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New amphiphiles for membrane protein structural biology

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Abstract

A challenging requirement for X-ray crystallography or NMR structure determination of membrane proteins (MPs), in contrast to soluble proteins, is the necessary use of amphiphiles to mimic the hydrophobic environment of membranes. A number of new detergents, lipids and non-detergent-like amphiphiles have been developed that stabilize MPs, and these have contributed to increased success in MP structural determinations in recent years. Despite some successes, currently available reagents are inadequate and there remains a pressing need for new amphiphiles. Literature examples and some new developments are selected here as a framework for discussing desirable properties of new amphiphiles for MP structural biology.

Keywords

detergents; lipids; membrane proteins; structure determination

1. Introduction

For decades, the structural determination of membrane proteins (MPs) has been an important challenge, one which has only recently been addressed with increasing success, primarily through X-ray crystallography [1]. The hydrophobic environment that MPs occupy necessitates the use of detergents for their extraction, solubilization and purification, a fundamentally different approach from the preparation of soluble protein samples. Conventional detergents resemble the overall phospholipid structure with polar groups at one end (head) and apolar hydrophobic chains at the other end (tail). Whereas cellular lipids assemble into planar bilayers, detergents form micelles of spherical or other shapes. These micelles solubilize MPs by surrounding the hydrophobic regions of the protein to form a protein detergent complex (PDC). Growing MP crystals directly in detergent micelles by vapor diffusion protocols (in-surf method), in essence analogous to procedures used for crystallizing soluble proteins, is the traditional and still by far the most popular method. Increased success has been obtained in recent years using novel bilayer-based crystallization protocols (in-meso method), in which detergent-purified MPs are reconstituted into bilayered micelle (bicelle) or lipidic cubic/sponge mesophases [2]. Because the use of amphiphiles is central to MP structural characterization, the selection of a suitable amphiphile, whether conventional “head-to-tail” detergents, lipids or non-detergent

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membrane mimics, represents both a critically important choice and a major bottleneck for successful sample preparation and structural studies.

Although many among the hundreds of commercially available detergents have been used in membrane biochemistry, the number used successfully for crystallography is quite limited [3, 4]. Historically, the majority of detergents were developed for other research areas or for industrial applications, and particular properties of these agents often disfavor their utility in MP structural studies. For instance, some industrial detergents such as the Triton and Tween series are impure in containing several components. Many detergents with charged polar groups (e.g. sodium dodecyl sulfate) are strong surfactants that tend to denature MPs. Currently, only certain detergents based on sugar, polyethylene oxide or amine oxide head groups prevail in the crystallization of MPs. The most popular among these, alkyl maltosides and glucosides, have so far contributed to solving over half of all α -helical MP structures. These sugar-based detergents are appealing because they have sufficient detergent action to solubilize membranes, yet are relatively mild and do not denature MPs. Improved procedures for the synthesis and purification of these valuable detergents contributed significantly to their ready availability, and consequently also to their broader application [5–7].

For more than two decades, there has been substantial interest in developing novel membrane mimics specifically targeted to the biochemical and biophysical characterization of MPs. Examples include new types of detergents, such as cycloalkyl maltosides (CYMAL detergents, www.affymetrix.com), hemi-fluorinated detergents [8], tripod detergents [9], and cholesterol-based CHOBIMALT detergents [10], as well as agents with structural features distinct from classical head-to-tail detergents, such as protein-based nanodiscs [11–13], amphiphilic polymers (amphipols [8, 14–16] and NVoy polymers [17], peptide surfactants [18, 19], helical peptides [20–22], cholate-derived facial amphiphiles (e.g. CHAPS and CHAPSO) [23, 24], and bolaamphiphiles [25], among others. Many of these amphiphiles have found broad applications in MP biochemistry and are used for the solubilization and stabilization of MPs for functional as well as solution NMR structural studies; for more information about these we refer the reader to the reports and reviews cited above. Of note, these newer amphiphilic structures have most often furthered the stabilization of MPs, clearly an important advance because it extends the window of opportunity to obtain functional proteins and crystals. However, the protein crystallization process imposes specific additional roles on the participating amphiphiles beyond mere stabilization, and protein crystallizability remains to be fully addressed.

