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Chemical Tools for Membrane Protein Structural Biology

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Abstract

Solving high-resolution structures of membrane proteins has been an important challenge for decades, still lagging far behind that of soluble proteins even with the recent remarkable technological advances in X-ray crystallography and electron microscopy. Central to this challenge is the necessity to isolate and solubilize membrane proteins in a stable, natively folded and functional state, a process influenced by not only the proteins but also their surrounding chemical environment. This review highlights recent community efforts in the development and characterization of novel membrane agents and ligand tools to stabilize individual proteins and protein complexes, which together have accelerated progress in membrane protein structural biology.

Introduction

Much has been written about the critical biological and biomedical significance of membrane proteins (MPs). Structural knowledge is crucial for understanding the underlying biological function and mechanism, as well as for structure-based drug design. However, membrane protein structural biology lags far behind that of soluble proteins [1–3]. Currently, X-ray crystallography, electron cryomicroscopy (cryoEM), and nuclear magnetic resonance spectroscopy are the major biophysical techniques for solving high-resolution structures, with each method having its own advantages and limitations [4–6]. Regardless of the technique, sample preparation is the most significant challenge for MP structure determination. Important tasks throughout this process are engineering of protein constructs and selection of the best expression platform. Stabilization of individual MPs or their complexes in a solubilized state is essential, requiring optimization of the chemical environment, including lipids, detergents, membrane mimetics and ligands [7,8]. The development of novel chemical and protein-centric tools together and significant

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Conflict of Interest Statement

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technological advancements in cryoEM and X-ray crystallography have accelerated progress in MP structural biology. This review describes chemical tools, including both membrane reagents and ligands (Figure 1), highlights recent achievements and discusses unmet challenges, with a perspective for more innovative tool development to impact both MP structural biology and drug discovery.

Detergents for MP Stabilization

MPs reside in cells in an anisotropic and heterogeneous membrane environment, where the lipid bilayer imposes geometric constraints on their structure and thermodynamic stability (Figure 1a). Specific lipid interactions are crucial for the function of many MPs [9,10]. Ideally, MP structures should be determined in their native biological membranes, where the proteins of interest are stable and fully functional, retaining their endogenous lipids, ligands and protein partners. However, very few MPs, such as bacteriorhodopsin [11], vertebrate rhodopsin [12], the acetylcholine receptor [13], and the sarcoplasmic reticulum Ca^{2+}-ATPase [14], are sufficiently abundant in their native membranes for direct electron crystallography, typically at low to subnanometer resolution. Most MPs are present in low copy numbers within cell membranes, necessitating purification and enrichment from either natural sources or overexpression using recombinant methods. A common practice is to extract and solubilize MPs using small-molecule detergents that can partition into and effectively disperse the membrane. Detergent micelles provide an artificial and less than ideal membrane-mimetic environment, in which MPs are prone to denaturation, aggregation and loss of activity. For structural studies, use of detergents may cause additional problems, such as the difficulty in crystallization of MPs with small soluble ectodomains that mediate protein-protein interactions in 3D crystals. A common vexation is the growth of low-quality MP crystals that are difficult to improve [15]. A challenge of cryoEM is the exceedingly low signal-to-noise ratio between the protein and the surrounding vitreous ice. Empty detergent micelles contribute to the background and interfere with single-particle image analysis, especially for MPs of similar particle size to the micelles. Nevertheless, despite all the challenges associated with their use, detergents were the first and remain the most versatile and invaluable tools for MP sample preparation. For both crystallography and cryoEM, a large fraction of MP structures are still determined in detergent micelles. Most other types of membrane mimetics (e.g. amphipols, nanodiscs, lipidic cubic phase (LCP)), as will be discussed in this review, do not effectively disperse cell membranes, and their use requires detergent solubilization and purification of MPs prior to exchange into the mimetic. A brief comment on the advantages and limitations of various membrane-mimicking reagents is included in Table 1.

Many new, less harsh detergents have been developed in recent years with improved properties, which enhance the stability of MPs for structural studies (Figure 1b). Of particular interest among these are molecules with improved properties as membrane mimetics, which include lipid-like branched or double alkyl chain detergents [15–19], as well as cholesterol-like, steroid-based detergents [20–24]. Although structurally distinct from each other, these detergents share common features, including enhanced hydrophobicity and compactness, allowing tighter association with MP surfaces than conventional single-chain detergents. New detergent designs that include an even larger

hydrophobic surface or contain more than two alkyl chains further increase MP stability [25–27]. Related to the development of various steroid-based detergents, many MPs can be stabilized by a combination of cholesteryl hemisuccinate (CHS) with common detergents such as dodecyl-β-D-maltoside (DDM) or lauryl maltose neopentyl glycol (LMNG). CHS may impart this important benefit by mimicking direct interactions of cholesterol with some eukaryotic MPs [28]. Structurally, the incorporation of CHS into DDM micelles induces flattened bicelle-like structures that better mimic a membrane setting [29].

