their integral participation in crucial biological functions, elucidating the characteristics of membrane proteins at a molecular level is imperative for propelling basic research progress and informing the rational design of innovative biotherapeutics.

With the development in the past two decades, native mass spectrometry (nMS) has emerged as a powerful tool for membrane protein characterization (Fig. 1)(Bolla et al., 2019). Unlike conventional denaturing MS approaches that bring protein denaturation, native mass spectrometry employs nondenaturing ionization conditions to transfer compounds from the aqueous phase to the gas phase with the preservation of noncovalent interactions and quaternary protein structure (Leney and Heck, 2017). The working mechanism of nMS involves

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several key components, including ionization sources, ion optics systems, fragmentation sources, and mass analyzers(Karch et al., 2022). The first module that the prepared MP samples encounter in a native mass spectrometer is the ionization source, where MP samples are transferred to the gas phase. Common ionization sources for nMS include Electrospray Ionization (ESI), Matrix-Assisted Laser Desorption/Ionization (MALDI), Desorption Electrospray Ionization (DESI), and Atmospheric Pressure Chemical Ionization (APCI)(Schachner et al., 2019). Among these, ESI is the most widely used method for nMS. It generates charged droplets from a solution containing analytes, effectively preserving the native conformations of MPs and their noncovalent interactions(Banerjee and Mazumdar, 2012). The ionized MP samples are then directed into the mass analyzer by Ion Optics like Ion Transfer Tubes, Quadrupole, and Hexapole systems under vacuum conditions. This module filters and focuses ions based on their mass-to-charge ratio (m/z), allowing for the selection of specific ions while removing unwanted ones and optimizing the transmission of the target ions(Bhanot et al., 2022; Savaryn et al., 2016). Fragmentation sources in nMS are designed to dissociate large biomolecular complexes, such as proteins, into individual molecules while preserving their native-like structures(Song et al., 2021). Common fragmentation sources include Collision-Induced Dissociation (CID) and Surface-Induced Dissociation (SID). CID involves colliding ions with a neutral gas, such as nitrogen or argon, while SID directs ions to collide with a surface, resulting in fragmentation without significant loss of the native state(Snyder et al., 2022). After ionization, fragmentation, and ion optics, the ions are transferred to a mass analyzer to determine their m/z. Common mass analyzers include Quadrupole Mass Filter, Time-of-Flight (TOF), Orbitrap, and Ion Trap(Tamara et al., 2021). Quadrupole Mass Filter is Commonly used for nMS, it allows for the selection of ions based on their m/z ratios and can be used in tandem with other mass analyzers for MS/MS experiments. TOF measures the time it takes for ions to travel a specific distance. TOF provides high-resolution mass measurements and is often used in combination with ESI. Orbitrap provides high mass accuracy and resolution by trapping ions in an electric field and measuring their oscillation frequencies. It is effective for detailed analysis of protein complexes and post-translational modifications. Ion Trap uses electric or magnetic fields, allowing for multiple stages of analysis (MSn) and is useful for studying fragmentation patterns(Li et al., 2024; Lössl et al., 2014; Mamyrin, 2001). Native MS's high-resolution accuracy demarcates protein constituents and stoichiometries with clarity unmatched by equilibrium or electrophoretic methods(Laganowsky et al., 2014; Qiao et al., 2020; Tamara et al., 2021). Native mass spectrometry provides the most reliable determination of membrane protein complex molecular mass. Traditional methods like SDS-PAGE and size-exclusion chromatography can only estimate mass via reference comparisons. Native MS directly measures intact complex mass-to-charge (m/z) ratios under native conditions, maintaining noncovalent interactions.(Leney and Heck, 2017).

Membrane proteins interact with lipids in their native environment, influencing their structure, stability, and function. Native mass spectrometry can identify lipid binding sites, determine the stoichiometry of lipid-protein interactions, and elucidate the role of specific lipid species in modulating protein function(Qiao et al., 2021a; Zhu et al., 2023). Instead of merely capturing a snapshot of the protein-ligand interactions, native mass spectrometry can monitor the dissociation of protein complexes and their ligands, it assesses their stability, conformational changes, and ligand binding kinetics. This dynamic information complements structural data obtained from other techniques, offering a more comprehensive understanding of protein behavior(Qiao et al., 2021b). The interaction between membrane proteins and other proteins can also be investigated by using native mass spectrometry. Analyzing intact complexes or introducing specific ligands can provide insights into binding affinities, stoichiometry, and the effect of ligand binding on complex stability(Keener et al., 2021a, 2021b). Native mass spectrometry has been successfully combined with structural biology

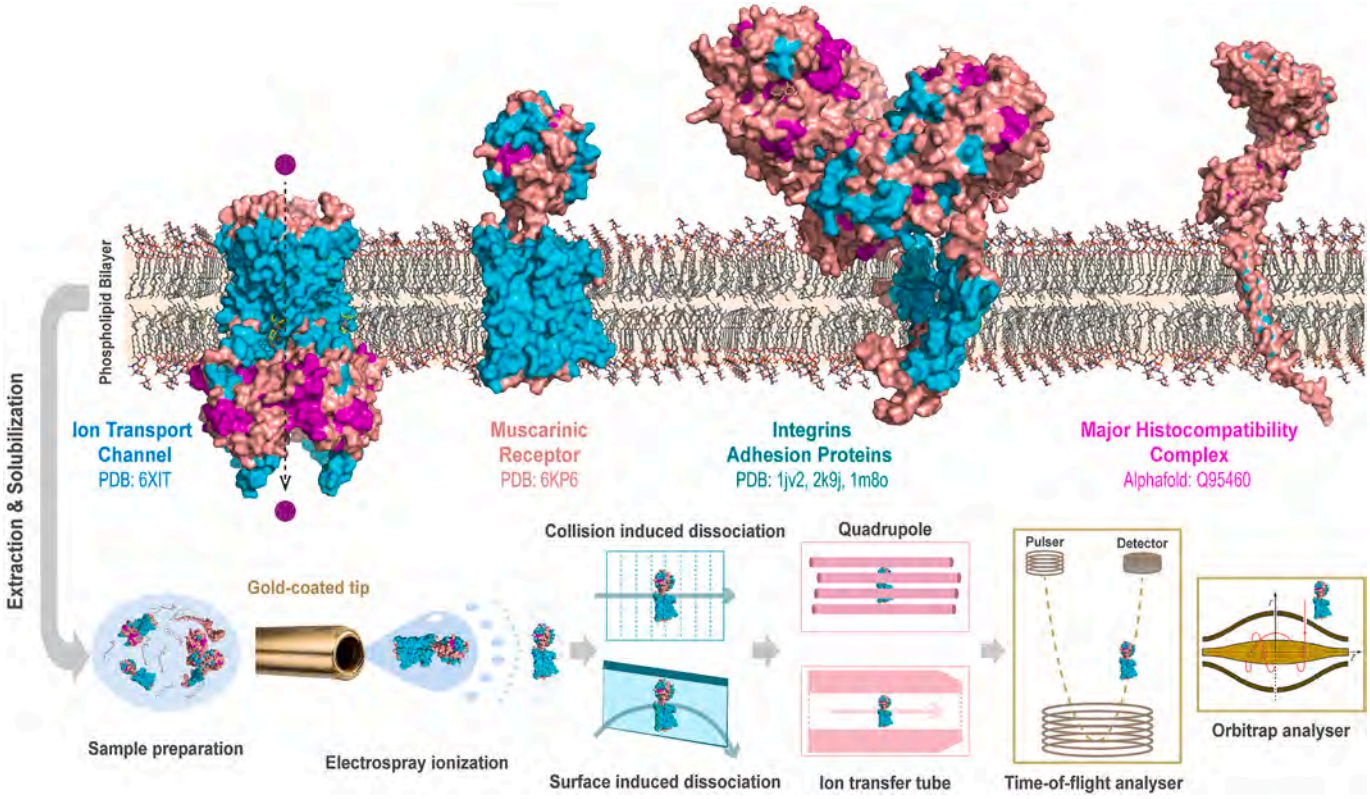

Fig. 1. A typical workflow for the characterization of membrane proteins (MPs) by using native mass spectrometry (nMS) with common ion optics, fragmentation sources, and mass analyzers.

techniques such as cryo-electron microscopy (cryo-EM) or X-ray crystallography. Cryo-EM or X-ray provides high-resolution molecular details while native mass spectrometry complements this with information like subunit organization and protein-ligand interaction(Tetreau et al., 2022).