It is not our intent in this article to exclusively review previously published work. The emphasis here will be to discuss the structure and function of amphiphiles designed to meet the requirements of MP structural biology, featuring examples from the literature and from our own laboratory, and to highlight future developments.

2. Balancing hydrophobicity and hydrophilicity in amphiphile design and selection

The definitive property of an amphiphile is its ability to self-assemble into structures in aqueous solution, driven by the hydrophobic effect [26]. The type and size of the polar and apolar parts of a detergent molecule determine its properties upon self-assembly. Hydrophobic interactions also drive the association of amphiphiles with the MP surface in the PDC. Resulting interactions between the alkyl chains and the protein side chains confer protein stability, while surface-exposed amphiphile polar groups are presumed to interact with solvent and bring the MP to a soluble state.

Conceivably, the balance between the hydrophobicity and hydrophilicity of an amphiphile may determine its ability to solubilize and stabilize a given MP. This balance is a concept embodied in almost every new amphiphile design. Well-behaved amphiphiles typically exhibit hydrophile-lipophile balance numbers (HLB) that fall within the range of 12–15 [27]. Amphiphiles with low HLB values tend to have low critical micelle concentration (CMC) values and limited solubility in water, and consequently are ineffective at solubilizing MPs, whereas those with higher HLB and CMC values solubilize membranes more completely but may provide less stability to the extracted protein. High-CMC detergents are also advantageous for solution NMR studies and for reconstitution of MPs into lipid bilayers. Protein extraction/solubilization, stabilization, purification, and crystallization procedures each represent distinct processes with varying constraints. For this reason, and because individual MPs vary widely in overall hydrophobicity, detergent selection is generally both protein- and application-dependent, and will continue to present a significant challenge.

As a component effort of the Joint Center for Membrane Protein Technologies, we have developed a library of more than 200 structurally diversified new detergents, including several classes of molecules that feature a variety of hydrophobic and hydrophilic characteristics [28–30]. Structural diversity was achieved through modular chemistry, a strategy best exemplified in our synthesis of a class of PEN-based amphiphiles derived from a versatile and inexpensive molecular scaffold, pentaerythritol. The four primary hydroxyl groups of pentaerythritol were decorated with one, two, or three alkyl chains (Figure 1). The remaining hydroxyl group(s) were retained, or were linked with polar head groups (glucosides, maltosides, or phosphocholine) that increase solubility. The PEN-based group covers a broad region of chemical space in terms of hydrophobicity and hydrophilicity, with an accordingly broad range of amphiphilic properties (CMC, micelle size, etc.). We found that the monoalkyl structures with a single polar head and the dialkyl structures with two polar heads (essentially a dimeric form of traditional detergents) demonstrated better capacity to solubilize and stabilize MPs than those with either large polar heads (2 or 3 functional groups) or large (trialkyl) apolar tails (unpublished results). These studies further reinforce the importance of balanced hydrophobicity/hydrophilicity in the design and selection of detergent molecules for the solubilization and stabilization of MPs.

New structures with two maltosides and two alkyl chains (MNG detergents), structurally similar to the dimeric detergents that we described above, were reported very recently by Chae et al. [31]. These detergents were shown to stabilize a number of test MPs for an extended period of time. After reconstituting β 2 adrenergic receptor-T4 lysozyme (β 2AR-T4L) fusion protein purified in one of the MNG detergents (MNG-3: 2,2-didecylpropane-1,3-bis- β -D-maltopyranoside) into lipidic cubic phase (LCP), larger and better-diffracting crystals have been generated, culminating in successful determinations of ligand-complexed structures [32, 33]. In addition, crystals of Cytochrome *b*₆f grown in MNG-3 micelles diffracted at up to 3.4 Å resolution, nearly as good as the reported 2.8 Å diffraction using DDM. Similarly, tests of several dimeric maltoside detergents (Figure 1) in our and collaborating laboratories also demonstrated stabilizing effects, although as yet no improvement in crystallization outcomes have been attained compared to commercial single-chain maltosides.

