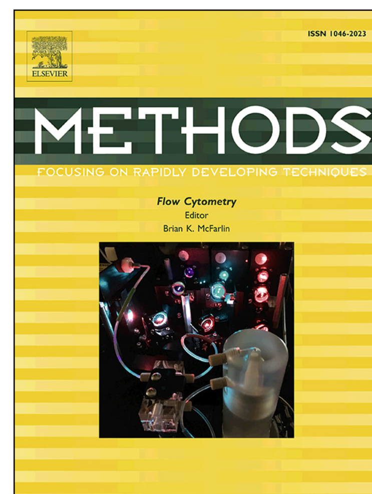
Accepted Manuscript



Methods of reconstitution to investigate membrane protein function

Ruth Skrzypek, Shagufta Iqbal, Richard Callaghan

PII: S1046-2023(17)30446-2
DOI: https://doi.org/10.1016/j.ymeth.2018.02.012
Reference: YMETH 4410

To appear in: *Methods*

Received Date: 9 January 2018
Accepted Date: 13 February 2018

Please cite this article as: R. Skrzypek, S. Iqbal, R. Callaghan, Methods of reconstitution to investigate membrane protein function, *Methods* (2018), doi: https://doi.org/10.1016/j.ymeth.2018.02.012

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Methods of reconstitution to investigate membrane protein function

Ruth Skrzypek, Shagufta Iqbal and *Richard Callaghan

Division of Biomedical Science & Biochemistry, Research School of Biology and Medical School, The Australian National University, Canberra, Australia

ACCEPTED MANUSCRIPT

Correspondence:

*Research School of Biology, Building 134, Linnaeus Way, The Australian National University, Acton, Canberra, ACT 2601 Australia

Tel: +61 2 6125 0824

E-mail: richard.callaghan@anu.edu.au

ACCEPTED MANUSCRIPT

ABSTRACT

Membrane proteins are notoriously difficult to investigate in isolation. The focus of this chapter is the key step following extraction and purification of membrane proteins; namely reconstitution. The process of reconstitution re-inserts proteins into a lipid bilayer that partly resembles their native environment. This native environment is vital to the stability of membrane proteins, ensuring that they undergo vital conformational transitions and maintain optimal interaction with their substrates. Reconstitution may take many forms and these have been classified into two broad categories. Symmetric systems enable unfettered access to both sides of a bilayer. Compartment containing systems contain a lumen and are ideally suited to measurement of transport processes. The investigator is encouraged to ascertain what aspects of protein function will be undertaken and to apply the most advantageous reconstitution system or systems. It is important to note that the process of reconstitution is not subject to defined protocols and requires empirical optimisation to specific targets.

KEYWORDS

Integral membrane protein; reconstitution; biomembranes, lipids, detergents, unilamellar vesicles, lipid bilayer, nanodiscs, liposomes

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

1 INTRODUCTION

The outer, or plasma, membrane of cells controls the import and export of nutrients, toxins, endo- and xenobiotics. It also regulates signalling to other cells or transduction of signals from the external milieu. The lipid bilayer structure enables it to form an adaptive and highly responsive barrier to the "outside world". Many of these key transport and signalling roles are undertaken by membrane proteins, which belong to a large variety of families. Membrane proteins may be classified as integral, peripheral, anchored and attached. The prominent functional roles of integral membrane proteins (IMPs) has led to the oft-quoted statistic that they comprise more than half of all "druggable" targets.

Investigating IMPs in their native cellular environment provides information on the cellular consequences of their activities. However, obtaining detailed information on their mechanism of action and their kinetic, or specificity, parameters is not straight-forward in a cellular system. Membranes provide a highly complex and integrated environment with competing or ancillary components to maintain organelle integrity and function. Consequently, investigating IMPs in isolation can provide important information on mechanism and by extension, therapeutic or translational intervention. Why then, do so many researchers baulk at the idea of isolating IMPs for functional analysis? There are many factors at play here including the notorious difficulty in achieving sufficient expression in heterologous systems. IMPs are large proteins and display high flexibility and unerring instability in isolation. With the advent of recombinant DNA technology the use of affinity purification systems greatly simplified their purification. However, due to their architecture and insertion within the lipid milieu, IMPs require extraction from their native environment to enable purification strategies. Extraction, typically by surfactants, is a considerable bottle-neck that requires much labour intensive empirical investigation.

