Artificial membranes for membrane protein purification, functionality and structure studies.

Mayuriben J. Parmar[*#], Carine De Marcos Lousa[†#], Stephen P. Muench[*], Adrian Goldman[*‡] and Vincent L.G Postis[*#1]

[*]Astbury Centre for Structural Molecular Biology, School of Biomedical Sciences, University of Leeds, Leeds, LS2 9JT, UK.

[#]Biomedicine Research Group, Faculty of Health and Social Sciences, Leeds Beckett University, LS1 3HE, UK

[†]Centre for Plant Sciences, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT, UK.

[‡]Division of Biochemistry, Department of Biosciences, University of Helsinki, Helsinki, FIN-00014 Finland.

[1]To whom correspondence should be addressed (v.l.postis@leedsbeckett.ac.uk)

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Abstract
Membrane proteins represent one of the most important targets for pharmaceutical companies. Unfortunately, technical limitations have long been a major hindrance in our understanding of the function and structure of such proteins. Recent years have seen the refinement of classical approaches and the emergence of new technologies that have resulted in a significant step forward in the field of membrane protein research. This review summarises some of the current techniques used for studying membrane proteins, with overall advantages and drawbacks for each method.

Micelles and classical detergent techniques
Membrane proteins account for ~30% of both prokaryotic and eukaryotic proteins[1]. Integral proteins such as transporters or ion channels, as well as peripheral membrane proteins such as G-proteins, all perform essential tasks in signal transduction, cell metabolism and transport of small molecules[2–4]. Integral and peripheral membrane proteins are respectively embedded in or closely associated with the phospholipid bilayer of cell membranes. Therefore, their function often relies on their precise lipid environment[5,6]. For instance, cardiolipin, which constitutes about 20% of the inner mitochondrial membrane, is essential to the function of many mitochondrial transporters such as the ADP/ATP carriers, the enzymes of the respiratory chain, and bacterial proteins[7–9]. This is because cardiolipin offers polar and electrostatic interactions that increase protein stability[10]; similar interactions have been observed for other lipids[11,12]. Unfortunately, classical techniques to study membrane proteins involve the use of detergents that solubilise the protein but also destabilise its interaction with membrane lipids. In many cases, membrane proteins may be stable in only a few detergents, limiting the range of conditions that can be used for crystallization trials[13]. Consequently, much time is spent testing various detergents in different ratios and concentrations in the hope of finding conditions that will mimic the essential interactions of the protein with its natural lipidic environment - and this way stabilise the protein and preserve its functional state. These problems have hindered membrane protein research for many years: both biophysical characterisation and structure solution have suffered due to difficulties of extracting proteins from membranes and keeping them stable away from their native environment. The past few years have seen the emergence of new techniques aimed at providing a membrane-like natural environment. These novel techniques include liposomes, bicelles, discs, polymer and lipids based strategies.

1. Liposomes
Reconstitution into liposomes is the oldest and one of the most widely-used methods for reconstituting membrane proteins into lipids. Liposomes contain a single bilayer consisting of phospholipids that assemble spontaneously in a vesicle. They are usually formed by hydration of dried lipids, which results in the formation of large multilamellar vesicles separated by a water layer. By adjusting the lipidic mixes and ratios, the nature and size of liposomes can be manipulated. Liposomes can be composed of pure lipids or mixed lipids and proportions can be adjusted to the protein requirements[14]. Liposomes are currently very popular in the biomedical field due to their ability to enter cells by phagocytosis, and so they are used for gene transfer and drug delivery.