3. Shrinking the size of protein-detergent micelles

A serious limitation of growing MP crystals in classical detergents is that they often diffract at low to moderate resolution which is difficult to be improved further. As a result, many structural projects stagnate for years and may ultimately be abandoned. Related to this observation, a large fraction of the MP structures currently deposited in the Protein Data

Bank (PDB) were determined at only moderate resolution that limits their usefulness both for detailed mechanistic understanding and for accurate modeling for the purpose of structure-based drug design.

Obtaining well-diffracting MP crystals suitable for X-ray analysis is made difficult by the relatively limited area of polar surface available to form crystal contacts. An integral MP is generally comprised of large areas of hydrophobic surface that, upon detergent solubilization, become solvated by hundreds of flexible, disordered detergent molecules with micellar or prolate monolayer ring arrangements [34]. This PDC is much larger than the protein itself and has high surface entropy. PDC bulkiness may reduce surface accessibility for protein-protein contact, leaving large channels in the crystal lattice occupied by detergents or aqueous solvent. Modifications to the amphiphile that reduce the size of the PDC should decrease the relative solvent content in crystals, thus facilitating hydrophilic protein-protein interactions to produce tighter protein packing in the lattice. Statistical analysis of a large number of PDB entries shows that higher packing density (lower solvent content) in a protein crystal correlates significantly with better resolution [35]. Detergent micelle size and PDC size appear to correlate [36], though more studies would be needed to extend the observed relationship to different classes of detergents and MPs. Statistically, detergents that form small micelles, such as OG (octyl β -D-glucoside) and LDAO (lauryldimethylamine oxide), tend to yield better-diffracting MP crystals [3, 4], whereas DDM (dodecyl β -D-maltoside), the most popular detergent for stabilization and crystallization, forms relatively large micelles and often gives lower resolution crystals. Conceivably, longer-chain detergents or those with large polar head groups may form larger micelles around the protein, giving rise to higher solvent content which often results in lower MP crystal quality. Thus, in addition to stability, the size of detergent micelles and PDCs may be informative criteria to guide detergent design and selection for high-resolution structural studies.

We recently introduced a class of branched-chain maltoside detergents in which short (1–3 carbon) alkyl branches were appended to the hydrophobic tails at the hydrophilic/hydrophobic interface [28]. We reasoned that short branches could be accommodated near the polar-apolar boundary of the detergent micelle, thereby reducing water content and increasing hydrophobicity in the micelle interior. By this approach, a branched-chain detergent would exhibit the same hydrophobicity as a longer straight-chain detergent, while also forming smaller diameter micelles. The behavior of branched-chain detergents is somewhat analogous to mixtures of detergents with small amphiphile additives that partition at the polar-apolar interface of the micelle, and serve to reduce micelle size [37, 38]. Our case studies indicated that addition of short alkyl branches enhanced protein stability compared to the corresponding straight-chain detergent [28] (and unpublished results), an effect which can be rationalized by the increased hydrophobicity of branched-chain maltosides. We have also demonstrated that use of branched-chain maltosides forming smaller micelles than DDM moderately improved the crystal diffraction of human connexin 26 [28]. Given the popularity of DDM and other maltosides in crystallization studies, we recommend the inclusion of branched-chain maltosides in trials to further optimize crystal diffraction.

Of note, for certain detergents the micelle size increases with concentration [28]. It remains uncertain whether this phenomenon would apply to PDC size if a protein were to be purified at different concentrations of such a detergent. It is generally recommended to use the lowest possible concentration of detergent for purification, and to use higher molecular weight cut-off filters during protein enrichment [39] to minimize detergent concentration in the purified preparation. These precautions might also be expected to help reduce the number of protein-

associated detergent molecules in the PDC, thereby increasing the likelihood of protein-protein interaction during crystallogenesis.

