

## Review Article

# Detergent-free systems for structural studies of membrane proteins

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Membrane proteins play vital roles in living organisms, serving as targets for most currently prescribed drugs. Membrane protein structural biology aims to provide accurate structural information to understand their mechanisms of action. The advance of membrane protein structural biology has primarily relied on detergent-based methods over the past several decades. However, detergent-based approaches have significant drawbacks because detergents often damage the native protein–lipid interactions, which are often crucial for maintaining the natural structure and function of membrane proteins. Detergent-free methods recently have emerged as alternatives with a great promise, e.g. for high-resolution structure determinations of membrane proteins in their native cell membrane lipid environments. This minireview critically examines the current status of detergent-free methods by a comparative analysis of five groups of membrane protein structures determined using detergent-free and detergent-based methods. This analysis reveals that current detergent-free systems, such as the styrene-maleic acid lipid particles (SMALP), the diisobutyl maleic acid lipid particles (DIBMALP), and the cycloalkane-modified amphiphile polymer (CyclAPol) technologies are not better than detergent-based approaches in terms of maintenance of native cell membrane lipids on the transmembrane domain and high-resolution structure determination. However, another detergent-free technology, the native cell membrane nanoparticles (NCMN) system, demonstrated improved maintenance of native cell membrane lipids with the studied membrane proteins, and produced particles that were suitable for high-resolution structural analysis. The ongoing development of new membrane-active polymers and their optimization will facilitate the maturation of these new detergent-free systems.

## Introduction

### The structural complexity of cell membrane environments

Cell membranes are composed of two fundamental components — lipids and proteins. Cell membrane systems are crucial for all living organisms to survive and flourish. In the course of evolution, the eukaryotic cell membrane system has developed a more complicated architecture than that of prokaryotes. For example, many organelles such as mitochondria, chloroplast, lysosome, and endoplasmic reticulum emerged in eukaryotic cells. The membrane surrounding these organelles is also uniquely featured by specific lipid components [1]. Furthermore, new types of lipids, such as cholesterol and sphingolipids, appeared in mammalian cell membrane environments [2]. Generally, biomembrane structure consists of two apposed leaflets formed mainly by phospholipids and a small portion of other lipids in the lipid bilayer; however, some archaeal membrane can be a monolayer composed of unique ether lipids [3]. The membrane proteins are embedded or adsorbed on the cell membrane system.

According to the fluid mosaic membrane model [4], the cell membrane is a laterally heterogeneous and anisotropic complex system. The complexity may be a result of coevolution between the cell membrane and membrane proteins [5]. The full picture of the cell membrane structure is more

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complicated when considering the diverse lipid components, various functional and structural membrane protein components, asymmetry of the lipid distribution, and how this asymmetry is employed to connect the cell membrane to the rest of the cell [6].

## The structures and functions of membrane proteins depend on the native cell membrane environment

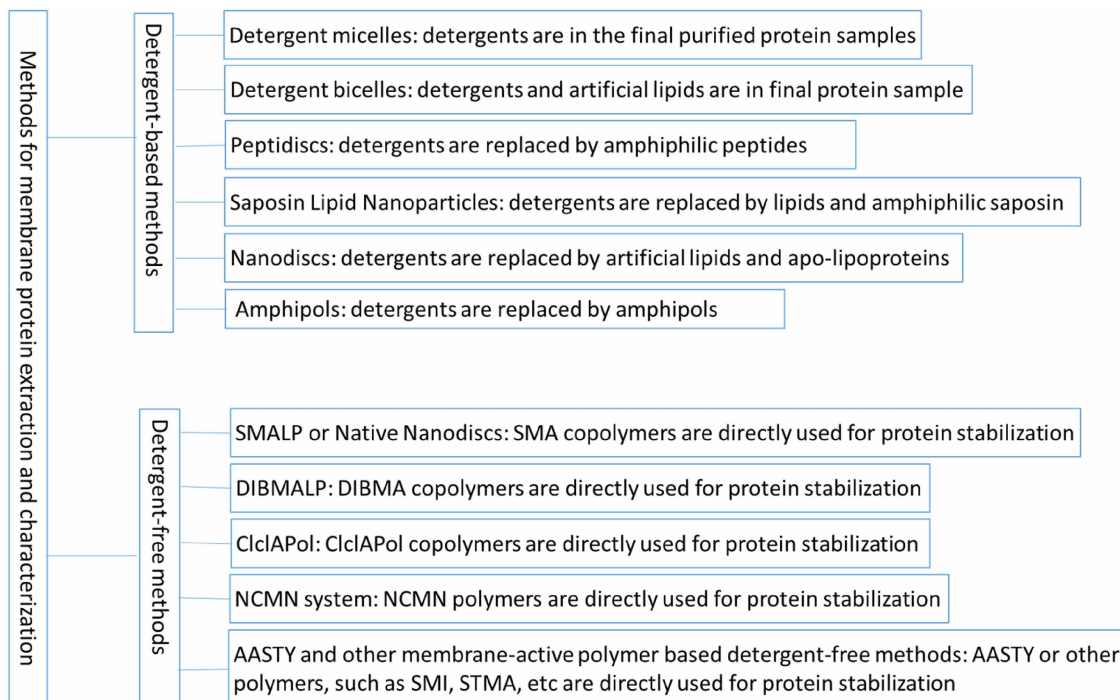
Native lipid environments are crucial for maintaining the natural structures and activities of many membrane proteins [7]. Therefore, how well we understand and can exploit the structure and function of those membrane proteins depends on how well we understand the interactions between membrane proteins and their associated native lipids. For example, receptors such as GPCRs often need cholesterol to maintain their natural structure and to elicit signaling [8–10]. Transporters, such as some members of the ABC family, need a cell membrane lipid environment to prevent uncoupled ATPase activity [11]. Channels, such as TRP and related proteins are regulated by specific lipids, such as phosphatidylinositol-4,5-bisphosphate (PIP2) [12]. Enzymes, such as respiratory complex I need lipid components to maintain their biological activity [13].