The size of liposomes can also be tuned by subsequent procedures such as swelling[17], extrusion[18], emulsion, sonication[19], electroformation[20], inkjet formation[21], microfluidic jet[22,23] freeze thaw cycles[24]. Sonication and extrusion are the two most popular techniques. Sonication usually results in small unilamellar vesicles (diameter 15-50 nm), whereas extrusion results mostly in large unilamellar vesicles (diameter > 100 nm). The latter procedure uses a set size of filters, resulting in a more homogeneous mean diameter of particles. For this reason, it is usually preferred to sonication. Extrusion through a 100 nm filter typically results in large unilamellar vesicles ranging between 120 and 140 nm, but can vary depending on the lipid composition. The subsequent particles can be analysed using techniques like Electron Microscopy (EM), light scattering, Atomic force microscopy and NMR. During liposome self-assembly, a small amount of water is generally encapsulated and separated from the extraliposome solution. This has many advantages in terms of membrane transporter function, as proteoliposomes with reconstituted transporters can be tested functionally by their ability to selectively concentrate substrate within the liposome. Similarly, if the proteoliposomes are formed in the presence of a substrate and washed, transport to the buffer can also be assessed[25]. In both cases, the leakage of the proteoliposomes and the orientation of the protein within the proteoliposomes should be assayed as controls.

2. Bicelles

Bicelles can be thought of as solubilised membrane bilayer disks. They are formed by mixing membranes with a short chain lipid (or a detergent) and a long chain lipid[26–29]. The long chain phospholipid forms the membrane protein-containing bilayer, which is then stabilised by the short amphiphilic chain positioning at the rim of the bilayer[30]. The most popular choice so far is a combination of DHPC (dihexanoylphosphatidylcholine) (short) and DMPC (dimyristoylphosphatidylcholine) (long). Bicelles adopt different conformations depending on the ratio of long to short lipids (the q-ratio), the temperature, pH and salt concentrations[30]. Simulations suggest that bicelles adopt a cigar-shaped structure that is converted into a disc-shaped structure when the integral membrane protein is added[30]. Protocols studying membrane proteins in bicelles have involved mixing bicelles with either purified proteins, when these are very stable, or detergent-solubilised proteins, but most bicelles are added as proteoliposome solutions. Bicelles are thus native-like environments for membrane proteins[30].

NMR studies have been the preferred downstream method to analyse membrane protein-containing bicelle particles, both in liquid-state or solid state NMR. Several membrane protein structures have been solved using bicelles in NMR[31,32]. Although bicelles are easy to handle, the need for a tight [DMPC]:[DHPC] ratio is often tricky and the detergent exchange step when adding bicelle can sometimes result in aggregation[33,34].

3. Artificial Discs

a. Nanodiscs

Nanodiscs were first introduced by Sligar and coworkers[35]. The Nanodisc is a non-covalent assembly of phospholipid and a genetically engineered “membrane scaffold protein” (MSP), which is itself based upon human serum apolipoprotein A-I[36]. Apolipoprotein A-I is the main protein found in high density lipoprotein: it helps shape the vesicles by forming an amphiphilic interface between lipids and water that acts like a scaffold [37]. MSP plays a similar scaffolding role to Apolipoprotein A-I around the phospholipid bilayer containing the membrane protein of interest[36]. Nanodiscs are made by mixing bicelles containing the protein of interest with MSP followed by slow detergent removal through techniques such as dialysis or BioBeads[38]. Several sizes of MSPs have been engineered to adapt to different membrane protein structures and shapes[39–42]. This feature is a great advantage for studying the oligomerisation of a protein or large protein complexes. Other advantages include solubility, stability and monodispersity[43]. The nanodisc technology however has some drawbacks. One is the ratio of phospholipid/MSP, which needs to be tightly controlled and which depends on the presence of the membrane protein: if the ratio is incorrect, the protein and the MSP aggregate[39]. The presence of large self-aggregates can usually be detected by size exclusion chromatography, although sometimes these elute near that of correctly formed Nanodiscs[36]. Another drawback is the multistep procedure required: the MSP has to be purified, and then mixed in the correct ratio with an already-purified membrane protein reconstituted into bicelles, with the attendant losses of both protein amount and activity even before reconstitution into nanodiscs.