4. Facial amphiphiles

The facial amphiphiles are a structurally unique class of detergents with promising properties for applications in MP structural biology and are worthy of comment here. Representatives include bile acids and their derivatives, as well as amphipathic helical peptides. Facial amphiphiles feature “side polarity” with polar and apolar segments on opposite faces of the molecule. Well known examples of facial amphiphiles that have been widely used in membrane biochemistry include cholate, CHAPS, and CHAPSO. These structurally rigid steroid amphiphiles are thought to be less denaturing for MPs than classical detergents and often effectively solubilize MPs in a functional state. It is not known why, in spite of more than a decade of effort, such steroid-based amphiphiles have yet to produce high-quality MP crystals. We have recently introduced a new cholate-based design with a short, flexible alkyl chain at one end so as to better mimic the structure of cholesterol, but with increased facial amphiphilicity achieved by the alignment of polar groups beneath the steroid nucleus (Figure 2) [30]. Facial amphiphiles based on this improved design have produced some notable successes in the crystallization of several monotopic and polytopic MPs (unpublished results, and published examples cited below). Several membrane-bound P450 enzymes including a number of drug/inhibitor complexes in multiple conformations were crystallized in the presence of facial amphiphiles [40–44]; in these cases the facial amphiphiles appeared to be required for the growth of well-diffracting crystals. A particularly interesting observation in the crystal structure of the ticlopidine complex of CYP2B4 is the presence of a bound facial amphiphile molecule (PDB entry 3KW4) [40]. In this 2-fold symmetry-related lattice, two facial amphiphiles mediate the crystal contacts between hydrophobic regions of the two CYP2B4 molecules. In the crystallization of cation-bound NorM, a multidrug/toxin exporter, addition of the facial amphiphile 231-chol, at 1×CMC, to the crystallization medium was found to improve protein crystallizability and to reduce background precipitation [45]. The best diffracting crystals of NorM (3.65 Å resolution) were grown in the presence of this facial amphiphile as well.

Depending on the identity and positions of the polar groups, steroid-based facial amphiphiles may form much smaller micelles than conventional detergents. With their distinct structural features, the facial amphiphiles may bind to MPs in a unique mode (Figure 2) that allows coverage of a larger hydrophobic area than classical detergents. Their greater hydrophobic association may lead to increased binding affinity, and thereby greater thermodynamic stability of the PDC. In addition, because of their efficient coverage of the hydrophobic patches on the MP surface, fewer facial amphiphile molecules are needed for solubilization so that smaller and more tightly packed PDCs can be formed. Increased protein stability and tighter PDC packing may be key to growing well-diffracting MP crystals. Very recently, facial amphiphile dimers derived from deoxycholate have been reported [46]. Similar to other steroid-based facial amphiphiles, these molecules form small micelles and are capable of stabilizing MPs. Unlike other steroid amphiphiles, these dimers are hypothesized to span the entire thickness of a membrane bilayer.

In addition to steroid-based facial amphiphiles, amphipathic α -helical peptides comprised of ~25 amino acids have been developed to stabilize MPs in solution [20]. A novel lipopeptide design appending alkyl chains at both ends of the helical peptide exhibited substantial stabilizing effect on several test MPs [21, 22]. Like steroid-based facial amphiphiles, these lipopeptides form small, tightly packed and rigid oligomers. Crystal structures of lipopeptides with bound DDM molecules showed a cylindrical bundle of eight helical peptides assembled with the hydrophobic chains buried inside, supporting a model proposed

for the sequestration of MPs in lipopeptide micelles [47, 48]. The lipopeptides are relatively costly, making their use impractical except in the final stages of sample preparation via detergent exchange. They could perhaps be tested as additives for optimizing the properties of PDCs extracted and purified using other detergents.