## The need for dedicated detergent-free systems for membrane protein structural biology

As discussed above, the preservation of membrane protein stability and function requires a native lipid environment. Currently, for high-resolution structure determination, membrane proteins often need to be extracted first by using detergents. Indeed, for the past few decades, the advancement of membrane protein structural biology can primarily be attributed to detergents, and structure determination of membrane proteins using detergent-based approaches has led to four Nobel Prizes directly or indirectly [14–16]. However, detergent-based approaches have significant drawbacks because they often lead to over-delipidation of membrane proteins, subsequent destabilization, and loss of biological activity [15,17]. Furthermore, membrane protein stability can also be compromised by detergent interactions with soluble domains [18]. Structure-based drug discovery requires accurate structural information, and membrane proteins are today the main class of drug targets [19]. Potential structural problems of membrane proteins determined using NMR and X-ray crystallography have been recently reviewed [15,20,21]. Single-particle cryo-EM studies of membrane proteins have recently generated many new membrane protein structures, but as of now, potential structural denaturation problems have not been carefully examined. However, considering the predominant application of detergents, structural issues are inevitable. Many novel technologies have been developed to overcome the limitations of detergent-based approaches, including Nanodiscs [22], Amphipol [23], Saposin [24], and Peptidisc [25] methods. These technologies are still reliant on detergents to initially extract the membrane proteins; therefore, they are not truly detergent-free procedures. Although these novel methods can overcome some of the limitations rendered by the purely detergent methods, they still suffer from altering the native association states between membrane protein and cell membrane lipids. Considering the importance of the protein–lipid interactions for membrane protein structure and function, dedicated detergent-free systems that can extract membrane protein and associated lipids as functional units are clearly needed for membrane protein structural biology. Membrane-active polymers, such as styrene-maleic copolymers (SMAs) [26], diisobutylene maleic acid copolymers (DIBMA) [27], new Cycloalkane-Modified Amphiphilic Polymers (CyclAPol) [28], and related detergent-free technologies, such as the SMALP technology [17,26,29] and the NCMN system [30–32] have recently emerged as alternatives to detergent-based approaches that possess the great promise. [Scheme 1](#) summarizes the current principal methods for membrane protein extraction and purification.

## Current status of the dedicated detergent-free systems

In contrast to detergents used for membrane protein structural biology, membrane-active polymers extract membrane protein but do not form micelles in aqueous solutions [33]. In 2009, the SMA was reported to extract membrane proteins [34], and thereafter considerable research was performed to solubilize and purify membrane proteins for enhanced stability and activity [35–40]. In 2017, the SMA was used to extract bacterial rhodopsin for crystallization in LCP [41]. One year later, styrene-maleic acid copolymers were demonstrated in successful high-resolution structure determinations of membrane proteins using single-particle cryo-EM [30,42]. Following this success, several high-resolution structures of different membrane proteins were reported, including the KimA potassium channel [43], acid-sensing ion channel 1 [44], and the Glycine receptor [45].



**Scheme 1. Main methods for membrane protein extraction and characterization.**

Simultaneously, novel membrane-active polymers such as DIBMA were also tested for membrane protein extraction and purification [46]. Recently, it has been reported that DIBMA could be used to determine the high-resolution cryo-EM structure of the mechanosensitive Channel YnaI from *E. coli* [47]. Moreover, the cycloalkane-modified amphiphile polymer (CyclAPol) has also been tested for the extraction of membrane proteins, and recent reports show that a novel CyclAPol, termed C<sub>8</sub>-C<sub>0</sub>-50, can be used for high-resolution structure determination of AcrB at 3.2 Å (Higgs A., et al., to be published). A high-resolution cryo-EM structure of human connexin 26 with associated cell membrane lipids using the NCMN system (Qiu, et al., to be published) was disclosed at the International SMALP Conferences 2020 (SMALP.net). Beyond these developments, other novel membrane-active polymers aiming to overcome the limitations of styrene-maleic acid copolymers have also been designed and demonstrated for membrane protein extraction [48,49].

To summarize, detergent-free technologies aim to overcome the limitations inherent in the detergent-based approaches, such as over-delipidation and destabilization of membrane proteins caused by detergents. In principle, a successful and dedicated detergent-free system should retain the natively associated lipids from the transmembrane domain of membrane proteins. It should also allow examining protein–lipid interactions to facilitate understanding of the structure–function relationships of membrane proteins. Correspondingly, the suitability of these new membrane-active polymers remains to be tested for high-resolution structure determination. The advance of detergent-free systems is quite promising, and clearly, all such methodologies generate some form of simulated biological construct. Thus, careful and critical examination of the structures solved with structural biological tools — single-particle cryo-EM, solid-state NMR, or X-crystallography — is required to assess the current status of detergent-free technologies.

This minireview will analyze five groups of membrane protein structures recently determined using four different detergent-free technologies. The analysis will focus on three themes: structural change, subunit association and protein–lipid interaction. The five groups of membrane proteins are AcrB, alternative complex III, KimA potassium channel, mechanosensitive channel YnaI and Glycine receptor. Relevant information on these five groups of proteins is summarized in Table 1. The four main detergent-free technologies include styrene-maleic lipid particles (SMALP), the native cell membrane nanoparticles (NCMN) system, diisobutylene maleic acid lipid particles (DIBMALP), and CyclAPols. All of these four detergent-free systems use membrane-active polymers to extract membrane proteins directly from cell membrane without using any detergent. The SMALP