Once purified, there are an extraordinary range of functional assays to investigate IMP function and all aspects are catered to. However, IMPs exist and function within a highly regulated or specific environment. Consequently, following their isolation, IMPs will require re-insertion into a lipid bilayer environment to undertake their activity. This process, known as reconstitution, may be considered by some as an art-form. There are very few rules governing reconstitution, the process remains inscrutable and there are no generic protocols. Reconstitution requires considerable empirical effort and there is a wealth of experience available in the literature.

This chapter presents the strategies and methods underlying many highly used reconstitution systems. The processes are from the perspective of reconstituting IMPs belonging to the transporter sub-class, although the methods are inter-convertible with enzymes and receptors. The slant

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

towards transporters led to the processes being divided into two categories; symmetric or compartmental. Advantages and drawbacks for each system are provided and numerous literature examples of each are cited. Our key message is that investigators will first need to ascertain what type of mechanistic information they require with the target IMP. This will lead to a decision on the downstream functional assays and the choice of reconstitution system will become apparent.

2 SYMMETRIC INVESTIGATIVE SYSTEMS

2.1 Detergent Micelle Preparations

In the vast majority of situations the investigation of isolated membrane proteins relies on their extraction from expression systems including bacteria, yeast, insect or mammalian cells. Detergents are amphipathic molecules and several distinct classes have been developed over the decades. The hydrophobic portion of detergents interacts with IMPs to form a micelle structure and the hydrophilic portion provides water solubility (1). However, putting this system into practice is not as simple as it sounds. The most effective detergents for solubilisation of a specific protein may not be the most effective at preserving its structure. Furthermore, detergent compatibility with protein function must be analysed on a case-by-case basis.

The choice of a specific detergent is also dependent on its physico-chemical properties. For example, to extract proteins from a membrane, the concentration of detergent must be kept above its critical micelle concentration (CMC) (1,2). For cost reasons, detergents with low CMC values are more desirable; however, they are notoriously difficult to remove prior to downstream functional assessment of the protein (3). Temperature also affects the extraction process and detergents must be kept above a critical temperature (Krafft point or critical micelle temperature) to ensure micelle formation. It is also advisable to use detergents with shorter alkyl chains since the increase in hydrophobicity with larger tails renders the detergent less soluble in aqueous phase. Generally, non-ionic detergents, which are characterised by uncharged hydrophilic head groups, are preferred. These mild detergents tend to disrupt protein-lipid and lipid-lipid interactions rather than intra-protein interactions (4). Zwitterionic detergents have also gained traction with membrane protein extraction. These detergents have both positively and negatively charged groups in the hydrophilic head and have high CMCs. The choice of buffer during solubilisation also plays an important part as the high salt concentration can lowers the CMC of detergents (5).

During the process of extraction, detergents strip membrane lipids away from proteins, which may lead to instability and inactivation. To maintain the functionality of the membrane proteins, exogenous lipids are added during the extraction process resulting in the formation of bicelles

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

and/or mixed micelles (5). The added lipids solvate the hydrophobic portion of membrane proteins and thereby render them more stable and functional. Many studies have reported the use of lipids for increased stability of purified membrane proteins; 1,2-dimyristoyl-*sn*-glycero-3-phophocholine (DMPC) for opsins (6), monogalactosyl diacylglycerol (MGDG) for bacteriorhodopsin (7), and cholesteryl hemisuccinate (CHS) for P-glycoprotein (8). Choosing the "correct" lipid additives is unfortunately an empirical process.