Nonetheless, nanodiscs have become popular for structural studies of membrane proteins because they maintain the stability of the protein, as long as the phospholipid/MSP ratio is correct. A number of membrane proteins, including cytochrome P450 monooxygenase[35,44], bacteriorhodopsin[45], rhodopsin[46,47], β2-adrenergic receptor[48,49], and the human mitochondrial voltage-dependent anion channel (VDAC1) have been studied using nanodisc technology[50].

b. Peptide disc

To simplify the nanodisc procedure while maintaining the stabilisation provided by nanodiscs, a new strategy, named peptide disc, has been developed. This is based on the use of synthetic 18 amino acid peptides mimicking the amphipathic helices of Apolipoprotein I. One of the most common peptides, called variously 18A or 2F, has the following sequence: DWLKAFYDKVAEKLKEAF[51]. These mimetic peptides have protective effects in high cholesterol patients and in atherosclerosis[52–54]. Used in a membrane protein purification context, mimetic peptides self assemble in discoidal particles like nanodiscs[55]. A large range of mimetic peptides can modulate the diameter of the discs. The main advantage of mimetic peptides is their ability to solubilise vesicles into nanodiscs at physiological conditions without detergent[55,56]. Due to the lack of stability of some peptide discs, a range of different mimetic peptides with various features is being developed with promising results[57].

4. Polymer-based strategies

a. Amphipols

Amphipols have been designed for the purification of membrane proteins. They are short amphiphilic polymers that contain a large hydrophilic moiety and closely spaced hydrophobic groups[58]. After solubilising the protein with detergents, the polymer forms a thin layer around the protein by interacting with the hydrophobic regions of membrane proteins, while allowing the complex to stay in aqueous solution due to the hydrophilic regions[59]. Amphipols have become increasingly successfully to study the function and structure of many membrane proteins by subsequent approaches such as mass spectrometry, electron microscopy and crystallography[60–63]. Recently, they have been used to purify the entire respiratory chain complex from bovine heart mitochondria and study its functionality[64,65]. However, it also presents the same disadvantage as bicelles and nanodiscs: the first step is based on detergent solubilisation, necessitating optimisation of the nature and the concentration of the detergent(s) used, creating potential difficulties in reconstitution and of course loss of protein activity.

b. SMALPs

Recently a novel technique, the styrene maleic acid (SMA) lipid particle system termed SMALPs, has been explored by Dafforn and colleagues[66–69]. Similar to discs and amphipols, SMA polymers assemble around the membrane protein to mimic the hydrophobic environment and stabilise the protein. However, unlike discs and amphipols, SMA can be used without the prior use of detergent, which is a major advantage. Not only does it reduce the time required for protein purification, as there is no need to find suitable detergent(s), concentrations and solubilisation conditions, but it can also be used directly on membranes. Membrane proteins can thus be purified in their native lipid environment[70], meaning that most membrane proteins purified in SMALPs retain activity[71]. The SMA polymer acts as a ‘’cookie cutter’’ directly in the membrane and forms a scaffold of nanodiscs around the membrane protein. The size of the most widely used SMALPs is such that they extract on average one protein per disc. The styrene groups provide hydrophobic character to the polymer, and the carboxylate groups, hydrophilic character. Because of their p*K*a, the carboxylate groups are uncharged at a pH below 6[72]. As a result, SMALPs are much more hydrophobic, and so they precipitate at acidic pHs. This pH dependency is a potential drawback for certain classes of proteins whose function depends on pH. However, this feature allows easy release of the protein from discs if necessary. The SMALP technique has successfully been used on various types of membrane proteins extracted from various organisms[66,67,73], though it was not successful for human equilibrative nucleoside transporter (Jaakola personal communication), indicating that, like all other techniques, the SMALP technology is not a universal solution for all membrane proteins.

5. Other strategies

a. Planar Lipid Membranes

Planar lipid membranes are artificial lipid membranes that can be used to assess electrophysiological properties and functions of channel molecules. Unlike the techniques described above, they are not means for purifying membrane proteins, but ways of reconstituting them. The source of the lipids could be natural, synthetic or mixed. There are three kinds of planar lipid membranes: Black lipid membranes (BLM), supported lipid bilayers (SLB) and tethered bilayer lipid membranes (tBLM).