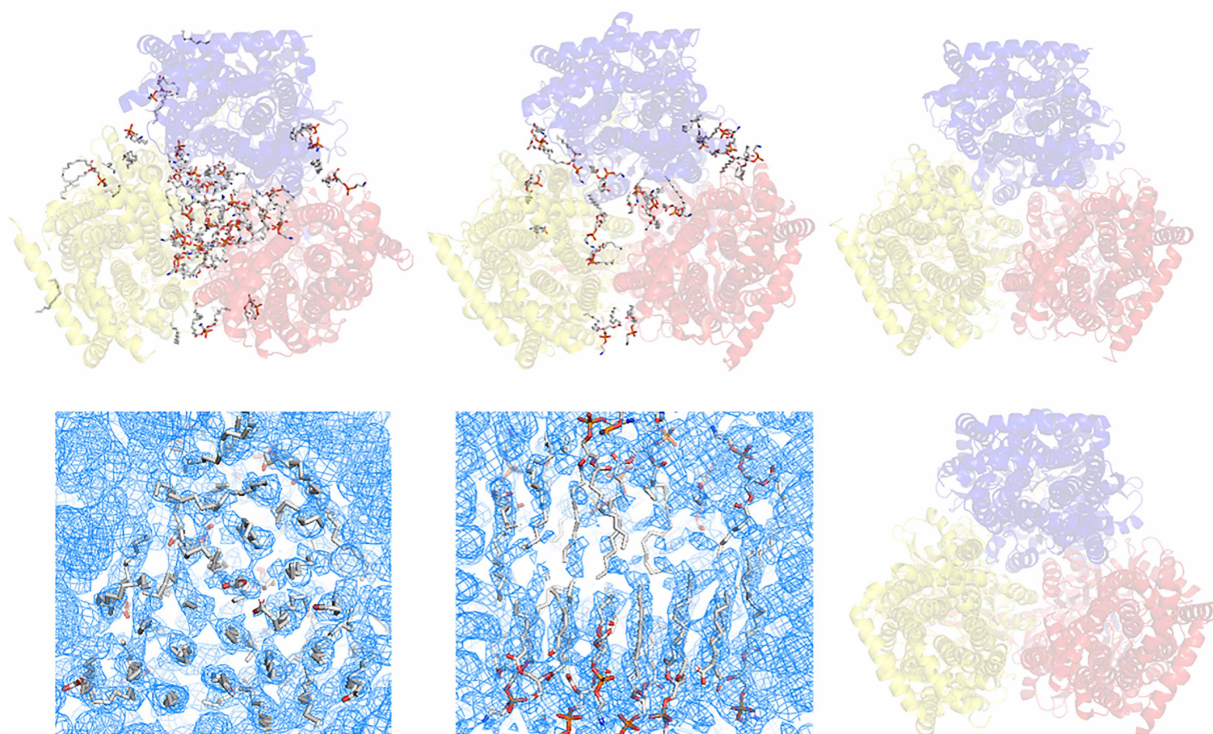
**Table 1. Summary of the structures of five group of membrane proteins determined using detergent and detergent-free systems**

Group of proteins	Proteins	PDB/EMD	Function	Oligomeric states	Expression system	Species	Methods
AcrB and homologs	AcrB	6BAJ/7074	Transporter	Trimer	<i>E. coli</i>	<i>E. coli</i>	NCMN system
	MtrDB	6VKS/21228	Transporter	Trimer	<i>E. coli</i>	<i>N. onorrhoeae</i>	Nanodisc
	AcrB	6Z12/4460	Transporter	Trimer	<i>E. coli</i>	<i>S. enterica</i>	SMALP
	AcrB	6SGU/10185	Transporter	Trimer	<i>E. coli</i>	<i>E. coli</i>	Saposin
	AcrB	7B5P/12043	Transporter	Trimer	<i>E. coli</i>	<i>E. coli</i>	CyclAPol
	AcrB	1IWG	Transporter	Trimer	<i>E. coli</i>	<i>E. coli</i>	Detergent
Alternative complexes	ACIII	6BTM/7286	Enzyme	Heterohexamer	<i>F. johnsoniae</i>	<i>F. johnsoniae</i>	SMALP
	ACIII	6LOD/0936	Enzyme	Heterohexamer	<i>R. castenholzii</i>	<i>R. astenholzii</i>	Detergent
	ACIII	6F0K/4165	Enzyme	Heterohexamer	<i>R. marinus</i>	<i>R. marinus</i>	Detergent
KimA	KimA	6S3K/10092	Transporter	Homodimer	<i>E. coli</i>	<i>B. subtilis</i>	SMALP
Ynal	Ynal	6ZYD/11557	Channel	Homoheptamer	<i>E. coli</i>	<i>E. coli</i>	DIBMA
	Ynal	6ZYE/11560	Channel	Homoheptamer	<i>E. coli</i>	<i>E. coli</i>	DIBMA
	Ynal	6URT/20862	Channel	Homoheptamer	<i>E. coli</i>	<i>E. coli</i>	Detergent
Glycine receptor	Glycine receptor	6PLZ/20381	Receptor/Channel	Homopentamer	<i>S. frugiperda</i>	<i>D. rerio</i>	SMALP
		6PXD/20518	Receptor/Channel	Homopentamer	<i>S. frugiperda</i>	<i>D. rerio</i>	Detergent
		6PLS/20374	Receptor/Channel	Homopentamer	<i>S. frugiperda</i>	<i>D. rerio</i>	Nonodisc

or alternatively Native Nanodiscs [50] is currently the most widely used detergent-free system. The NCMN system is distinct from SMALP technology: *first*, the NCMN system has a unique membrane-active polymer library. All the membrane-active polymers are purposely and individually developed for high-resolution structure determination of membrane proteins with diverse characteristics. *Second*, the NCMN system has a collection of protocols developed and optimized for each of the NCMN polymers for making high-quality NCMN particles. *Third*, the NCMN particles are only made from the native cell membrane, emphasizing the membrane proteins expressed by the cells from the same species. For example, human membrane proteins expressed in bacteria, yeast, or insect cell lines will not be appropriate for making NCMN particles and, therefore, will be avoided. *Fourth*, except for cryo-EM tomographic analysis, all downstream NCMN particle analyses, such as enzyme assays, single-particle cryo-EM, mass spectrometry, and proteoliposome reconstitution, require the use of homogenous samples that are confirmed with the electron microscope. *Fifth*, the NCMN system is not suitable for LCP crystallization because mixing NCMN particles with artificial lipids will disturb the native lipid–protein interaction. NCMN particles might be used for crystallization, just like soluble proteins. For comparison and evaluation of the detergent-free methods with detergent-based methods, each group of membrane proteins will be discussed in separate sections for the convenience of analysis.

## Acrb and homologs

AcrB is a model membrane protein used for method development in several laboratories [24,30,51,52]. Structures of AcrB have been extensively investigated using detergent-based methods with both X-ray crystallography and cryo-EM [53–55]. However, even at a very high resolution of 1.8 Å [55], the lipid bilayer structure was lost in the detergent-prepared sample. The first single-particle cryo-EM structure of AcrB determined using SMALP technology only reached a poor resolution of 8.8 Å [56]. No lipid molecule density was observed in the reconstructed EM density map. In 2018, the first high-resolution single-particle cryo-EM structure of a lipid bilayer patch within AcrB was determined using the prototype NCMN system (Figure 1A) [30]. As the figure indicates, very robust EM density for native lipids can be observed in this structure. A novel amphipol, CyclAPol (C<sub>8</sub>-C<sub>0</sub>-50) was recently used to determine a high-resolution structure of AcrB at 3.2 Å (Higgs A., et al., to be published); yet, no lipid density was observed in the EM density. A single-particle cryo-EM structure of an *E. coli* AcrB homolog, gonococcal multidrug efflux pump, was solved at a high resolution of 2.7 Å using *E. coli* total lipids reconstituted nanodiscs (Figure 1B) [57]. In this structure, the hexagonal hydrocarbon pattern in the lipid plug's inner leaflet position is observable; nevertheless, it is not complete, and the outer leaflet lipids were not well determined. A *Salmonella* AcrB cryo-EM structure at a resolution (4.6 Å) determined using SMALP technology has also been reported [58]. Again, no associated native lipid molecules were found in the EM density (Figure 1C). Similar to the lipid reconstitution using nanodisc technology, an *E. coli* AcrBZ complex reconstituted into saposin discs using *E. coli* lipids and saposin as