As it was elaborated above, native MS provides crucial insights into membrane protein structure-function relationships and roles within cellular contexts. However, analyzing membrane proteins via nMS is significantly more challenging than established denaturing MS techniques. Unlike soluble proteins analyzed under denaturing conditions using standardized workflows like LC-MS, membrane proteins do not readily conform to a one-size-fits-all native MS protocol. Both membrane protein sample preparation and nMS instrumentation setup demand multiparametric optimization on an individual basis. Detergent selection, additive screening, tuning of buffers, ionization conditions, and mass analyzer parameters all require careful evaluation and adjustment to reproducibly preserve native-like states during the ionization and fragmentation processes of native MS. This customized optimization effort is necessary to generate high-quality nMS signals amenable to downstream structural characterization. This review focuses on the sample preparation step for membrane protein characterization by using native MS.

2. Membrane protein solubilization

The prerequisite for the employment of native mass spectrometry in the study of membrane protein is the solubilization step. Unlike soluble or globular proteins that reside in the cytoplasm freely, membrane proteins are often associated with the cellular membrane structure, especially integral membrane proteins(Hunte and Richers, 2008). Thus, the cellular membrane proteins need to be extracted from the cellular membrane and solubilized in aqueous solution before being subjected to further characterization (Fig. 2). Besides, the hydrophobic domain in membrane proteins hinders their solubilization in water, direct exposure to a polar system would lead to protein unfolding and denaturation. As a result, membrane mimetics are also required for the stabilization of membrane proteins. The common agents used for membrane protein extraction and solubilization are detergent micelles, fluorinated surfactants, nanodiscs, amphipols, lipid vesicles, and styrene-maleic acid-lipid particles (Fig. 2)(Arachea et al., 2012; Lee et al., 2016a, 2016b; Popot, 2010).

2.1. Detergent micelles

Historically, detergent-based extraction is the most frequently used method for the separation of membrane proteins(Stetsenko and Guskov, 2017). Detergents are amphipathic molecules bearing both a hydrophilic (polar) headgroup and hydrophobic (apolar) tails. By this nature, detergent molecules spontaneously form micelles with the polar headgroups facing outside and the apolar tails toward the inside when they reach the critical micelle concentration (CMC) at a temperature higher than the critical micellar temperature (CMT). If their concentration were lower than the CMC, detergent molecules would present as monomers. Upon contact, detergent molecules could insert their hydrophobic tails into the cellular membrane constituted with a lipid bilayer(Calabrese and Radford, 2018; Quick and Javitch, 2007). As a consequence, the lipidic membrane is disrupted, and the originally embedded membrane proteins are released and encapsulated in the detergent micelles (Fig. 2). The process of detergent-based membrane protein extraction often starts with protein hyperexpression. Host cells that have expressed target membrane proteins could be harvested by centrifugation and lysed with a microfluidizer. Then, the cell membrane pellets are precipitated by

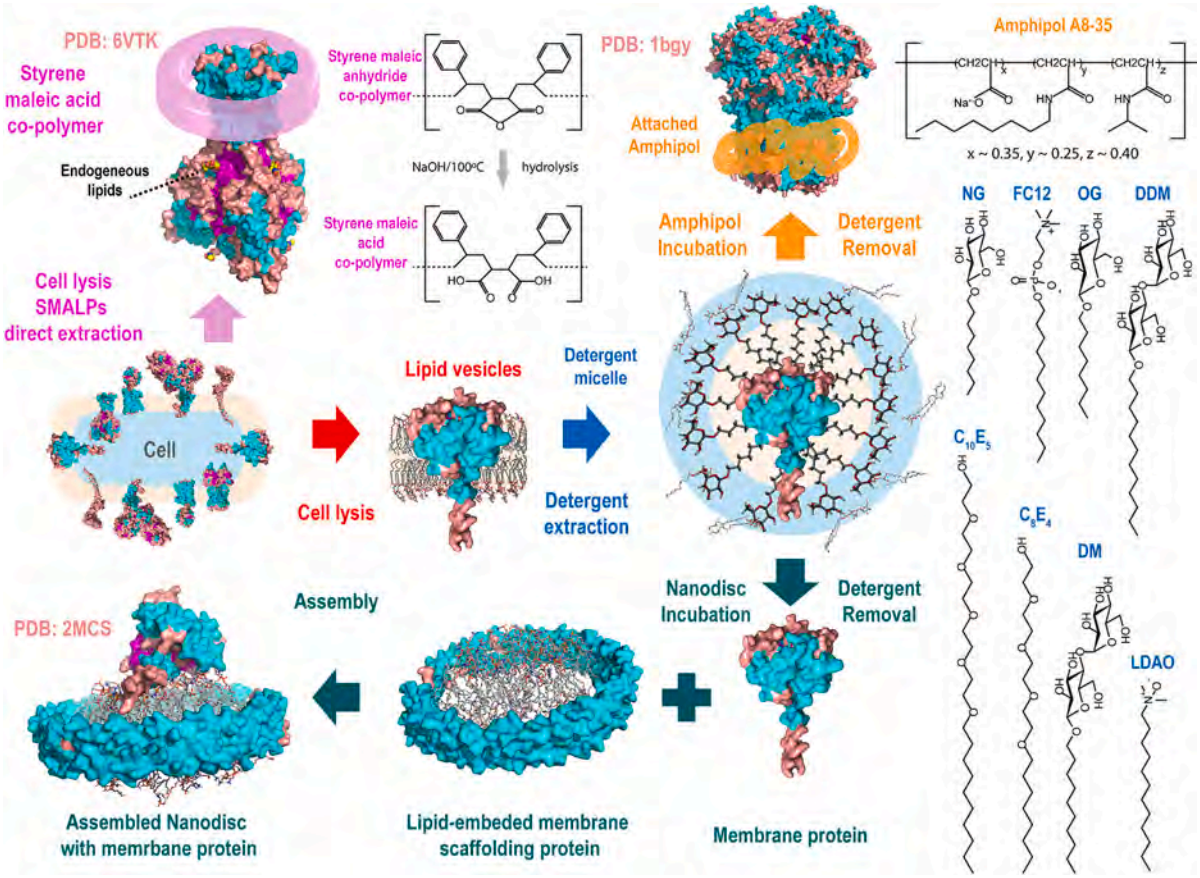

Fig. 2. Commonly employed membrane protein solubilization strategies.

ultracentrifugation and resuspended by homogenization. The opaque membrane resuspension is then supplemented with detergents and incubated at 25 °C or lower temperature.