It is also worthwhile to note recent successful cryoEM MP structures using digitonin or similar molecules (glyco-diosgenin/GDN [23]). Digitonin has had a long history of use in membrane biochemistry, but to the best of our knowledge, it has yet to succeed in supporting crystallization of MPs. The reason for its lack of success in crystallography might be attributed to its mixed (and variable) chemical composition, its complex phase properties, or the large size of micelles that it forms. Digitonin is, nevertheless, among the best detergents for stabilization of challenging eukaryotic MPs or MP complexes, as it has been used in the structure determination of some notoriously difficult targets, such as γ-secretase [30], the cystic fibrosis transmembrane conductance regulator (CFTR) [31], and the multidrug resistance protein 1 (MRP1) [32]. Digitonin has also been demonstrated to retain phospholipids during the solubilization of the transporter associated with antigen processing (TAP) [33]. The unique steroid moiety of digitonin along with extreme structural rigidity may confer its particularly mild properties compared to other detergent types. Given the known limitations of digitonin for crystallographic use and the scarcity of digitonin analogs, it is worthwhile to expand this promising class of detergents by synthesizing new digitonin-like molecules with improved chemical and physical properties. To this end, DGN, a synthetic digitonin, has now been commercialized (Anatrace).

Detergent-free Solubilization of MPs

Dramatic advances made in cryoEM methodology over the last few years have greatly expanded the utility of non-detergent membrane-mimetic systems for high-resolution MP structural studies. Prominent examples for single-particle cryoEM are phospholipid bilayer nanodiscs [34] and amphipols [35] (Figures 1c and 1d), but they have thus far eluded applications in MP crystallization. In contrast to traditional detergent molecules, amphipols are amphiphilic polymers decorated with multiple alkyl chains that can wrap around the hydrophobic surfaces of MPs to form a less dynamic protein-polymer assembly (Figure 1d). Nanodiscs are usually prepared by mixing detergent-solubilized MPs, lipids, and membrane scaffold proteins (MSPs) at controlled ratios, followed by removal of detergent. Individual MPs or MP complexes become embedded in the resulting lipid bilayer discs, which are encircled by the MSP “belt” of defined size (Figure 1c). MSPs were originally designed based on the differently truncated sequences of high-density apolipoproteins [36–38]. More recent variations include covalently circularized MSPs, designed to achieve more precise control over the nanodisc size [39], and an alternative scaffold-protein, called saposin [40]. The various nanodisc systems offer a wide range of disc diameters (from 6 nm up to 80 nm), capable of accommodating small to large MPs or MP complexes at different stoichiometries, for structural and functional studies. We refer readers to more extensive reviews on the applications of protein-based nanodiscs [34,41].

Unlike MSPs and aforementioned amphipols, styrene-maleic acid (SMA) copolymers have been recently shown to have the ability to solubilize MPs directly from cell membranes [42,43]. The membrane solubilization efficacy of SMA polymers, which randomly display hydrophobic styrene and hydrophilic maleic acid moieties along their linear hydrocarbon chains, varies with the polymer length, styrene/maleic anhydride ratio, and pH [44]. An important feature of direct solubilization with SMAs is its co-solubilization of endogenous lipids together with MPs into nanosized lipid particles termed SMALPs (Figure 1c). Along with bypassing the use of conventional detergents, this is an ideal strategy to capture MPs in a nearly native environment for subsequent purification and structure determination. But at present, the solubilization efficacy and stabilizing benefits of SMAs remain less well established than many other widely adopted chemical tools for MP structural and functional studies. In some cases, SMAs can be difficult to utilize. For instance, UV absorbance of the styrene moieties in the polymer presents an inconvenience. Chelation of divalent cations (e.g. Ni^{2+}, Ca^{2+} and Mg^{2+}), owing to the density of carboxylate anions, constrains the use of SMAs for some chromatographic purifications (e.g. immobilized metal affinity chromatography) and for certain functional assays (e.g. ATPases). As such, various structural modifications in both polar and apolar segments of the polymer have been made in order to improve the properties of these polymers [45–47].