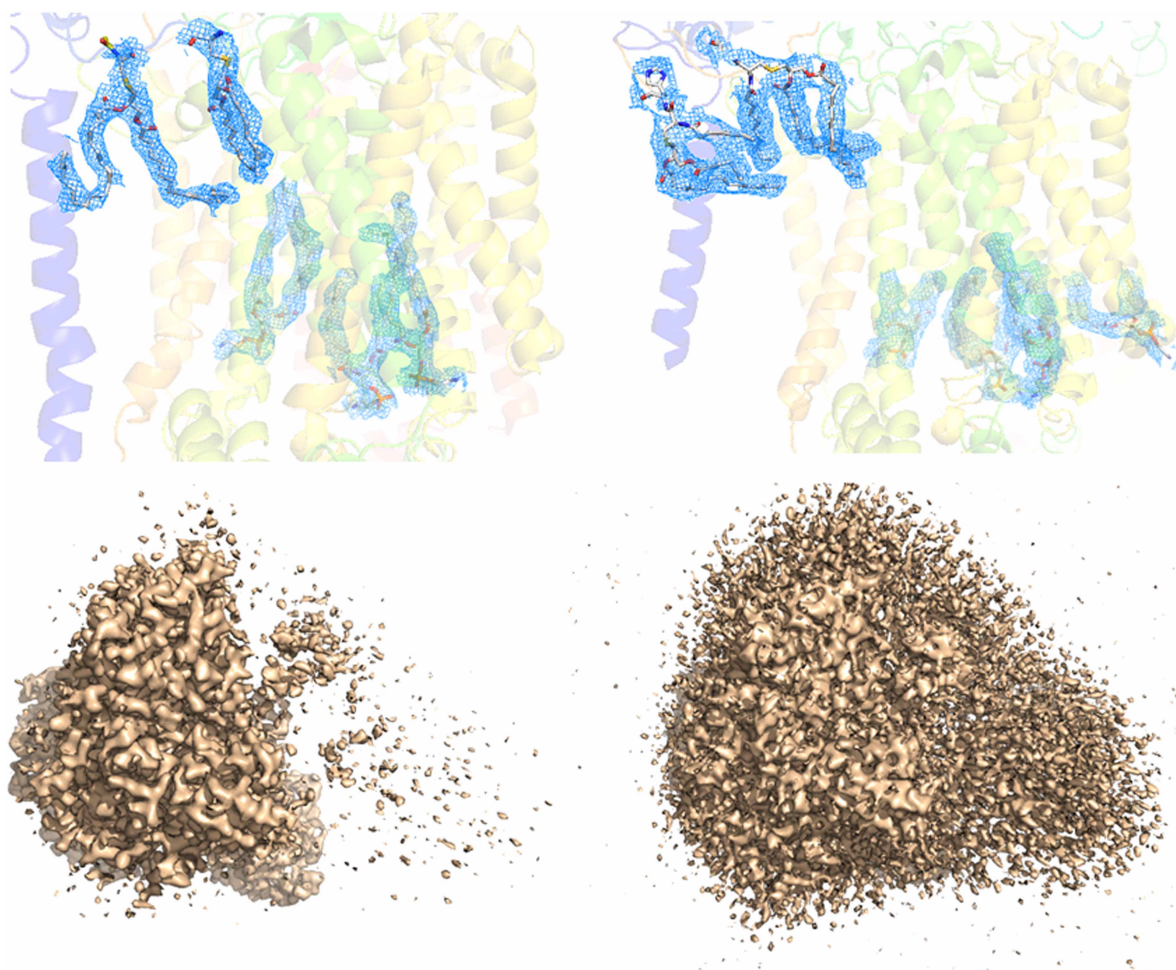
**Figure 1. Structures of AcrB and homolog solved in detergent-based and detergent-free systems.**

(A) Single-particle cryo-EM structure of *E. coli* AcrB determined using NCMN system (PDB: 6BAJ). The NCMN system retains native cell membrane lipids associated with AcrB, a native lipid bilayer patch composed of 24 lipid molecules can be unambiguously determined. (B) Single-particle cryo-EM structure of *Neisseria gonorrhoeae* MtrD (AcrB homolog) determined using Nanodisc technology (PDB: 6VKS). Nanodisc reconstitution using artificial lipids or total *E. coli* lipids could not reconstruct the native lipid bilayer patch within the lipid cavity of the transmembrane domain. (C) Single-particle cryo-EM structure of AcrB from *Salmonella enterica subsp. enterica* (PDB: 6Z12) determined using SMALP technology. (D,E) Single-particles cryo-EM density of *E. coli* AcrB determined using Saposin-lipid reconstitution (EMD:10185). Close-up view of the lipid plug (D) bottom view of the inner leaflet lipid-pattern with a similar orientation to the structure in panel A, but with a clockwise rotation around the Z-axis about 30° for a better view; (E) side view of the lipid bilayer plug, turn 90° clockwise around the X-axis relative to the density in panel (D); top half: outer leaflet, bottom half: inner leaflet). Saposin-lipid reconstitution can partially reconstitute the lipid bilayer patch within the lipid cavity of the transmembrane domain of *E. coli* AcrB. However, it still could not recover the native state of the lipid bilayer structure. (F) Crystal structure of *E. coli* AcrB using detergent-based method. In this structure, almost all native lipids were washed away by detergents. In all the figures above, cartoons show the AcrB protein. Sticks show the lipid molecules.

scaffolding protein shows a similar hexagonal hydrocarbon tail pattern in the inner leaflet (Figure 1D,E) [59]. However, the outer leaflet is not well resolved, even at the same resolution of 3.2 Å. In both the lipid reconstitution cases using nanodisc and saposin technologies, although the lipids can be reconstituted to some extent, they were not the naturally associated lipids from the cell membrane. Therefore, artificial bias could be a potential concern when interpreting the protein–lipid interactions using the reconstituted system.