The detergents commonly used for membrane protein extraction and solubilization have been changing with the development of native mass spectrometry during recent decades. The popular detergents in the early stages are saccharides like n-dodecyl-β-d-maltopyranoside (DDM), Decyl β-D-maltopyranoside (DM), n-octyl-β-D-glucopyranoside (OG), and n-nonyl-β-D-glucoside (NG), especially in structural biology (Laganowsky et al., 2013). These detergents, although very efficient in preserving structural integrity, are not very suitable for native MS spraying as they form stable micelles that are hard to dissociate during ionization (Laganowsky et al., 2014). The MS spectra collected at present of these detergents manifest broad peaks that represent low resolutions(Qiao et al., 2020; Qiao et al., 2021a; Zhu et al., 2023). To obtain a better native MS signal, membrane protein samples have been exchanged for detergents that can overcome these defects. The detergents with polyethylene glycol hydrophilic head groups like tetraethylene glycol monooctyl ether (C_8E_4), pentamethylene glycol monodecyl ether ($C_{10}E_5$), and lauryldimethylamine-N-oxide (LDAO) have a weak affinity with membrane proteins and can be easily removed during the ionization, making them suitable for native MS studies. It is worth noticing that the micelles formed by these detergents are relatively weak and some membrane proteins are not as stable in these detergents as they were in DDM. As a result, recent studies have investigated the potential of detergent mixtures in the solubilization of membrane proteins for native MS analysis. The membrane proteins on the mixed-detergent micelles could interact with the ideal detergent molecules and form a stable complex. For example, the adenosine A_{2A} receptor ($A_{2A}R$) and beta 1 adrenergic receptor (β1AR) are exchanged into mixed micelles made of DDM and Fos-choline (FC12) to generate better native MS spectra(Chen, 2022). Optimization of detergent mixture formulations for membrane proteins requires tailored approaches as the ideal formulations vary among different proteins.

Besides detergent mixtures, researchers have also explored other types of detergents or surfactants. A novel library of modular oligoglycerol detergents (OGDs) was developed for native MS(Urner et al., 2020a, 2020b). A typical OGD molecule consists of a hydrophobic tail, a hydrophilic headgroup, and a linker group, each of which can be chemically modified for further characterization such as protein purification, ligand binding, and charge reduction(Urner et al., 2018). OGDs are proven to be effective in purification and native MS characterization of membrane proteins like G-protein coupled receptors, and neurotensin receptor type I (NTSR1), for example. (Lee et al., 2016a, 2016b). Detergent analogs such as fluorinated surfactants (FSs) are also utilized in the solubilization of membrane proteins. FSs are not typically categorized as detergents as they do not partition well into lipid bilayers and lyse cells, although they form micelles just like detergents. The molecular structure of FSs is similar to classic detergents with fluorine atoms on the hydrophobic tails (Rodnin et al., 2008). Due to the poor miscibility of perfluorinated alkanes and regular alkanes, the interaction between the methyl group-covered transmembrane surfaces of membrane proteins and the perfluorinated chains on FSs is weak. To enhance this hydrophobic interaction, a hydrogenated tip is commonly grafted onto the perfluorinated tail on FSs, generating hemifluorinated surfactants (HFSs)(Park et al., 2007). Based on the current observation, FSs or HFSs are likely to form relatively huge polydisperse micelles with or without membrane proteins, and this seems to be under the regulation of the size of the polar group on the surfactants(Breyton et al., 2009). There has been no report on native MS characterization of membrane proteins solubilized with FSs or HFSs yet. We speculate that such trials have been made but high sample heterogeneity of the complex has hindered the further deconvolution of native MS spectra.

Despite the successful application of detergent micelles in preparing membrane proteins for native MS, detergents have the potential to disrupt and destabilize membrane proteins during the extraction process. In many cases, according to the latest evidence, the extracted membrane proteins are in a detergent-lipid-protein complex where the coexistence of lipids drastically alters their characteristics(Qiao et al., 2020; Qiao et al., 2021a; Qiao et al., 2021b; Zhu et al., 2023). The lipids bound to the membrane proteins are not merely contaminants. On the other hand, the removal of the lipids by detergents could induce protein destabilization and inactivation possibly due to the increased hydrophobic mismatch or decreased lateral pressure(Parton et al., 2011; van den Brink-van der Laan et al., 2004). Ensuring the preservation of the native, folded state of membrane proteins is of utmost importance in native mass spectrometry. Therefore, it is crucial to exercise caution when selecting detergents for sample extraction and stabilization. This inherent limitation has resulted in the endeavor to find alternative membrane mimetics that resemble natural lipid bilayers with higher fidelity.

2.2. Nanodiscs

The most popular detergent substitute widely used for membrane protein solubilization is nanodiscs, also known as lipid particles(Lee et al., 2016a, 2016b; Marty et al., 2013; Pettersen et al., 2023). Nanodiscs are biomembrane mimetics that mimic the natural lipid environment for membrane proteins(Rouck et al., 2017). They usually consist of a patch (130 to 160) of organized lipid molecules as a bilayer surrounded by membrane scaffolding proteins (MSPs). MSPs are originally derived from ApoA-1, the human high-density lipoprotein with genetic modifications(Brouillette et al., 1984). The length of MSPs is designed to be longer to increase the perimeter of the nanodisc(Bayburt and Sligar, 2002; Rouck et al., 2017). Typically, to assemble nanodiscs, detergent-solubilized membrane proteins, phospholipids, and MSPs must be incubated with a specifically controlled ratio(Bayburt and Sligar, 2010; Borch and Hamann, 2009). The advantages of nanodiscs include low sample heterogeneity, high sample stability, and constant protein oligomeric state(Denisov and Sligar, 2017). Due to these merits, nanodiscs have been successfully utilized in membrane protein characterization with native mass spectrometry(Marty et al., 2016). Since the incorporated nanodiscs are multicomponent complexes, other ingredients' characteristics have substantial impacts on the ionization process of native mass spectrometry (Fig. 2). Thus, parameters like nanodisc diameter, lipid composition, chemical additives, and instrumental tuning have to be carefully optimized to obtain resolvable native mass spectra(Keener et al., 2018). In this study, Keener et al. have successfully tuned the instrumental settings and applied chemical supercharging agents to directly measure the subunit architectures of model membranes proteins aquaporin Z (AqpZ) and ammonium transporter B (AmtB) on nanodiscs. The binding preference of AmtB toward palmitoyl-oleoyl-phosphatidylglycerol (POPG) over palmitoyl-oleoyl-phosphatidylcholine (POPC) was also observed upon controlled activation within the nanodiscs constituted with POPC/POPG (50/50 *v*/v) (Zhang et al., 2020). Despite their incontrovertible availability in the characterization of model membrane proteins, it is important to recognize that nanodiscs may not be universally suitable for all membrane proteins. One limitation of nanodiscs is their compatibility with unstable membrane proteins. Some membrane proteins exhibit inherent instability, even in mild detergent solutions. In such cases, the detergent-stripping step during the incorporation of membrane proteins into nanodiscs can lead to denaturation or destabilization of the proteins, hindering further characterization (Qiao et al., 2020). Another limitation of characterizing membrane proteins in nanodiscs with native mass spectrometry is the difficulty in the observation and deconvolution of complex ligand binding events. Since the assembled nanodiscs themselves contribute to a complex signal in the mass spectra, the specific interactions between membrane proteins and ligands are covered in the background noise, generating a signal hump that is unresolvable.

2.3. Amphipols

Detergents are commonly considered inactivating due to their delipidating properties, regardless of their efficacy in extracting membrane proteins(Lee et al., 2018). Consequently, there have been ongoing endeavors to explore and develop alternative substitutes that can solubilize and stabilize membrane proteins in aqueous buffers, aiming to overcome the limitations associated with traditional detergent-based methods (Fig. 2). For example, amphipols are amphipathic polymers that carry a lot of hydrophobic chains that have high binding affinity for the transmembrane surface of membrane proteins(Popot, 2010). Amphipols offer a clear advantage over detergents when it comes to stabilizing membrane proteins, particularly in terms of concentration independence. Detergents exist in an equilibrium between monomers and micelles, and if the concentration falls below the critical micelle concentration (CMC), detergent micelles can collapse, releasing membrane proteins into the surrounding aqueous solution. In contrast, amphipols tightly associate with membrane proteins through multiple contact points and do not form micelles. This unique characteristic prevents potential collapse when the system encounters unexpected fluctuation in concentration which is common in the structural characterization of membrane proteins(Le Bon et al., 2018). The strong association between membrane proteins and amphipols, which is beneficial for stabilization purposes, presents a drawback when it comes to characterizing membrane proteins using native mass spectrometry. The tight binding between amphipols and membrane proteins hinders the efficient release of proteins from these complexes, thereby impeding their ionization and restraining the deconvolution of native mass spectra, leaving alone further analysis(Keener et al., 2021a, 2021b). Besides, the occupation of amphipols on the transmembrane domain of membrane proteins could also interfere with the interaction between membrane proteins and their ligands or regulators(Laganowsky et al., 2014; Qiao et al., 2020).