5. Reagents for bilayer-based crystallization methods

Compared to detergent micelles, LCP creates a more native-like bilayer environment for the stabilization and crystallization of MPs [49, 50]. A single-chain monoacylglycerol lipid, monoolein, presently accounts for the majority of successes of the in-meso approach to crystallization [32, 33, 51–56]. Monoolein self-assembles into a stable cubic phase in aqueous solution and acts as the lipidic host for MPs. A key feature of success in the LCP approach is tight packing of protein subunits in the bilayer, giving lower solvent content and higher resolution diffraction. Misquitta et al. have designed monoolein homologs with varying alkyl chain length and *cis*-double bond position, and certain shorter-chain variants have proven useful in LCP-based crystallization of MPs [57–59]. We have recently developed a new procedure for the efficient synthesis of these molecules to address the pressing need for new LCP lipids [60]. The characterization of branched isoprenoid alkyl chain molecules as new LCP lipids represents another notable development [61–63]. To further expand the LCP approach to studies of MPs with large soluble domains or with soluble protein complexes, it will be very useful to continue the development and characterization of new lipids with properties different from monoolein - in particular, “sponge phase” lipids forming a swollen cubic phase containing a larger aqueous compartment [64, 65].

The bicelle-based crystallization of bacteriorhodopsin (bR) was first reported in 2002 by S. Faham and J. Bowie using dimyristoyl phosphatidylcholine (DMPC)-CHAPSO bicelles [57]. Subsequent studies demonstrated that DMPC-CHAPSO bicelles were superior to DMPC-DHPC (dihexanoyl phosphatidylcholine) bicelles in the crystallization of bR [66, 67]. Since then, several other MP structures, including voltage-dependent anion-selective channel protein 1 (VDAC1) [68], β 2AR-Fab complex [69], and rhomboid protease [70], have been determined using DMPC-CHAPSO bicelles. An exception is the use of DMPC-nonyl maltoside bicelles in the crystallization of xanthorhodopsin [71]. The structures of lipid/detergent mixed bicelles, generally regarded as a compromise between detergent micelles and lipid bilayers, are not well defined under crystallization conditions. Bicelles form a gel phase above the transition temperature (T_m) of the lipid component and remain in solution state below the T_m . The structures and phases may also vary depending on compositions and conditions. It has been demonstrated that the detergent selection and the ratio of detergent to lipid in the bicelle mixture significantly affect protein stability [72]. The molecular composition of bicelles, including the lipid component, may affect the membrane thickness, flexibility and fluidity, and therefore the crystallogenes of proteins as well. Currently, very few bicelle systems have been explored in MP crystallization. Compared to the cubic phase formed by monoolein, bicelle phases are relatively unstable. Characterization of stable bicelle formulations will facilitate further progress with this novel crystallization technique.

6. Solution NMR studies

Solution NMR spectroscopy lags behind X-ray crystallography for structural determination of MPs. One major limitation is that reconstitution of MPs with amphiphilic molecules gives rise to a substantial increase in mass of the NMR target. As the smallest membrane-replacing agents, detergent micelles would appear to be the prime choice for MP reconstitution for solution NMR analysis. The recent determination in detergent micelles of

solution NMR structures for VDAC (19 β -strands) [73], the homotrimeric diacylglycerol kinase (DAGK, 9 transmembrane helices) [74], and the seven-helix sensory rhodopsin II (pSRII) [75] represent major advancements in NMR-based structural solution of larger MPs. Several zwitterionic detergents (e.g. DHPC; dodecylphosphocholine, DPC; LDAO) or lysophospholipids (e.g. lyso-myristoyl phosphatidylglycerol, LMPG; lyso-palmitoyl phosphatidylglycerol, LPPG) are the most popular choices for NMR [76]; these are nevertheless fairly aggressive detergents and often do not sufficiently stabilize the MP. Novel stabilization reagents (e.g. amphipols) and those forming small PDCs (e.g. facial amphiphiles, lipopeptides) offer favorable properties for solution NMR applications. Other bilayer mimics such as bicelles and lipid-protein nanodiscs have produced high-quality NMR spectra of several MPs that are comparable to those obtained in detergent micelles. Although they tend to form particles larger than traditional PDCs, these nonmicellar systems, conceivably as better membrane mimics, have excellent potential for NMR applications; on this topic we refer the reader to a recent review [77]. Overall, selection of amphiphiles for solution NMR studies is as challenging as in X-ray crystallography, but the requirements of intermolecular association differ. Thus, experience from MP crystallization is of little help, and an agent that is successful for crystallizing a given MP is often unsuitable for its NMR characterization and vice versa. Further development of amphiphiles specific for NMR will be of great value.