## Alternative complex III and homologs

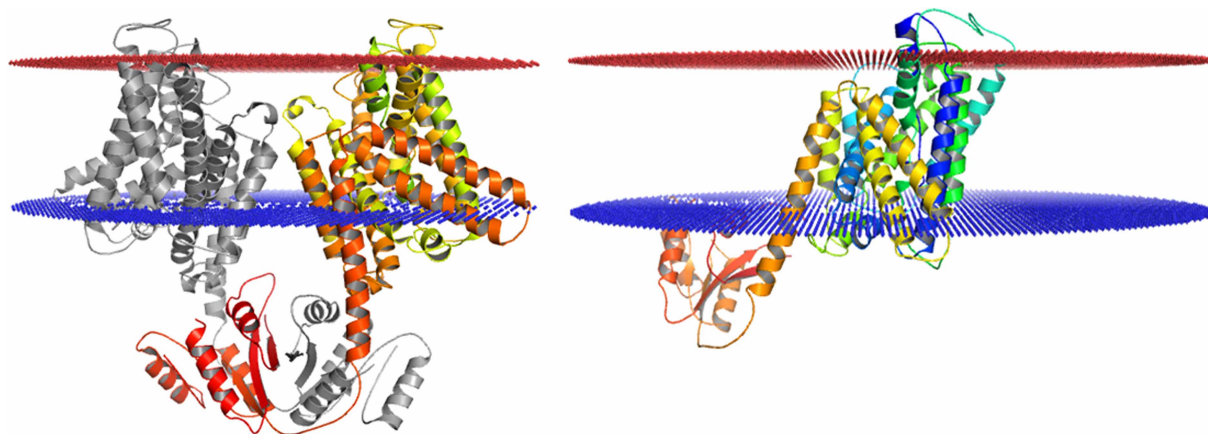
Alternative complex III is an essential membrane protein component within the respiratory and/or photosynthetic electron transport chain in many bacteria [60]. Both detergent-based approaches and SMALP technology have been used to investigate the high-resolution structure of alternative complex III. The reported single-particle cryo-EM structure determined using SMALP technology was shown to retain some natively associated lipid from the cell membrane and to retain the supercomplex of alternative complex III and aa3-type cytochrome c oxidase (Figure 2A,D). Interestingly, the other high-resolution single-particle cryo-EM structure of alternative complex III prepared in detergents also shows essential structural native lipids in equivalent



**Figure 2. Structures of alternative complex III/Cyt-aa3 supercomplex and homolog solved in detergent-based and detergent-free systems.**

(A) Single-particle cryo-EM structure of alternative complex III SMALP technology (PDB: 6BTM). The tightly bound lipids within the complex were well solved. Blue colored EM density in mesh shows the location of lipids. (B) Single-particle cryo-EM structure of alternative complex III determined using detergent (PDB: 6LOD), in the structure, even with detergent, the tightly bind lipids within the complex were well resolved. Blue colored EM density in mesh shows the location of lipids. (C,D) Single-particle cryo-EM density of alternative complex III/Cyt-aa3 supercomplex determined using SMALP technology. The density maps shown in (C,D) are in the same orientation, vertical to the plane of cell membrane. The density maps are displayed at contour levels 5 and 3 (in sigma units), respectively, using PyMol. At level 5, only alternative complex III is well resolved; at level 3, although EM density is observable, the density is too noisy to build the Cyt-aa3 structure.

positions (Figure 2B). In the SMALP-derived structure, it was reported that alternative complex III forms Cyt-aa3 complex at a 1 : 1 ratio; however, this may not be the case. EM density displayed at contour level 5 (in sigma units) (Figure 2C) only shows a well-solved EM density of complex III but not Cyt-aa3. EM density displayed at contour level 3 (Figure 2D) does show EM density of Cyt-aa3 but is very noisy. There are two possibilities here. One is that Cyt-aa3 is structurally flexible; the other possibility is that in the SMALP prepared sample, only a small portion of particles contain Cyt-aa3, i.e. the alternative complex III/Cyt-aa3 ratio is much larger than 1. However, the natural complex III/Cyt-aa3 complex ratio on the cell membrane could be close to 1. Besides, because the Cyt-aa3 structure was solved at high-resolution separately [61], this structural flexibility can also be excluded; therefore, we can conclude that the poor density of Cyt-aa3 is because of the failure of SMALP technology to retain the alternative III/Cyt-aa3 supercomplex if these supercomplexes are predominant in the native cell membrane. Furthermore, in other research, an alternative complex III prepared in detergent also shows the same supercomplex [62].