2.4. Lipid vesicles

Lipid vesicles (small liposomes with endogenous lipids and membranes) are generated by sonication on the large membrane fragments obtained from the cell lysis after ultracentrifugation(Chorev et al., 2018; Chorev et al., 2020). The key advantage of this strategy is to stabilize membrane protein with the lipid molecules from native membranes, by which the natural membrane environment is better represented (Fig. 2). Besides, this method could characterize endogenous membrane proteins in their natural cell environment as it does not require protein overexpression or purification. Typically, higher activation energy is utilized for the ionization of the membrane protein samples, as well as the dissociation of liposomes and adducts(Chorev et al., 2018). As a consequence, the protein samples that could be characterized by this method should have high stability. Since the membrane proteins are directly incorporated in the native liposomes, the high sample heterogeneity could impede the mass assignment on the native mass spectra (Hirst et al., 2019). This seems to be a shared disadvantage with nanodiscs. However, with the development of native mass spectrometry instruments, lipid vesicles will be more available in the characterization of proteins that cannot be overexpressed or purified in vitro.

2.5. SMALPs

Unlike nanodiscs that rely on the initial extraction of membrane proteins by detergents, styrene-maleic acid-lipid particles (SMALPs) do not need any detergent participation(Overduin and Esmaili, 2019; Ravula et al., 2019). As an established method, the SMALPs solubilization starts with the generation of styrene maleic acid (SMA) *co*-polymer. Afterward, the SMALPs are formed by titrating a lipid suspension with SMA co-polymer until the cloudy solution becomes clear. To isolate membrane proteins from the expression hosts, *E. coli*, *Saccharomycetes cerevisiae*, and *Pichia pastoris* cells for example, the cell membrane suspension should be prepared as described in the detergent section. The membrane suspension is supplemented with SMA co-polymer until the cloudy solution becomes less opaque, and the mixture is incubated at room temperature until further purification. The peculiarity of the SMALPs method is the preservation of partial native membrane structure during the extraction of membrane proteins (Lee et al., 2016a, 2016b). This feature, however, hinders its application in native mass spectrometry since the assembled SMALPs are highly heterogeneous with countless unknown components in the complex(Keener et al., 2021a, 2021b), generating unresolvable signals. This method is similar to nanodiscs with the MSPs protein belt substituted by SMA co-polymer, but it appears that the dissociation of membrane protein from SMALPs is difficult to achieve by using conventional collision-induced dissociation (CID). Although a novel laser-induced liquid bead ion desorption (LILBID) was successfully employed to ionize SMALPs and evaluate the protein oligomeric state, the mass spectra signal of SMALPs was broad and unresolvable, restraining its application in measuring the ligand binding events with membrane proteins (Fig. 2)(Hellwig et al., 2018). SMALPs are potential substitutes for detergent in the extraction of membrane proteins. Attempts have been made to extract membrane protein with SMALPs and exchange the protein for detergent (DDM) or amphipols, but the MS spectra only offer broad peaks with low resolution(Hesketh et al., 2020). It is worth noticing that DDM is not a charge-reducing or delipidating detergent, membrane proteins extracted with DDM were observed as broad humps on MS spectra(Liu et al., 2019; Qiao et al., 2024a, 2024b; Qiao et al., 2021a). This means that the extraction efficiency of SMALPs has to be further characterized.

3. Membrane protein heterogeneity

Membrane protein solubilization is only the initial step for native mass spectrometry (MS) analysis. Once the membrane proteins are solubilized, further purification is necessary to obtain samples suitable for native MS characterization. Sample heterogeneity poses a significant challenge during membrane protein sample preparation. Heterogeneous protein samples often result in broad peaks in the mass spectrum, and the signals from different charge states can be too close together, preventing accurate determination of parameters such as protein oligomeric distribution, ligand binding interactions, and even thermodynamic properties (Qiao et al., 2020; Qiao et al., 2024a, 2024b; Qiao et al., 2021a). This is because native MS relies on preserving the native-like structural features and oligomeric states of membrane proteins during the transition from the solution phase to the gas phase. Heterogeneity in protein size, charge, and conformation can obscure the interpretation of native MS data, limiting the insights that can be gained about the functional and structural characteristics of these important biomolecules. The factors that contribute to the sample heterogeneity of membrane proteins include post-translational modifications, ligand binding interactions, and the degradation of membrane proteins. Developing effective strategies to enhance the proteoform homogeneity of membrane protein samples is crucial for enabling higher-resolution native MS analysis and efforts could be made in these aspects (Fig. 3).

3.1. Conformational stability

The conformational stability of membrane proteins plays a crucial role in the effectiveness of native mass spectrometry (nMS) characterization(Terral et al., 2016). Mass spectrometers are commonly fine-tuned to preserve the conformation of protein complexes(Laganowsky et al., 2013). Practically, stable membrane proteins are more likely to retain their native, folded structures during the ionization and transfer processes, leading to stronger and more reliable signals in nMS. Conversely, proteins with low conformational stability are prone to unfolding or aggregation, which can result in weak, noisy, or inconsistent spectra, making accurate characterization difficult. Maintaining the native conformation of these proteins is essential for reliable data