2.1.1 Method of membrane protein purification and reconstitution with mixed micelles

The example method below describes the process for the extraction and purification of the multidrug transporter, P-glycoprotein (P-gp)

i) Lipid films are prepared from a solution of total *E. coli* lipid extract and cholesterol 4:1 (w/w) in chloroform:methanol (2:1). The lipids are stored in sealed vials and the film prepared in glass test-tubes using gentle evaporation under nitrogen, then residual solvent is removed by vacuum evaporation (1-2 hr)
ii) The films are re-suspended in solubilisation buffer (20mM MOPS, 200mM NaCl, 20% glycerol pH 6.8) containing 1% (w/v) n-Dodecyl-β-D-Maltopyranoside (DDM) to a final lipid concentration of 0.4% (w/v). Re-suspension is achieved by sonication until the solution turns from cloudy to clear or translucent
iii) Insect cell membranes are resuspended in solubilisation buffer to a final total protein concentration of 5mg/ml. This protein concentration was selected by prior empirical testing (9). The mixture is subsequently treated in a Dounce homogeniser
iv) The suspension is incubated at 4˚C for 1-2 hours with gentle stirring and the insoluble fraction (pellet) removed by ultra-centrifugation. The polyhistidine-tagged P-gp is purified from the soluble fraction (supernatant) using immobilized metal affinity chromatography with Ni-NTA resin
v) The soluble fraction is incubated with Ni-NTA resin for an hour at 4˚C on a rocker platform. The resin is then transferred into a gravity column and washed with buffer (20mM MOPS, 200mM NaCl, 20% glycerol pH 6.8) containing a lower concentration of detergent (0.05% w/v) and lipid (0.1% w/v) to maintain the mixed micelles. A step-gradient of imidazole (10 to 20 to 80mM) is used to remove contaminants and pure P-gp is eluted in 400mM
vi) The purified P-gp is subsequently reconstituted by adsorption of detergent to SM2 Bio-beads. The BioBeads are pre-treated sequentially with 10 volume equivalents of H_2O, 50% methanol, 100% methanol, 50% methanol and H_2O

ACCEPTED MANUSCRIPT

vii) The pre-washed BioBeads are added to the purified protein (9) for removal of detergent at ambient temperature over a 2-3 hr period. Adsorption of detergent by BioBeads is greatly reduced at 4°C and may take 1-2 days for complete removal

2.1.2 Pros and cons of using detergents

Detergents are a near indispensable first choice to extract membrane proteins; however, there are some issues associated with their use. Firstly, micelles represent a poor mimetic system of a native membrane. For example, the alkyl chain length of detergents is lower than membrane lipids and many proteins require the lateral pressure found in native membranes, which is absent in micelles. Finally, detergents can destabilise the target protein by disrupting vital internal protein-protein interactions (10,11). Moreover, screening for a suitable detergent class, protein concentration, buffer and temperature conditions is a time-consuming process and requires cross-validation of protein function (12). Finally, the presence of detergent monomers in the solution can lead to the aggregation of protein.

Development of novel detergents with the ability to overcome the problems outlined above remains a continuing objective. The introduction of amphipols (13) has been one of the major advance in this respect. Amphipols are amphiphilic molecules that surround the hydrophobic transmembrane portion of membrane proteins, thereby stabilising them. Novel detergents including glucose neopentyl glycol (GNG), maltose neopentyl glycol (MNG), and calixarene have been developed which have additional hydrophobic, hydrophilic and aromatic groups to stabilize and solubilize the membrane proteins at the same time. These detergents have been successfully used to purify functional transporter proteins (14-16). GNG and MNG have helped determining the high resolution structures of G-protein coupled receptors (17), aquaporin 2 (18) and Na^+ pumping pyrophosphatase (19).