7. Conclusion

Engineering the chemical environment of MPs by introduction of carefully designed amphiphiles can be as important, if not more so, as engineering protein constructs in order to obtain stable samples suitable for high-resolution structural studies. Many of the amphiphiles that have been developed in the past are valuable for solubilizing and stabilizing MPs. However, the number of useful amphiphiles for X-ray crystallography and NMR structure determination is still limited. As a consequence, determination of MP structure at high resolution remains a formidable challenge in general.

The selection of detergents for MP structural studies has historically proceeded by a process of trial-and-error. We still know little about how and why a MP crystallizes well in a given detergent, even though some empirical rules, such as protein stability, homogeneity and monodispersity, have emerged. Oftentimes, a specific MP can be crystallized in one detergent but not in others, indicating that the crystallization must be closely associated with the properties of the detergent and the PDC. A deeper understanding of the structure-activity relationship of detergents and other amphiphiles, however, has been impeded by infrequent publication of negative data and by the common habit in the majority of laboratories of focusing on a single technique and/or protein family. Thus, the collective effort of chemists, biochemists and structural biologists studying MPs will be needed to expand our armamentarium of amphiphiles, and enable the accumulation of new knowledge and understanding, beyond mere empirical guidelines, to guide the design and selection of even better amphiphiles.

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Figure 1.

Schematic diagram illustrating the modular synthesis of structurally diverse detergents derived from pentaerythritol (PEN). Structures with three alkyl chains and one polar head exhibit low solubility and are not depicted. MG (one alkyl chain, one glucoside polar head) and DM (two alkyl chains, two maltoside polar heads) species display well-balanced hydrophobicity and hydrophilicity, and are characterized as performing well in the solubilization and stabilization of MPs. The attached table summarizes the micelle properties of MGs and DMs. Longer-chain detergents (e.g. DM-4 and DM-5) form large micelles that enlarge with concentration.

Figure 2.

(A) Facial amphiphiles derived from cholate. Top: known facial amphiphiles (e.g. cholate, CHAPS, CHAPSO) resemble classical detergents in retaining the main polar groups at one end, and have weak facial amphiphilicity. Bottom: a new design omits the terminal polar head groups, leaving a short alkyl chain similar to cholesterol, and attaches "side" polar groups below the steroid nucleus to increase facial amphiphilicity. (B) Different arrangements of polar (green) and nonpolar domains (black) in classical "head-to-tail" detergents and in novel facial amphiphiles. Facial amphiphiles are proposed to bind more tightly to MP surfaces and to form relatively smaller PDCs than classical detergents.

Table

Detergent	Alkyl chain	CMC (mM) ^a	Micelle size (R _h) ^b
MG-2	n-C ₉ H ₁₉	2.83	2.9 ± 0.1 nm
MG-3	n-C ₁₀ H ₂₁	0.80	3.0 ± 0.1 nm
MG-4	n-C ₁₁ H ₂₃	0.24	3.1 ± 0.1 nm
MG-5	n-C ₁₂ H ₂₅	0.043	4.1-4.6 nm
DM-1	n-C ₈ H ₁₇	0.099	2.8 ± 0.1 nm
DM-2	n-C ₉ H ₁₉	0.029	3.1 ± 0.1 nm
DM-3	n-C ₁₀ H ₂₁	0.016	3.4 ± 0.1 nm
DM-4	n-C ₁₁ H ₂₃	0.0091	4.2-8.6 nm
DM-5	n-C ₁₂ H ₂₅	0.0045	20-30 nm

^a CMC determined by 8-anilino-1-naphthalenesulphonic acid fluorescence in H₂O at 20 °C.

^b Micelle size was estimated by the hydrodynamic radius, R_h, measured by dynamic light scattering over a range of detergent concentrations (0.1–0.6% wt/vol) in H₂O at 20 °C.