BLMs refer to a lipid bilayer formed in an aperture created in a thin layer of hydrophobic material[74] that is part of the wall that separates two chambers filled with (different) aqueous solutions. The setup thus allows for example, transport measurements across the membrane by making the two solutions different. The term ‘’black lipid layer’’ refers to the fact that the bilayer appears black in reflected light[75]. Detergent solubilised membrane proteins added to the solution spontaneously incorporate into the preformed BLM. The main drawback of BLMs is the lifetime of the membrane which does not exceed one hour, thus limiting long experiments.

SLBs are an alternative to BLMs, where a planar bilayer is placed on a solid support. The main advantage is the stability of the bilayer, which lasts for weeks or more, making long experiments feasible. In addition, experiments using SLBs are much more reproducible than those using BLMs because multiple runs can be done on the one SLB, rather than having to make a new SLB each time, and multiple experiments can be performed using various conditions, such as different ligands in a binding or transport assay. In this technique, however, only the upper face of the bilayer is accessible to substrates. Moreover, introduction of membrane proteins with large lumenal domains can be hindered by the relatively small space between the bilayer and the solid support, which leads to protein denaturation. This has been overcome by the introduction of tethered bilayer lipid membranes (tBLM), which are similar to SLBs but the lipids are chemically anchored to the support, usually gold, *via* the introduction of a spacer[76]. This introduces a space between the lipids and the gold support to allow for lumenal domains of membrane proteins to be accommodated below the bilayer[76,77]. Although some optimisation is still necessary, t-BLMs represent a promising technique to study membrane protein functionality and structure[78,79].

b. Lipidic cubic phase

Lipidic cubic phase (LCP, also referred to as *in cubo*) has become a popular method for the crystallization of membrane proteins, particularly G-protein coupled receptors (GPCRs), by extracting them out of detergent solution into a lipid-water mixture. LCP is a specific region in the phase diagram of certain lipid-water mixtures where the lipid forms a bilayer that, because it is highly curved, consists of a completely-connected three-dimensional sheet penetrated by an equally-interconnected system of aqueous channels[80]. Both the lipid sheet and the aqueous channels have crystallographic “cubic” symmetry, hence the name. Because the lipid is made up of single connected bilayer, a membrane protein in it can in principle diffuse freely. This method uses a mixture of monoolein lipid and

purified membrane protein which diffuses in the lipid to form and feed crystal nuclei. The lipid bilayer environment maintains the stability of proteins and removes the protein aggregates and other impurities. Numerous membrane proteins, including bacteriorhodopsin and many different GPCRs, have been successfully crystallized and their structures solved using this approach. Because of the high curvature and small aqueous channel size of the first lipid used, monoolein, which is the preferred lipid, it seemed that this technique might be only suited to relatively small membrane proteins like GPCRs. This does not appear to be the case, and crystallisation in LCP has become one of the standard membrane protein techniques, and especially for membrane proteins with small soluble domains[81,82].

LCP crystallisation has one major advantage over conventional crystallisation in detergent: the protein remains in a more native environment. Nevertheless, the high viscosity of the phase making it difficult to extract and difficulties visualising the crystals represent significant hurdles. Recent new technologies, like using commercial robots and pre-crystallization assays like LCP-FRAP (Fluorescence recovery after photobleaching)[82], have made this technology more accessible and improved the success rate.

Conclusion:

For a decade now, membrane protein studies have benefited from advances in artificial membranes. Although many studies on membrane proteins still use classical methods based on detergent solubilisation, emerging alternative detergent free techniques present many advantages. Firstly, they are less time consuming and more cost effective. The crucial advantage, however, is the preservation of a native/native-like lipid environment, which usually contributes to conservation of functional and structural properties. Although the field still requires technical improvements, the developments in the way we can now stabilise and study membrane proteins will make many more systems tractable through structural and biochemical means.

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Figure Legend:

Figure 1: Comparison of structures of various artificial membranes.