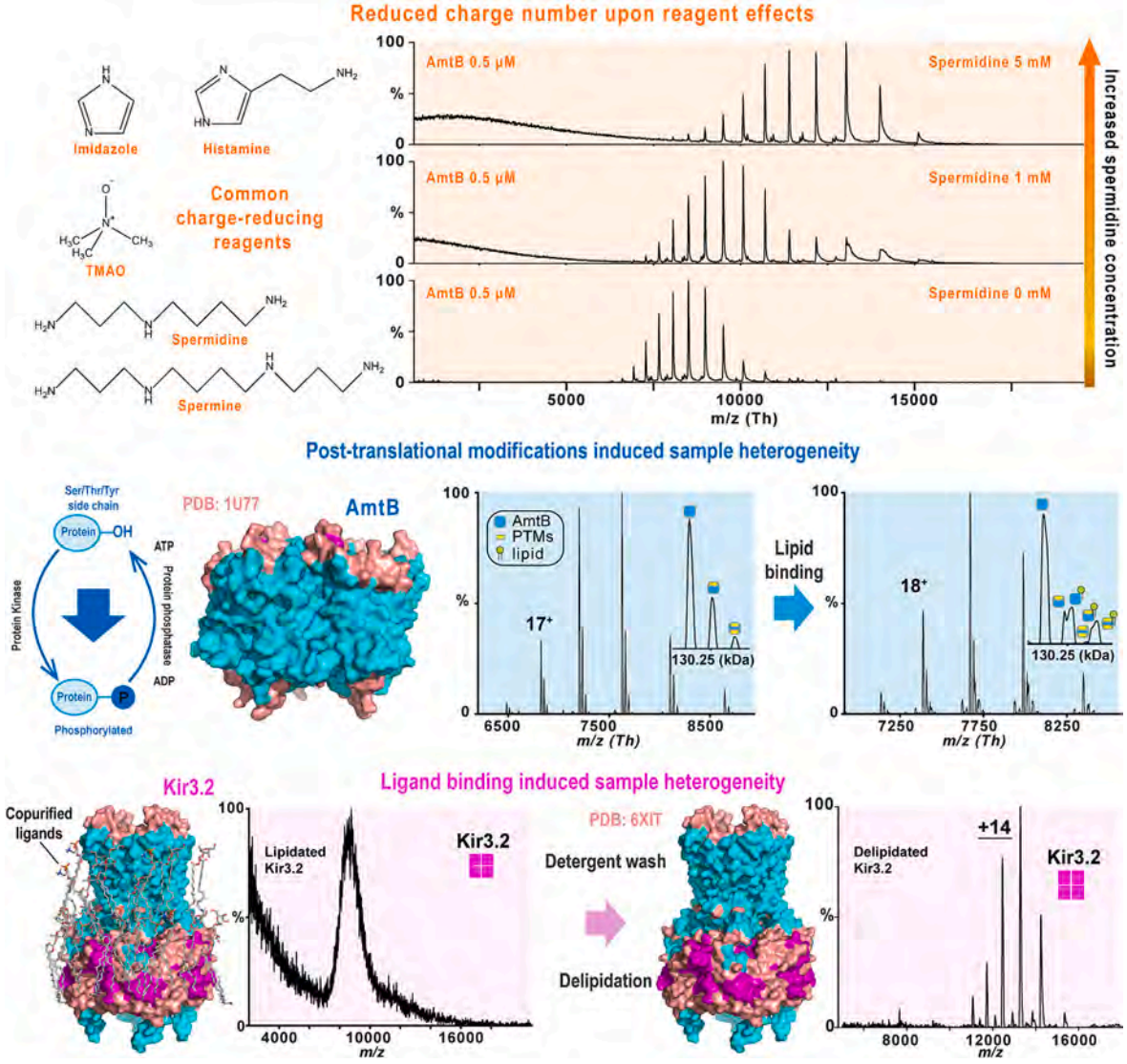

Fig. 3. Common factors that impact membrane protein sample heterogeneity. Native mass spectrometry spectra are adopted with permission (Lyu et al., 2022; Lyu et al., 2020; Qiao et al., 2021a; Zhu et al., 2023).

acquisition, as it ensures the preservation of critical structural and functional details(Lyu et al., 2020). Thus, the inherent stability of a membrane protein directly influences the quality and availability of nMS data, highlighting the importance of optimizing sample preparation and experimental conditions to support the native state of the protein throughout the analysis (Fig. 3).

Several strategies are employed to help preserve the native structure of membrane proteins during mass spectrometry (MS) analysis. The first and most widely used is optimizing buffer and solvent conditions. Volatile buffers like ammonium acetate are commonly used because they can be easily removed during ionization, minimizing the risk of protein denaturation(van Schaick et al., 2022). A promising method for preserving noncovalent interactions involves reducing the charge number of protein molecules. This reduction lowers their internal energy and minimizes Coulombic repulsion, thereby decreasing the likelihood of unfolding or activation(Hall et al., 2012; Townsend et al., 2019). The pivotal role of detergents in modulating the charge states of membrane proteins has been firmly established(Laganowsky et al., 2014). Notably, N-Dodecyl-β-D-maltopyranoside (DDM), a commonly employed detergent for membrane protein purification and solubilization, often yields high charge states that present challenges in minimizing activation energy, potentially leading to membrane proteins unfolding(Borysik et al., 2013; Qiao et al., 2020). However, the paradigm shifted with the advent of charge-reducing detergents such as tetraethylene glycol monooctyl ether (C_8E_4), pentaethylene glycol monodecyl ether ($C_{10}E_5$), n-dodecyl-*N*,*N*-dimethylamine-N-oxide (LDAO), and synthetic oligoglycerol detergents, marking a revolutionary advancement in native mass spectrometry (MS) methodologies(Laganowsky et al., 2014; Qiao et al., 2021a; Urner et al., 2020a, 2020b). These innovative detergents have enabled the characterization of compact, native-like complexes via ion mobility (IM) techniques, resulting in collision cross-section (CCS) values that correlate closely with those calculated from atomic coordinates(Lyu et al., 2020). Furthermore, they facilitate the preservation of native-like states in protein complexes while concurrently increasing the separation between bound adducts. This augmentation has proven advantageous in elucidating the various lipid-bound states of membrane proteins, thus enriching our understanding of their structural dynamics and functional implications(Patrick and Laganowsky, 2019).

The next strategy for charge state reduction is the addition of charge-reducing chemicals to protein samples during ionization, such as trimethylamine N-oxide (TMAO), a small colorless amine oxide generated from choline, betaine, and carnitine by gut microbial metabolism (Kaldmäe et al., 2019; Velasquez et al., 2016). Imidazole and its derivatives are also shown to marginally reduce the charges (by 2–3 charge

states) of membrane protein complexes (Townsend et al., 2019). The most effective charge-reducing chemicals for membrane proteins appeared to be histamine, spermidine, and spermine (Lyu et al., 2020). The impact of histamine, spermidine, and spermine on the charge states of membrane protein AmtB solubilized was compared specifically. Histamine, an amine-functionalized imidazole, previously studied for its involvement in the immune and nerve system, significantly lowered the average charge state compared to the control. Spermidine and spermine, both natural polyamines, also showed strong charge-reducing properties and resulted in narrower charge state distributions. Unlike TMAO, which produced a broader range of charge states, histamine, spermidine, and spermine maintained high-quality mass spectra with fewer charge states, indicating better preservation of the protein's native structure without significant adduction (Fig. 3).

Ion mobility spectrometry (IMS) allows the separation of ions based on shape and size, aiding in the identification of native-like conformations, and providing valuable structural information. Incorporating gas-phase fragmentation techniques, such as electron-capture dissociation (ECD) or electron-transfer dissociation (ETD), alongside traditional collision-induced dissociation (CID), can provide complementary structural insights while minimizing denaturation effects (Mihalca et al., 2004). Gentle ionization techniques, such as native electrospray ionization (nESI) and laser-induced liquid bead ion desorption (LILBID), reduce the likelihood of denaturation, particularly for fragile membrane protein complexes (Young et al., 2020). Optimizing collision energies minimizes fragmentation, maintaining the protein's native structure and facilitating the analysis of intact protein complexes (Hinneburg et al., 2016). By combining these strategies and leveraging advancements in MS technology, researchers can enhance the stability of membrane proteins during MS analysis, leading to more accurate and reliable data regarding their native structures, interactions, and functions, ultimately advancing our understanding of cellular processes at the molecular level.

3.2. Post-translational modifications

Post-translational modifications (PTMs) play a critical role in expanding the functional proteome and allowing proteins to dynamically respond to intracellular and extracellular cues (Ramazi and Zahiri, 2021; Virág et al., 2020). PTMs are covalent modifications that occur after a protein has been translated, involving the addition of moieties such as phosphate, acetyl, methyl, or ubiquitin groups (Shah et al., 2024). These modifications are catalyzed by specialized enzyme families and can regulate diverse properties including a protein's activity, stability, cellular localization, and interaction with other molecules. One of the most prevalent and well-studied PTMs is phosphorylation, which frequently acts as a molecular on/off switch that controls key processes like enzyme activation, protein complex assembly, and subcellular transport (Schastnaya et al., 2021). Other important classes of PTMs include glycosylation, which influences protein folding, stability, and targeting to specific membrane compartments, as well as lipid modifications that anchor proteins such as kinases and Raf to cell membranes (Esmail and Manolson, 2021; Lien et al., 2013). It is estimated that over half of eukaryotic proteins undergo some type of PTM, underscoring their widespread regulatory roles in cellular physiology (Schjoldager et al., 2020).