**Figure 3. Single-particles cryo-EM structures of KimA potassium channel determined using SMALP technology.**

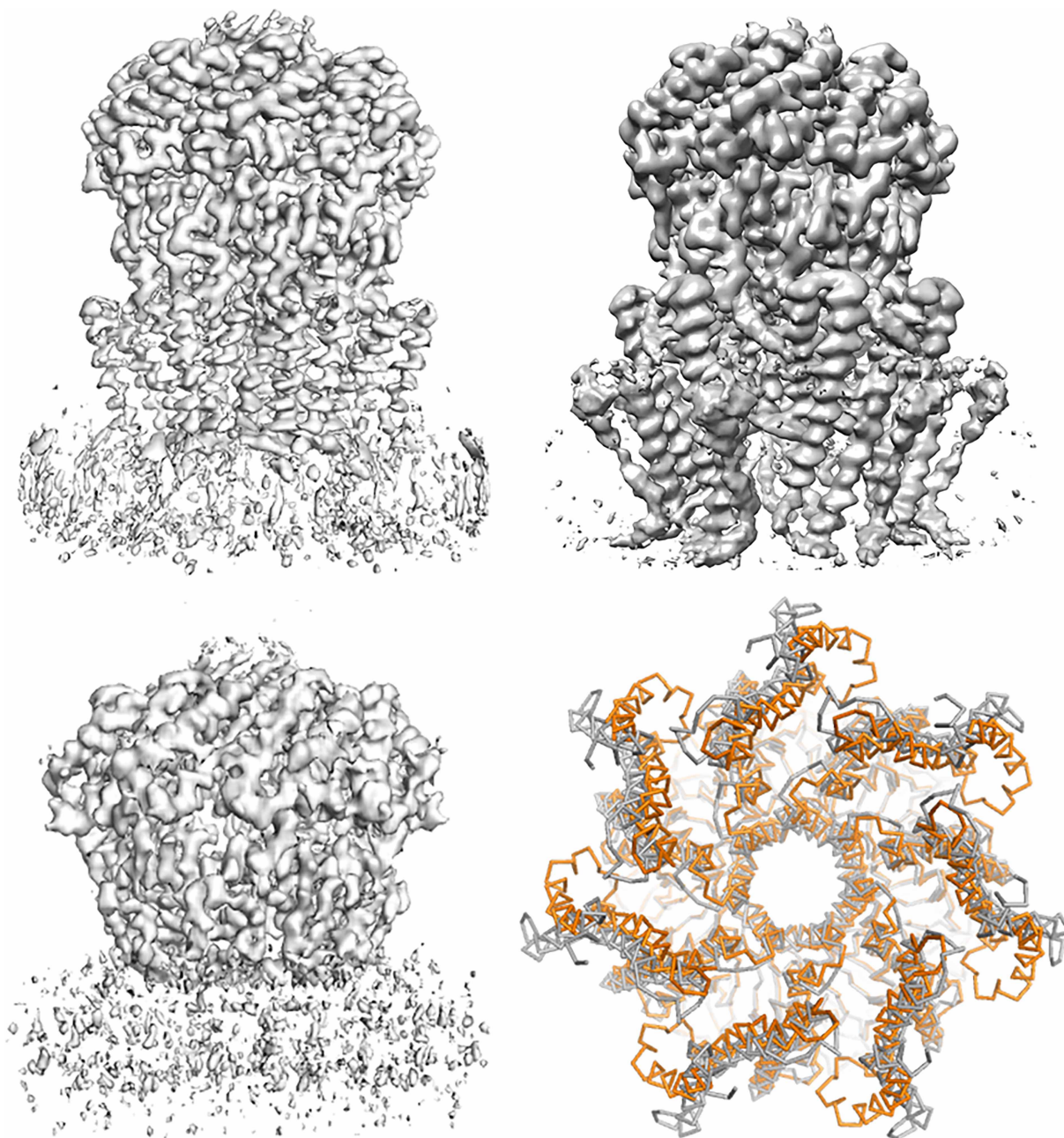
(A) Single-particle cryo-EM structure of KimA potassium channel dimer (PDB: 6S3K), in the structure, even without detergent, no lipids were resolved in the transmembrane domain. Transmembrane region analysis using PPM server showing the transmembrane domain of the dimer composed of two tilted subunits. (B) Transmembrane region analysis of a single subunit of KimA potassium channel dimer (PDB: 6S3K). The transmembrane domain region calculated from the monomer is drastically different from that of the KimA dimer.

### ***Bacillus subtilis* KimA potassium channel**

*Bacillus subtilis* KimA potassium channel is a member of the KUP family. KUP transporters facilitate potassium uptake through the co-transport of protons [63]. Recently, a high-resolution single-particle cryo-EM structure of KimA has been solved using SMALP technology with the membrane-active polymer Xiran SL30010 P20 [43]. The solved single-particle cryo-EM structure does not have any associated native cell membrane lipids. However, molecular dynamic simulation analysis results suggest that the transmembrane domain of KimA dimer is associated with cell membrane lipids between the two subunits [43]. The KimA dimer structure might collapse once the lipid molecules were washed away by the membrane-active polymers, SZ30010. The KimA monomer was extracted with PyMol from the KimA homodimer coordinates to examine if the transmembrane region of the KimA dimer is consistent with the KimA monomer. Transmembrane region calculations of the dimeric and monomeric structure of KimA did show an apparent discrepancy: the tilting angle difference between the transmembrane helices in the KimA dimer and monomer is about 30° when calculated with the cell membrane as reference (Figure 3A,B). All the pieces of evidence indicate that SMALP technology failed to retain the lipids associated with the KimA potassium channel's transmembrane domain and led to a non-natural structure of the KimA dimer.

### **Mechanosensitive channel YnaI**

*E. coli* YnaI is a mechanosensitive channel [64] where protein–lipid interaction is crucial for gating [65,66]. Recently, DIBMA was used for high-resolution structure determinations for both the closed- and open states (Figure 4A,C) [47]. Examination of the EM density of YnaI solved with DIBMA revealed that in the claimed open state, the transmembrane domain has very poor EM density; no lipid molecule density can be assigned, and even the transmembrane helices could not be assigned unambiguously (Figure 4C). In the closed state, the (outer) transmembrane helices TM1 and TM2 EM density were not well resolved, and many of the native lipid molecules that are expected to be associated with the transmembrane domain were lost. Recently, the same YnaI channel single-particle cryo-EM structure determined using LMNG as detergent has also been reported (Figure 4B) (PDB: 6URT, EMD-20862, Hu, et al., to be published). In the detergent-based YnaI structure, all the transmembrane helices are well solved. Comparison between the cryo-EM structures of YnaI determined using DIBMA and LMNG shows drastic conformational differences in the transmembrane domain, especially for the transmembrane helices TM1 and TM2 (Figure 4D). Compared with the structure isolated via the detergent-based method, the DIBMA structure failed to show the advantage of retaining the natively associated lipid and maintain the native structure of membrane proteins. Here, it is also worth pointing out that current