SMALP nanoparticles appear to have distinct properties compared to MSP nanodiscs. In MSP nanodiscs the lipid movement is largely confined within individual discs, providing a relatively static, stable lipid environment. In contrast, fast lipid transfer (occurring within seconds) has been observed between SMALP particles [48]. In this way SMALP nanoparticles are a dynamic system similar to detergent micelles. Direct crystallization of SMA-solubilized MPs will likely be challenging, although bacteriorhodopsin crystals have been grown in LCP after detergent-free SMA solubilization and purification [49]. In a recent marked success, SMA-solubilized nanoparticles yielded a 3.4-Å resolution cryoEM structure of the *Flavobacterium johnsoniae* alternative complex III (ACIII), together with a structure of its supercomplex with an aa_3-type cytochrome c oxidase [50]. The cryoEM map revealed a surprisingly thin layer of density contributed by SMA and lipids, following the contours of the protein. The structural flexibility of SMALPs was also manifested in another recent cryoEM structure of the bacterial multidrug exporter AcrB, where a putative thin layer of lipid was described that followed the contours of the protein [51].

Membrane-Mimetic Mesophases for Crystallization

MPs can readily crystallize directly from lipid bilayers, provided that the bilayers are interconnected to form a 3D network, such as the arrangement that exists in LCP or in mixed lipid-detergent, perforated lamellar phases, often referred to as bicelles. Since the high-resolution structure determination of bacteriorhodopsin in 1996 [52], LCP has become one of the most successful membrane-mimetic matrices for stabilization and crystallization of MPs. MPs have also been crystallized in bicelle systems [53–55], but their popularity appears to be waning.

LCP spontaneously self-assemble upon mixing of specific lipids with an aqueous buffer to create a periodic structure with cubic symmetry. Topologically, LCP consists of a single

lipid bilayer dividing the space into two networks of interwoven water channels (Figure 1e). The most commonly used and least expensive host lipid for LCP crystallization is monoolein, a monoacylglycerol with a double bond in the middle of its 18-hydrocarbon chain (9.9 MAG) [56]. An efficient synthesis of variable chain MAGs has been described [57,58]. In particular, the shorter chain MAGs that support larger diameter solvent channels were essential for crystallization of several challenging MPs, including GPCRs bound to their heterotrimeric G protein signaling partners [59,60], a proton-translocating transhydrogenase enzyme [61], and other MPs [62,63]. As an alternative to MAGs, a series of isoprenoid-chain lipids have been developed, and one of them (β-XylOC$_{16+4}$) supports MP crystallization in LCP [64].

Native lipids of biological membranes do not spontaneously produce LCP. However, certain types of phospholipids, cholesterol, and other natural lipids can be doped into an LCP mixture to tune such properties as the membrane thickness and curvature, or to provide specific lipid-protein interactions. Despite the wide adoption of the LCP crystallization method, the number of available host lipids and their properties remain limited. Among recent developments to expand the LCP host lipid repertoire, the double bond of monoolein was replaced with a cyclopropyl group (monodihydrosterculin, MDS), extending the temperature range of LCP to enable low-temperature crystallization [65]. The relatively low chemical stability of MAGs was recently addressed by the development of non-hydrolysable lipids (e.g. GlyNCOC$_{15+4}$), which also support MP crystallization at 20 °C and 4 °C [66].

Custom Ligands for Stabilization of MPs

In addition to engineering a membrane-like environment, selecting or designing tightly bound ligands can provide synergistic MP stabilization (Figure 1f). The early structural studies of bacteriorhodopsin and rhodopsin took advantage of the stability conferred by the covalently-bound retinal ligand. Similarly, many recent successful structural studies of GPCRs have benefited from wide-ranging medicinal chemistry efforts that produced a wealth of high-affinity antagonists and agonists. These ligands substantially enhance MP thermal stability and conformational homogeneity [67]. To overcome generally low affinity or a brief residence time of natural or synthetic agonists, covalent agonists have also been designed to trap receptors in active conformations [68,69]. The design and synthesis of stabilizing antagonists also contributed to the successful crystallization of full-length smoothened [70] and cannabinoid receptor 1 [71]. Nevertheless, there is a pressing need to generate ligand tools for many other GPCRs that have thus far defied high-resolution structural determination, such as the largest subfamily of ~400 olfactory receptors.