Post-translational modifications (PTMs) present a significant challenge for the analysis of protein complexes and quaternary structures by native mass spectrometry (Fig. 3). Different PTMs on a protein can bring mass heterogeneity that broadens peak widths in the mass spectrum. Even homogeneous PTMs shift peak centroids, necessitating their identification and modeling for proper assignment. For example, variable phosphorylation patterns could result in different charge state envelopes that overlap and interfere with the accurate deconvolution of oligomeric states. This could be observed on the membrane protein samples at high charge states. Even if the phosphorylation did not make the signal of protein at different charge states overlap with each other, the mass distribution raised by it would compromise the accuracy of the measurements made in the binding between membrane proteins and their potential ligands. For example, native mass spectrometry (nMS) and surface-induced dissociation (SID) were combined on a high-resolution Q Exactive UHMR spectrometer to show that phosphorylated aquaporin-0 (AQP0) protein monomers were only singly phosphorylated, instead of multiply phosphorylated (Harvey et al., 2022). It is worth noticing that this observation was made on the sample being subjected to strong SID impacts. Uniform PTMs on membrane proteins facilitate the native spectrum of AQP0 without the influence of SID manifested a big ratio of phosphorylation on the membrane protein, inhibiting potential further binding characterization (Harvey et al., 2022). Native mass spectrometry could faithfully reflect the interaction between membrane protein with its ligand when the protein is homogeneously modified. The G-protein coupled inwardly rectifying potassium Kir3.2 was successfully characterized by using native mass spectrometry, while the sample was found phosphorylated at multiple positions (Liu et al., 2019). Only signals of sharp single peaks were observed on the high-resolution mass spectrometer and detailed lipid-interaction patterns were successfully examined.

Protein glycosylation involves the addition of a variety of sugar moieties to proteins, forming glycans with heterogeneous compositions, linkages, and structures. Glycans play an extensive regulatory role through a wide range of biological functions such as modulation of receptor signaling, mediation of immune recognition, and facilitation of cell-cell communication (Wu and Robinson, 2022). As a result, glycosylation is also heterogeneous and increases protein mass inconsistently. This added layer of sample heterogeneity can obscure signals and limit resolution. Protein modifications must therefore be mapped and quantified to interpret native spectra. Advances in PTM-resolving capabilities are improving deconvolution and allowing native mass spectrometry to probe how PTMs like phosphorylation regulate multiprotein complex assembly and dynamics central to cellular regulation. Orthodoxly, the native mass spectrometry characterization on glycosylated membrane proteins focused on deciphering the glycan compositional heterogeneity, which was often used for antibody analysis (Rosati et al., 2012). Recombination of exoglycosidases and endoglycosidases was employed to determine the monosaccharide residues on a human tumor-necrosis-factor inhibitor (etanercept). The results manifested that the overall heterogeneity of etanercept was significantly reduced after treatment with single and non-interfering peaks observed on the native mass spectrum (Fig. 3) (Wohlschlager et al., 2018). Unlike antibodies or other soluble proteins, membrane proteins are mostly glycosylated on the extracellular domains that protrude from the cell surface (Chandler and Costello, 2016). These regions are accessible to luminal glycosyltransferase enzymes residing in the endoplasmic reticulum and Golgi apparatus through which nascent proteins traffic (Kellokumpu et al., 2016). In contrast, transmembrane domains embedded in the lipid core of the membrane are not typically glycosylated. However, occasional glycosylation sites may occur on recessed extracellular loops proximal to the membrane surface or within intracellular membrane compartments. Glycans and copurified glycolipids were recently discovered on a human V-type ATPase (membrane-embedded protein complex) by using high-resolution native mass spectrometry (Wang et al., 2020). While glycosylation on membrane proteins is generally less extensive than on soluble proteins, deglycosylation could potentially improve the quality of native mass spectrometry signals from membrane protein samples. However, glycans play important physiological roles in membrane protein folding, stability, assembly, and ligand recognition. Therefore, arbitrary, or non-specific removal of glycans risks unintended structural and functional consequences by altering the membrane protein's behavior. Further method development is still needed to better resolve the effects of heterogeneous glycosylation on membrane protein analysis by native mass spectrometry.

3.3. Ligand binding events

Another major source of sample heterogeneity in membrane protein preparations stems from ligand binding. Membrane proteins frequently interact with diverse binding partners like lipids, ions, and triacylglycerol in physiological settings. In practical applications, ligand binding contributions tend to introduce greater heterogeneity than PTMs when analyzing membrane protein samples by native mass spectrometry (Liu et al., 2019). Membrane proteins of interest are commonly expressed in heterologous systems such as *E. coli*, yeast, and insect cells. While these model organisms naturally involve fewer PTMs or artificial means are used to reduce PTMs relative to native mammalian systems, the biochemical environments still differ significantly. As a result, ligand interactions with the heterogeneously expressed membrane proteins are more variably affected. The lipid environment on the expression hosts could also impact the membrane protein sample heterogeneity that hinders the nMS characterization (Qiao et al., 2024a, 2024b). Thorough characterization and optimization of ligand binding states is therefore particularly important when working with membrane protein preparations from non-native host systems, to obtain higher quality native mass spectrometry data (Fig. 3).

Phospholipids are among the most prevalent adducts that membrane proteins can acquire during purification procedures. Given their abundance in biological membranes, phospholipids frequently co-purify with membrane proteins of interest. Two main experimental approaches can help remove co-purified lipids before native mass spectrometry analysis. The first involves fine-tuning mass spectrometer parameters to selectively ionize the protein over any weakly-bound lipids. The second approach (named delipidation) is to intentionally delipidate the sample during the purification process, using techniques like detergent exchange, chromatography, or treatment with lipases or organic solvents (Fig. 3).

Delipidation early in the workflow aims to strip away lipids that could otherwise contribute to sample heterogeneity and compromise mass spectrometry resolution. Together, selective instrumentation setup and optimized purification protocols aimed at reducing phospholipid carryover can improve the quality of native mass spectra for membrane protein studies (Lyu et al., 2020; Zhu et al., 2023). The detergent exchange has emerged as a popular method for membrane protein delipidation before native mass spectrometry. Techniques such as affinity chromatography or size-exclusion chromatography allow the facile exchange of membrane proteins from their initial solubilizing detergent into a second, non-ionic detergent. This promotes the dissociation of weakly bound lipids while maintaining protein stability. Compared to other delipidation approaches involving lipases, chaotropic, or organic solvents, detergent exchange is a relatively simple and mild method. It provides good protein yields without the risk of precipitation or denaturation. For these reasons, detergent exchange through affinity or size-exclusion columns has gained prominence as an effective delipidation strategy before high-resolution native MS analysis. The gentle steps preserve protein quaternary structure while removing phospholipid contaminants that could otherwise compromise mass spectrometry resolution. Take the human G protein-coupled inwardly rectifying potassium channel Kir3.2 that we have extensively studied as an example (Liu et al., 2019; Qiao et al., 2020; Qiao et al., 2021b). A membrane protein sample of heterologously expressed mammalian Kir3.2 that was purified from *Pichia pastoris* or insect cell Tini demonstrated the impact of co-purified lipids on native mass spectrometry. When analyzed directly in non-delipidating detergents via native MS, the spectra exhibited broad, unresolved peaks and smearing features (Fig. 3). However, after subjecting the sample to a delipidation procedure to remove co-purified lipid adducts, the quality of the native MS spectra improved dramatically. Individual protein complexes were resolved at different charge states, with peaks separated (Liu et al., 2019; Qiao et al., 2020). The delipidated Kir3.2 sample was then successfully applied in the characterization of the selective binding of Kir3.2 with phosphorylated phosphatidylinositol lipids (PIPs) (Fig. 3) (Qiao et al., 2020; Qiao et al., 2021b). This highlights how even weakly associated phospholipids can compromise native MS resolution and restrain the further study of membrane proteins if not stripped away before analysis. This delipidation effect on improving the native MS spectra is universal. It has been observed in many other membrane proteins, such as G protein-coupled inwardly rectifying potassium Kir3.4, ammonia channel protein AmtB, ATP-binding cassette transporter MsbA, and potassium channel of Streptomyces lividans (KcsA)(Cong et al., 2016; Liu et al., 2019; Lyu et al., 2022; Qiao et al., 2021a). One of the key benefits of delipidating membrane protein samples before native mass spectrometry is that it provides cleaner analytes. With delipidated samples, the native MS instrumentation can be tuned to more gentle conditions without needing to activate the ionization of the membrane protein solely for contaminant removal. Under milder source conditions, the observed protein-ligand interactions are likely to better represent the intrinsic binding dynamics under native physiological scenarios. By stripping away phospholipid adducts and other co-purifying components before analysis, delipidation allows native MS to probe membrane protein systems under instrument settings that maximize the preservation of quaternary structure and native-like conformations. This enhances the biological relevance of the collected interaction parameters. Overall, delipidation facilitates more faithful insights into membrane protein structure and ligand recognition by enabling native MS under gentler conditions optimized for preserving inherent solution behavior.