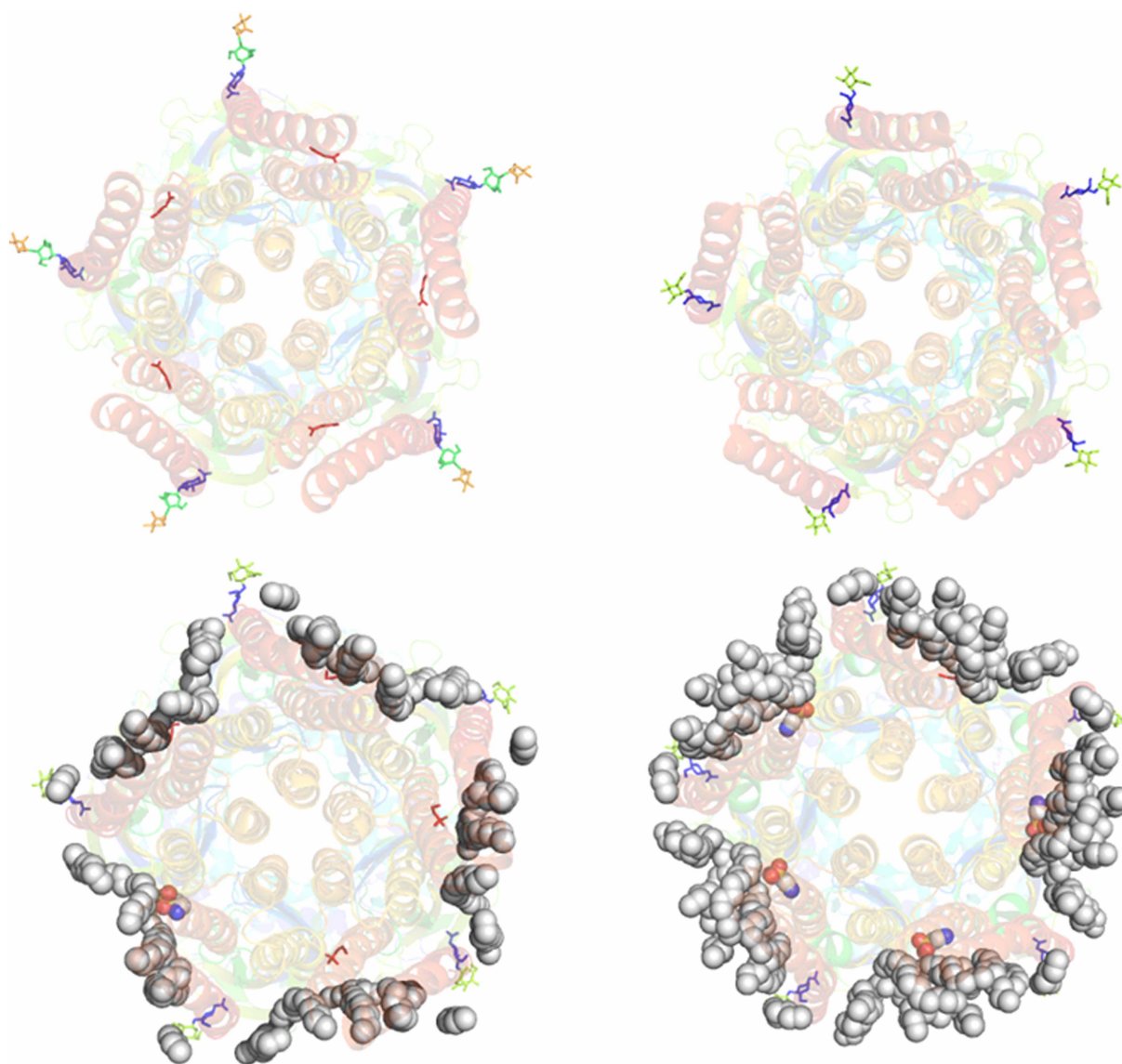
**Figure 4. Single-particle cryo-EM analysis of *E. coli* mechanosensitive channel YnaI.**

(A) Single-particle cryo-EM density of YnaI in a closed state. (EMD-11557) determined using DIBMA as a detergent-free method. The EM density in the transmembrane domain was not completely resolved; the EM density of TM1 and TM2 are not clear. (B) Single-particle cryo-EM structure of YnaI determined using detergent LMNG with addition of fluoridated Fos-choline 8 (EMD-20862). The transmembrane helices within the transmembrane domain are well resolved, however, no native lipid molecules were seen. (C) Single-particle cryo-EM density of YnaI in the claimed open state (EMD-11560). The EM density of transmembrane domain is not resolved at all, although the soluble domain is. (D) Structural comparison between YnaI determined using DIBMA (PDB: 6ZYD, orange colored) and LMNG (PDB: 6URT, gray colored). The two structures are significantly different for the TM1 and TM2 paddle, although the other portion is very similar; this reflects the potential bias of the structure determined using DIBMA resulting from the poor EM density in the transmembrane domain.

SMALP technology also failed initially in determining the cryo-EM structure of YnaI before the authors switched to DIBMALP. Related to this, it has also been reported that for human pannexin 1, samples prepared with SMALP technology produced a lower-resolution structure than samples prepared using detergent [67].

## Glycine receptor

The glycine receptor belongs to ligand-gated ion channels, which mediate signal transduction at neural synapses in neurotransmitters' responses [68]. Recently Eric Gouaux and colleagues reported 19 single-particle cryo-EM structures of the zebrafish glycine receptor in various states using a detergent-based method (1 structure), Nanodisc technology (5 structures), and SMALP technology (13 structures) [45]. Therefore, cryo-EM of glycine receptor structures serves as an excellent case for evaluation of the SMALP technology. Careful



**Figure 5. Single-particle cryo-EM analysis of zebra fish glycine receptor.**

(A) Single-particle cryo-EM density of glycine receptor in a closed state (PDB: 6PLZ) determined using SMALP technology. No well-solved lipid molecule EM density observed in the transmembrane domain. (B) Single-particle cryo-EM structure of glycine receptor in apo state determined using detergent (PDB: 6PXD). No well-solved EM density was observed in the transmembrane domain. (C) Single-particle cryo-EM density of glycine receptor in a closed state determined using Nanodisc technology (PDB: 6PLT). Some well-resolved lipid density was observed in the transmembrane domain and lipid molecules can be built in the structural model. (D) Single-particle cryo-EM density of glycine receptor in a desensitized state determined using Nanodisc technology (PDB: 6PLS). Significant well-resolved lipid density was observed in the transmembrane domain and lipid molecules can be built in the structural model. The gray spheres show the lipid molecules. The colored spheres show taurine molecules. Sticks show the sugar moieties at the glycosylation sites. Cartoons show the glycine receptor protein.

examination of the EM density maps corresponding to the reported 13 cryo-EM structures of glycine receptor reveals that none of them showed observable associated native cell membrane lipids density, although some structures were determined at high-resolution  $\sim 3.0$  Å or better. **Figure 5A** shows a representative glycine receptor structure determined using the SMALP technology. **Figure 5B** shows the structure of glycine receptors determined using detergent. Both detergent-based and SMALP technology failed to retain native cell membrane lipids naturally associated with the glycine receptor. By contrast, all five cryo-EM structures of glycine receptors determined using Nanodisc technology show clear lipid density within the transmembrane domain. Although those lipids are not natively associated with cell membrane lipids, it indicates that glycine receptors are naturally associated with cell membrane lipid molecules. **Figure 5C,D** show cryo-EM structures determined using the Nanodisc technology in closed and desensitized states, respectively. The glycine receptor case study also tells us that current SMALP technology could not retain those associated native cell membrane lipids. In terms of high-resolution determination, SMALP technology does not show the expected advantage of retaining native cell membrane lipids that are removed by detergents. However, the glycine receptor structures in the open and desensitized states solved using SMALP technology are consistent with the expected physiological conformations, despite the lack of resolved lipid density.