(A) Bicelles are formed by mixing 2 different lipis: One long chain phospholipid (yellow) which will interact with the protein, and one short chain phospholipid (red) which will arrange in the rim of the disc. (B) Nanodiscs are derived from Apolipoprotein I and assemble around detergent-solubilized membrane proteins. (C) Peptide discs composed of the assembly of 18 amino acids peptides, assemble around the membrane proteins similarly to nanodiscs. (D). SMALPs are polymer based particles which act as cookie cutters and cut out portions of membranes containing membrane proteins.

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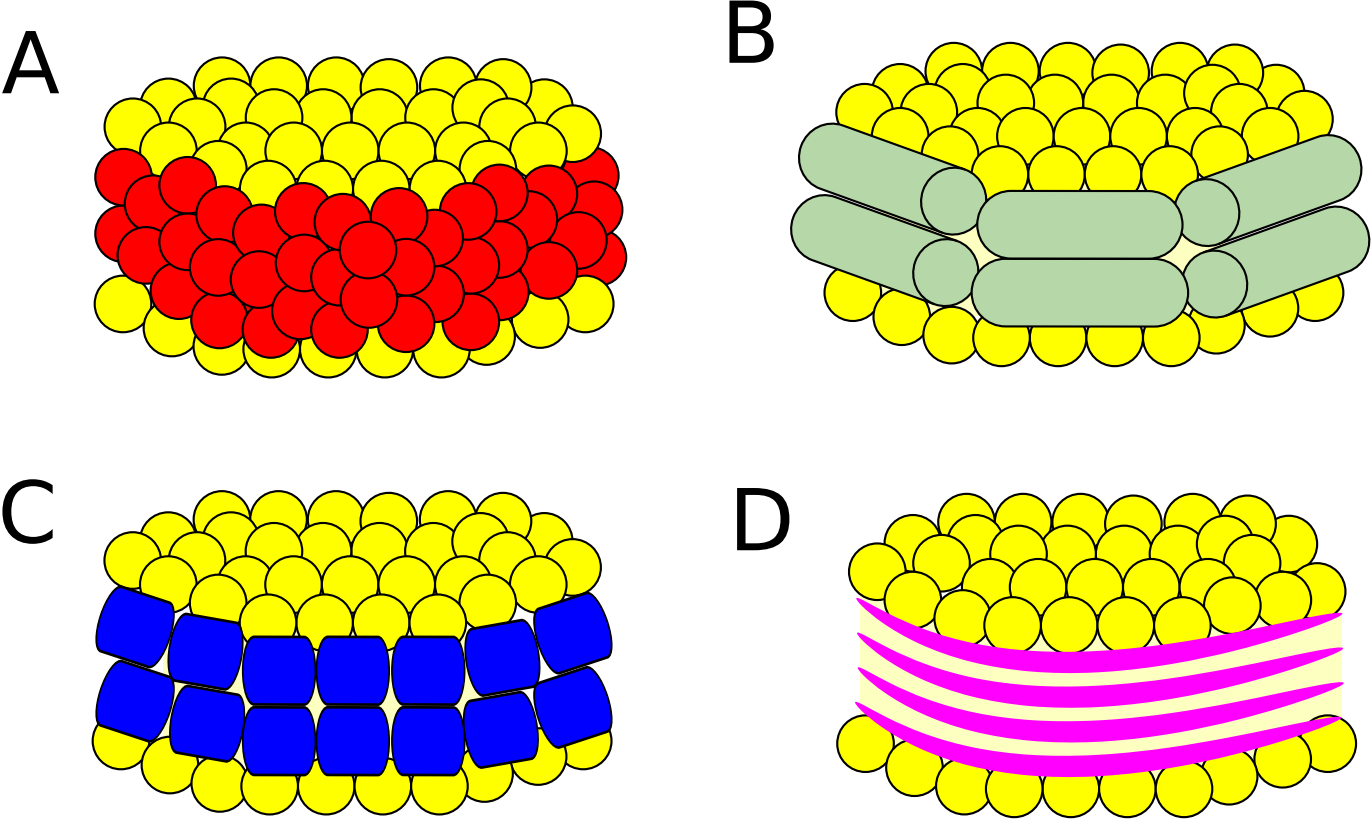
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