4. Membrane protein purification

For effective membrane protein characterization using native mass spectrometry (MS), careful purification of protein samples is essential to obtain high-resolution spectra. Protein purification encompasses a variety of methodologies, including protein precipitation, chromatography, electrophoresis, and membrane filtration (Hunte et al., 2003; Pandey et al., 2016). In practice, the preparation of membrane proteins for native MS primarily relies on various types of chromatography, as this step aims to preserve the proteins' folded state and eliminate impurities. Chromatography is particularly favored because it can be fine-tuned to maintain the delicate balance between protein stability and purity, which is crucial for native MS analysis. The strategies previously discussed, such as the use of charge-reducing agents and specific buffer compositions, are often integrated into the chromatography purification process for membrane proteins (Fig. 4). Chromatography techniques used for membrane protein purification can be broadly categorized into two groups: those employing fusion tags and those exploiting the inherent properties of the proteins.

4.1. Fusion tag-based purification

Fusion tag-based purification is also named affinity chromatography since it is based on the affinity binding between the resin and the fused tag on the protein (Pandey et al., 2016). This method involves the genetic fusion of a tag to the target protein, which can be a polyhistidine tag (His-tag), a FLAG tag, or a maltose-binding protein tag. These tags facilitate the purification process by providing a specific binding site for affinity chromatography. For instance, His-tagged proteins can be purified using immobilized metal affinity chromatography (IMAC), which binds the histidine residues. The primary advantage of this method is its high specificity and efficiency in isolating the target protein from complex mixtures. The affinity tags commonly used in the purification of membrane proteins are polyhistidine (HisTag), glutathione-S-transferase (GST), maltose-binding protein (MBP), streptavidin binding peptide (Strep-Tag, StII), FLAG octapeptide (Flag-Tag), (Kimple et al., 2013). The general purification principle and the molecular basics of these affinity tags have been extensively utilized and elaborately described in countless research articles and reviews, the interested readers could turn to these great works (Hartmann et al., 2017; Pandey

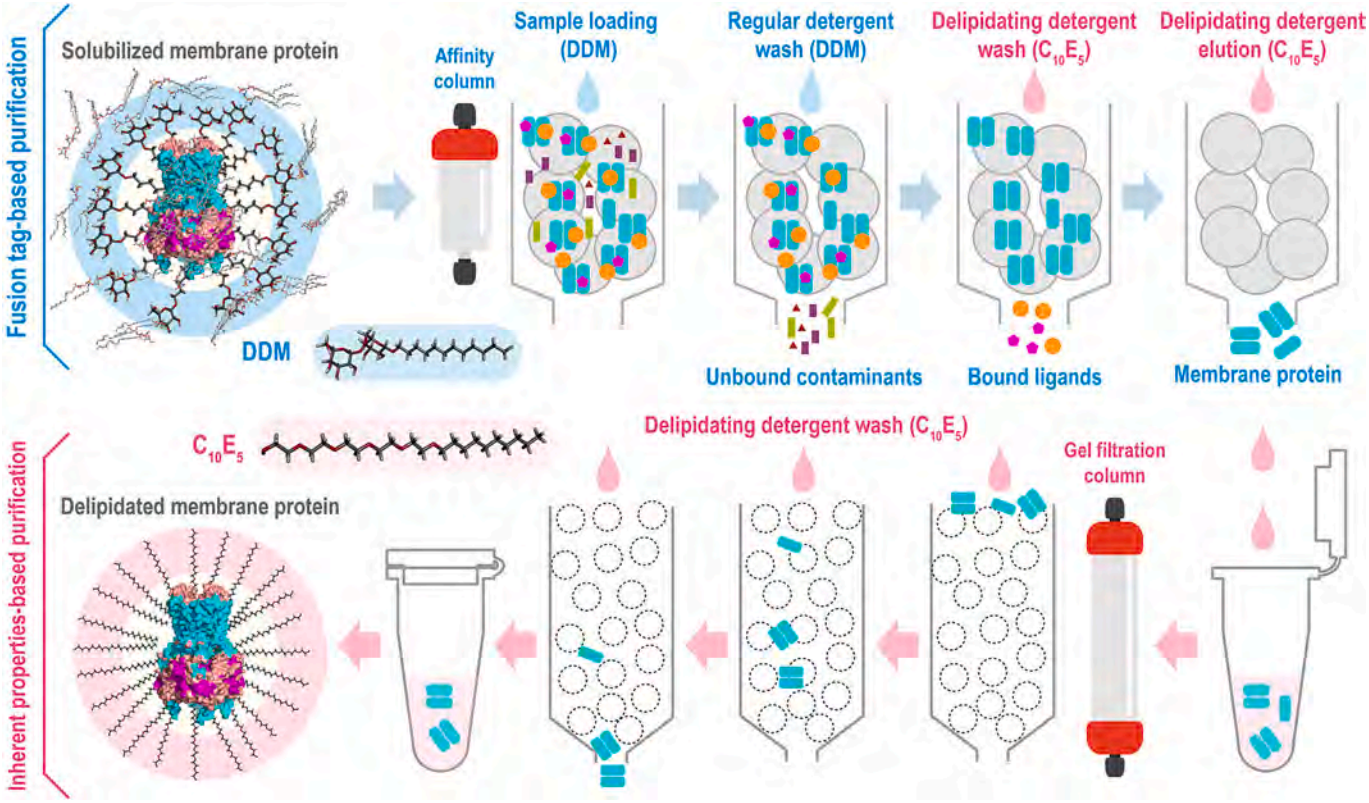

Fig. 4. Standard purification workflow of a membrane protein for native MS characterization.

et al., 2016; Pettersen et al., 2023; Urner et al., 2020a, 2020b). The present review will be focused on the unique procedures for membrane protein preparation specifically for native mass spectrometry (Fig. 4).

As elaborated above, sample heterogeneity significantly affects whether the signal of a membrane protein can be deconvoluted from the MS spectrum. High-resolution native mass spectra, essential for determining the exact molecular weight of a membrane protein and its binding kinetics with ligands, can be obtained if the membrane protein sample has low heterogeneity. Affinity chromatography is widely used to reduce the heterogeneity of membrane proteins. Affinity chromatography leverages the specific binding interactions between a target protein and an immobilized ligand on the chromatography resin. This method is particularly effective for membrane proteins, which often require meticulous handling to maintain their functional and structural integrity. By using affinity tags, such as polyhistidine, FLAG, or maltose-binding protein, researchers can selectively isolate the target membrane proteins from complex mixtures. This selective binding not only purifies the protein but also significantly reduces sample heterogeneity by removing contaminants and non-specifically bound proteins. For instance, membrane proteins such as AmtB, Kir3.2, Kir3.4, and MsbA have been bound to affinity resins and treated with delipidating detergents to remove adducts (Lyu et al., 2022; Lyu et al., 2020; Qiao et al., 2020; Qiao et al., 2021a; Qiao et al., 2021b; Zhu et al., 2023). Delipidating detergents are crucial in this context as they help to strip away extraneous lipid molecules that can form adducts, which contribute to sample heterogeneity. Removing these adducts is vital for achieving a uniform sample, as it minimizes the variation in mass and charge states that can complicate mass spectrometric analysis. Moreover, the use of affinity chromatography can be combined with other purification steps to further enhance sample homogeneity. For example, after initial affinity purification, size exclusion chromatography (SEC) can be employed to separate protein complexes based on their size, ensuring that only the correctly assembled protein complexes are analyzed. This two-step purification process ensures higher purity and homogeneity, which is crucial for obtaining high-resolution MS data.