## Future directions

### Significance of detergent-free technologies and current status

Protein–lipid interactions are crucial for the natural structure and the normal function of membrane proteins. Detergents have been predominantly used for membrane protein structural biology since 1985, when the first membrane protein crystal structure was reported [69]. Detergents have played a primary role in advancing membrane protein structural biology [14,16]. However, laborious detergent screening to find the best detergent for each study is performed by membrane protein structural biologists, and, unfortunately, this is often not successful. Furthermore, the detergent selected after such screening may still be deleterious to the study because of the over-delipidation of the membrane protein. Thus, detergent-free methods that can extract membrane proteins and the associated native cell membrane lipids are preferred. Considerable progress in detergent-free method development has been made during the past decade or so since the first report of using the styrene-maleic acid copolymer for membrane proteins extraction [34]. This was accelerated following the high-resolution structure determination of bacterial rhodopsin, AcrB, and alternative complex III using these copolymers. Some dedicated detergent-free systems such as SMALP, NCMN, DIBMALP technology, and CyclAPol have emerged as promising alternatives. In examining the recently reported single-particle cryo-EM structures of five groups of membrane proteins, it can be concluded that currently, detergent-free systems for membrane protein structural biology, especially for high-resolution structure determination, are still in their infancy. Except for the evolving studies using the NCMN system, the current detergent-free technologies failed to show many of the expected advantages over detergent-based approaches. Whether the NCMN system also works for other membrane proteins, especially for human homologs, is yet to be determined.

Below, we present some suggested guidelines for obtaining and studying structures with detergent-free methods.

### The requirement for quality control of membrane-active polymers

All detergent-free systems depend on membrane-active polymers. Among these membrane-active polymers, SMA, DIBMA, and amphipols are most popular. Many derivatives based on these polymer backbones have also been developed [70–75]. Furthermore, new polymers with novel backbone structures have also been reported or are in development [48,49]. However, the suitability for high-resolution structure determination and the capability of retaining naturally associated and functionally important lipids remain to be examined for the many new membrane-active polymers. Systematic characterization and screening of these membrane-active polymers with model membrane proteins using single-particle cryo-EM analysis will be an excellent way to examine the quality of the new polymers. Only high-quality membrane-active polymers should be selected and used for membrane protein structural biology to guarantee the accuracy of the structural information of protein–lipid interactions.

Quality control of single-particles prepared with membrane-active polymers is also crucial in evaluating other biochemical and biophysical analysis results. Single-particle cryo-EM or at least negative stain EM should also be used to check the prepared sample's homogeneity. For example, lipid analysis of membrane protein

samples using the detergent-free method could be misleading if the particles prepared are not of good quality and crucial native lipids were not successfully retained in the particles. In theory, membrane-active polymers should maintain the native cell membrane lipids and the membrane protein supercomplex; however, this may not be true in practice, as in the cases of AcrB, alternative complex III, KimA, YnaI, and the glycine receptor described in this minireview.

Systematic SAR (structure-activity relationships) studies of membrane-active polymers in conjunction with single-particle cryo-EM analysis will be the best way to accumulate experimental data for theoretical research and subsequent rational design of membrane-active polymers.

## Protocols for the application of membrane-active polymers have to be optimized

Because of the structural diversity of membrane-active polymers, each polymer will have its unique set of properties. Therefore, an individual protocol should be developed for the application of each polymer for membrane proteins. Because of the diversity of cell membrane systems and local membrane protein–lipid interactions, it is probably impossible to have a single membrane-active polymer that can work well for all membrane proteins. However, some membrane-active polymers may be able to work broadly for many membrane proteins. Developing and screening such membrane-active polymers using model membrane proteins are urgent goals for developing the technology of detergent-free systems.

## Detergent-free technology in conjunction with proteoliposome for functional studies

Proteoliposome is a powerful platform for functional studies of membrane proteins, especially for transporters and channels. Both SMALP technology and the NCMN system have been reported for successful proteoliposome reconstitution for the functional study of membrane proteins [32,50,76]. In theory, the detergent-free technology's advantage is its capability to retain the native lipids with their membrane proteins and maintain the protein in its natural and functional state. However, it does not always work well, as in the case study of KcsA proteoliposome reconstitution where SMA2000 did not work well, and a novel NCMNP7-1 polymer was used for functional proteoliposome reconstitution [32], although SMA2000 was previously reported to be successful for reconstitution of KcsA onto a planar lipid bilayer [50]. This suggests the requirement that there be diversity of membrane-active polymers in practice.

Finally, I would acknowledge the remarkable achievements that have been made in understanding membrane protein structure and function using detergents. With caution, detergents will continue to make significant contributions, despite the drawbacks highlighted in this minireview.

## Perspectives

- Importance of the field: Membrane proteins play crucial roles in many biological processes and serves as the primary drug targets of modern drugs. Membrane protein structure biology can provide accurate structural information of membrane proteins, but only when membrane proteins can be studied within the cell membrane lipid environment. Dedicated detergent-free technologies have been explicitly developed for the membrane protein structural biology within the cell membrane lipid environment.
- Current thinking: The essentiality of detergent-free technology for membrane protein structural biology is well established as it is well known that detergent-based approaches have significant drawbacks. The detergent-free technologies such as SMALP, NCMN, DIBMALP, and CyclAPol are all promising platforms; however, they are all in their infancy.
- Future directions: Many diverse membrane-active polymers will be required for the detergent-free technology to be universally applicable. High-level standards for quality control need to be established to select high-quality membrane-active polymers.

## Competing Interests

Y.G. is the inventor of the NCMN system and a co-director of the SMALP network and has filed patents on NCMN polymers and corresponding protocols.

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## Abbreviations

Cryo-EM, cryogenic electron microscopy; CyclAPol, cycloalkane-modified amphiphile polymer; Cyt-aa3, cytochrome aa3; DIBMALP, diisobutyl maleic acid lipid particles; EMD, electron microscopy data resource; GPCR, G protein-coupled receptor; KUP, the potassium (K<sup>+</sup>) uptake permease; LCP, lipidic cubic phase; LMNG, lauryl maltose neopentyl glycol; NCMN, native cell membrane nanoparticles; NMR, nucleic magnetic resonance; PDB, protein database bank; PIP2, phosphatidylinositol-4,5-bisphosphate; SAR, structure-activity relationships; SMALP, styrene-maleic acid lipid particles.

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