Unlike the rich pharmacology of GPCRs, there is a general lack of high-affinity ligands for transporters and channels. Conformational heterogeneity of transporters poses a significant challenge for their high-resolution structure determination, and obtaining ligand-bound structures will be especially important to define ligand binding sites in the context of a dynamic conformational pathway for substrate transport. For instance, several crystal and cryoEM structures of the multidrug resistance P-glycoprotein (ABCB1) were determined at 3.4–3.8 Å resolution in complexes with several ligands bound within a V-shaped transmembrane cavity [72–74]. These ligands have only moderate affinities (> 200 nM) and

do not appear to stabilize P-glycoprotein in a single conformation. As such, development of higher affinity ligands may lead to higher-resolution structures of this multidrug transporter. To this end, a 2.9 Å-resolution crystal structure of the bacterial P-glycoprotein homolog MsbA was recently solved in a complex with both the lipopolysaccharide substrate and a potent inhibitor, which was discovered in a screen of ~ 3 million compounds [75]. In this case, the use of a novel facial amphiphile [21] also contributed to stabilization of MsbA.

Assaying a Large Chemical Space

It can be a daunting task to screen a vast number of chemical variables including membrane mimetics, ligands, as well as other buffer conditions and additives to identify a stabilizing matrix for structural studies of a given MP. Various stability assays, such as those based on protein activity, thermal stability, aggregation, or chemical denaturation, have been frequently employed. Among these, several fluorescence-based thermal stability assays, including, for example, fluorescent protein-based size-exclusion chromatography (FSEC) [76,77] and a protein unfolding assay using a cysteine-reactive fluorescent dye (CPM) [78] are appealing because of their high sensitivity and easy adaptability to different MPs. The use of only nanogram to microgram protein samples in these assays allows screening of many conditions typically in a medium-to-high throughput format. Despite these advances, it is still imperative for the community to develop MP-specific sparse matrix type high-throughput screens, such as those recently reported for studies of soluble proteins [79]. Complications involving a membrane matrix in MP assays limit the application of many fluorescent dye-based stability assays because of the high fluorescence background that is amplified in a hydrophobic environment. The advantage of using a CPM dye that becomes highly fluorescent upon conjugation with free cysteines partly addresses this issue. On the other hand, the application of CPM assays may require careful engineering of free cysteines embedded in the protein core, which could be especially challenging for multidomain proteins and complexes. Binding of CPM to cysteines in MP ectodomains may also complicate the interpretation. Lastly, non-specific CPM reactivity limits its application for broad screening of chemical libraries, as well as some buffer and high pH conditions. In this regard, miniaturized label-free differential scanning fluorimetry that measures the changes of intrinsic protein fluorescence represents a promising and relatively new development for thermal stability assays of MPs [80].

Conclusions and Future Perspectives

Preparing high-quality samples amenable to high-resolution structural studies continues to be a major challenge in MP structural biology. The fundamental issue is stabilization of MPs while faithfully maintaining their native activity through solubilization, purification and structural studies. Over the last two decades, many innovative reagents have been developed that solubilize and stabilize MPs in a more membrane-like environment. However, despite the exciting progress, the selection of the most efficient combinations of such chemical tools for a new MP target remains largely a process of trial-and-error.

Direct solubilization of MPs into a nearly native environment, as embodied in the concept of amphipols such as SMAs, can make the handling of MPs more convenient and economical,

which would significantly impact a broad range of MP research beyond structural applications. In addition, access to large libraries of chemical or biological ligands, with high affinities and high propensity for thermostabilization would have an enormous impact on structural studies of MPs. New developments in synthetic chemistry could be applied to the discovery of such ligands. For example, the rapidly evolving DNA-encoded library (DEL) synthesis has enabled the generation of an unprecedented number of compounds (> 10^8), which can be screened efficiently [81]. In a recent example, the DEL strategy was used to identify the first allosteric antagonist of the β_2-adrenergic receptor, which was subsequently co-crystallized with the receptor [82,83]. Covalent *in situ* attachment of a ligand, such as by biorthogonal SuFEx chemistry [84,85], may be attempted to stabilize the numerous MP targets that currently lack high-affinity binders. Looking forward, we envision exciting opportunities for synthetic chemists to play an important role in advancing MP structural biology and pharmacology.