4.2. *Inherent properties-based purification*

This approach leverages the unique characteristics of membrane proteins, such as hydrophobicity, charge, size, or specific binding affinities, using techniques like ion exchange chromatography (IEC), size exclusion chromatography (SEC), hydrophobic interaction chromatography (HIC), and reversed-phase high-performance liquid chromatography (RP-HPLC). Ion exchange chromatography separates proteins based on their net charge, with positively charged proteins binding to negatively charged stationary phases and vice versa. Size exclusion chromatography, on the other hand, separates proteins based on their size, allowing smaller molecules to penetrate the stationary phase's pores and emerge later than larger molecules (Fig. 4). Hydrophobic interaction chromatography exploits the hydrophobic regions of membrane proteins, with interactions between hydrophobic patches on the protein and hydrophobic ligands on the stationary phase facilitating protein separation. Reverse-phase high-performance liquid chromatography (RP-HPLC) separates proteins based on their hydrophobicity, making it particularly useful for purifying small membrane proteins that are stable at exposure to organic solvent (Langelaan et al., 2013).

However, RP-HPLC is less suitable for native MS analysis due to its tendency to strip detergent micelles during elution, leading to protein denaturation in the aqueous solution. Consequently, proteins purified using RP-HPLC often exhibit broad and discontinuous elution profiles, indicative of protein misfolding or conformational differences (Pandey et al., 2016). This renders proteins unsuitable for native MS analysis, as they are not in their native state. To improve sample quality, sample heterogeneity-removal reagents are often applied during chromatography purification steps. These reagents help to remove contaminants and non-specifically bound proteins, enhancing the purity of the protein sample. However, direct exposure of membrane proteins to these reagents, such as delipidating detergents, can disrupt protein structure and

increase the unfolding rate (Fig. 4). Therefore, while these methods are effective for separating denatured protein samples after treatment to reduce sample heterogeneity, they are not ideal for preparing samples for native MS analysis. Instead, alternative purification strategies that preserve the native state of membrane proteins, such as affinity chromatography and gentle elution conditions, are preferred for native MS analysis.

5. Future perspectives and potential solutions

5.1. Improvements in detergent systems and lipid-based approaches

Recent advancements in detergent systems and lipid-based approaches have significantly contributed to the field of membrane protein characterization using native MS. Detergents play a crucial role in solubilizing membrane proteins while maintaining their native structure and function. Traditional detergents like DDM have been widely used, but they can sometimes lead to protein denaturation or aggregation. Newer detergent systems, such as fluorinated surfactants or amphipols, offer improved stability and compatibility with MS analysis. These detergents form smaller micelles, providing better coverage and stabilization of membrane proteins during purification and MS analysis.

Lipid-based approaches, such as the use of nanodiscs or lipid nanodisks, offer an alternative method for stabilizing membrane proteins. Nanodiscs consist of a lipid bilayer surrounded by a belt of membrane scaffold proteins, providing a more native-like lipid environment for membrane proteins. This approach has been shown to improve the stability and activity of membrane proteins in solution, making them suitable for native MS analysis. Additionally, lipid-based approaches allow for the incorporation of specific lipids or lipid mixtures, mimicking the lipid composition of biological membranes and providing insights into the role of lipids in protein structure and function. Overall, improvements in detergent systems and lipid-based approaches have expanded the toolkit available for membrane protein characterization using native MS, enabling more accurate and comprehensive analysis of membrane protein complexes and their interactions.

5.2. Emerging sample preparation methodologies

In addition to traditional sample preparation methods, several emerging methodologies are being developed to address the challenges associated with membrane protein characterization using native MS. One such approach involves the use of microfluidic devices for sample preparation and analysis (Shao et al., 2023; Su et al., 2023). Microfluidic systems offer precise control over sample handling and manipulation, allowing for the integration of multiple sample preparation steps in a single device (Ha et al., 2021). This enables rapid and efficient sample processing, reducing sample loss and improving overall sensitivity. Another emerging trend is the development of label-free sample preparation methods that minimize sample handling and reduce the risk of sample contamination. These methods rely on the intrinsic properties of membrane proteins, such as their charge or hydrophobicity, for purification and analysis. For example, charge-based purification methods, such as isoelectric focusing or electrostatic repulsion chromatography, selectively isolate membrane proteins based on their charge, eliminating the need for exogenous labels or tags (Kwok et al., 2023; Shen et al., 2021).

Furthermore, advancements in mass spectrometry instrumentation, such as the development of high-resolution mass analyzers and ion mobility spectrometry, are enabling more detailed and accurate analysis of membrane protein complexes (Christofi and Barran, 2023; Deschamps et al., 2023). These technologies offer improved resolution and sensitivity, allowing for the detection of subtle structural changes and dynamic interactions within membrane protein complexes. In summary, emerging sample preparation methodologies, coupled with advancements in mass spectrometry instrumentation, are driving innovation in the field of membrane protein characterization using native MS. These developments hold great promise for advancing our understanding of membrane protein structure, function, and dynamics in biological systems.

6. Conclusion

Native mass spectrometry (nMS) has emerged as a powerful tool for studying the structure and interactions of membrane proteins in the native-like states. However, sample preparation for such analyses poses inherent challenges that should be addressed. The key hurdles include maintaining native conformation, minimizing sample heterogeneity, and preserving protein-ligand interactions. Membrane proteins are characterized by their hydrophobic motifs, as they are embedded within lipid bilayers in their native cellular environments. Solubilizing and maintaining the structure integrity of MPs during sample preparation is the critical first step for nMS characterization. Existing strategies for MP solubilization and stabilization include the use of detergent micelles, nanodiscs, amphipols, lipid vesicles, and styrene-maleic acid-lipid particles. After the initial step, future purification is often required to stabilize conformations, reduce post-translational modifications, and minimize ligand binding events. It is worth noting that the presence of bound lipids from expression, residual detergents after extraction, or other contaminants during purification can severely interfere with MS analysis, further complicating sample preparation. These co-purified species can suppress ionization, mask signals, and introduce heterogeneity, ultimately compromising the quality and reliability of the nMS data.

To address these challenges, researchers are developing innovative MP sample preparation strategies. Advanced purification techniques, such as affinity chromatography, can help stabilize membrane proteins and minimize sample heterogeneity. Additionally, the exploration of label-free methods that leverage intrinsic protein properties, like charge or hydrophobicity, may streamline sample processing and reduce contamination risk. Furthermore, advancements in mass spectrometry instrumentation, including high-resolution mass analyzers and ion mobility spectrometry, offer new opportunities to improve the sensitivity, resolution, and accuracy of nMS measurements. These technological developments, coupled with innovative sample preparation approaches, are crucial for advancing our understanding of membrane protein structure, function, and dynamics in biological systems. Overall, optimizing sample preparation is a critical step for ensuring the reliability and quality of native mass spectrometry data on membrane proteins. Continued research and innovation in this area will be essential for unlocking the full potential of nMS as a tool for membrane protein characterization.

Disclosure statement

All the authors have agreed to the publication of this manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

This review does not contain unpublished data.

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