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•• of outstanding interest

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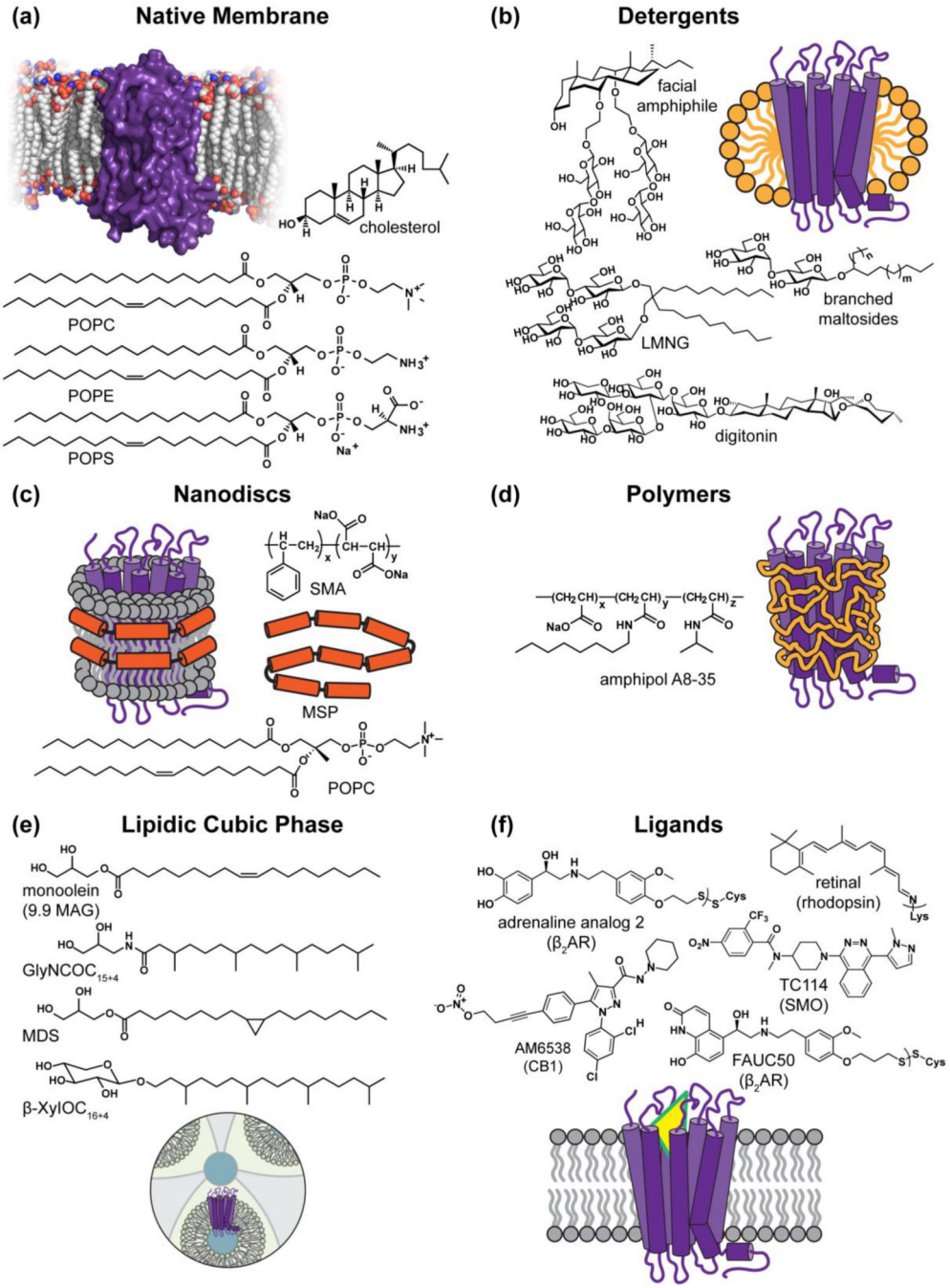

Figure 1.
Chemical tools for MP stabilization outside of their native membrane environment.

Table 1.

Advantages and limitations of different chemical tools for MP structural studies.

	Advantages	**Limitations**
Detergents	- Highly versatile	- Often too harsh - Less than ideal membrane mimic - High background in cryoEM - Poor crystal quality for MPs with small soluble domains
Polymers	- Gentler alternative to detergents	- Most do not directly extract MPs
MSPs	- Used for encompassing nanodiscs - Most native-like membrane mimic - Great for cryoEM	- Do not directly extract MPs - Not suitable for crystallography
Lipid Mesophases	- Membrane-like environment - Support crystallization	- Not suitable for cryoEM - Limited range of host lipids
Ligands	- Provide synergistic stabilization - Can be covalently attached	- Not available for many MPs