2.2 Membrane protein reconstitution into nanodiscs

Mixed detergent-lipid micelles are an essential element of the purification process for membrane proteins. X-ray crystallography based structural studies with membrane proteins are amenable to micelle based systems. However, the function of membrane proteins is frequently impaired in a micelle system (10). The previous section outlined a method to reconstitute membrane proteins from micelles to liposomes. Moreover, the use of compartmental systems is discussed in section 3. Reconstitution into liposomes is affected by the orientation of inserted protein, which may also affect functional studies. Non-compartment reconstitution systems offer the advantage of complete access to both faces of a membrane protein and nanodiscs provide one such system.

ACCEPTED MANUSCRIPT

Sligar and co-workers (20) coined the term “nanodiscs” in 2002 for the structures formed by reconstitution of an integral membrane protein into phospholipids in the presence of apolipoprotein A1. Nanodiscs are typically 8-16nm in diameter (21), composed of a discoidal phospholipid bilayer encircled by a belt made up of two molecules of a helical protein termed “membrane scaffolding protein” (MSP). MSPs are peptides derived from human serum apolipoprotein A1, typically consisting of 22 residues forming helical repeats of proline and glycine, although recombinant MSPs of different sizes are also commercially available. Membrane proteins within nanodiscs are stable owing to a strong interaction between the hydrophobic residues of the MSP and the acyl chain of lipids (22,23). The reconstitution of a membrane protein into nanodiscs involves more parameters than insertion into liposomes. Specific parameters include (a) the stoichiometric ratio of MSP and lipids to the target membrane protein, and (b) the size of the MSP (24). Ratios for each target protein need to be optimised empirically. If the ratio is sub-optimal, the size range of the nanodiscs formed will be broad, and the stability of the membrane protein may be compromised (25). In addition, larger MSPs tend to generate large nanodiscs that may not be compatible with biophysical techniques, particularly those involving spectroscopy (26).

2.2.1 Method for reconstituting into nanodiscs

Nanodiscs can be prepared using one of two strategies:

Strategy 1: Detergent extracted proteins are first chromatographically purified and then added to the mixture of MSP and lipids at an appropriate ratio

Strategy 2: Cell membrane proteins are solubilised with detergent and then directly added to the mixture of MSP and lipids in an optimal ratio for set period of time. The target protein within nanodiscs is subsequently purified by chromatography

The only difference between the two methods is the point at which the purification step is undertaken and a generic protocol follows (23,27-31):

i) The poly-histidine-tagged recombinant MSPs (e.g. MSP1E3D1) are expressed in *E.coli* using an IPTG inducible expression system (24). Our preferred method to harvest the protein is through physical disruption using a Constant Systems Cell Disruptor at approximately 30 kpsi.
ii) Following centrifugation at 22,000g, the supernatant is collected and the MSP purified by affinity chromatography using imidazole for washing and elution
iii) Membranes containing the target proteins are solubilised with appropriate detergent and purified by affinity chromatography. (refer to section 1 for an example procedure)
iv) A lipid film is generated and resuspended in aqueous buffer as described in *Section 2.1.1*

ACCEPTED MANUSCRIPT

v) Purified MSP and lipids are added to the purified protein at specific ratios dependent on the protein. For example, for $MalFKG_2$ the optimal molar ratio) of protein:MSP:lipid is 1:3:60 (28). This step is carried out at 4˚C overnight on a rocking platform

vi) Pre-washed SM2 Bio-beads (Section 2.1.1) are added to the mixture for 1-2hr (gentle rocking) to remove the detergent by adsorption and generate nanodiscs. The beads are then allowed to settle by gravity and the supernatant is removed

vii) Any protein aggregates formed during the reconstitution process are removed by centrifugation at 2000g for 5 minutes

viii) A portion of the final suspension is analysed by gel permeation chromatography. Successful reconstitution into nanodiscs is validated by co-migration of the target protein and MSP during chromatography

2.2.2 Advantages of Nanodiscs

Nanodiscs offer stability, lateral pressure and the presence of a full length lipid bilayer arrangement, compared to the more heterogenous environment found in detergent micelles and bicelles. The monodispersity of nanodisc size is advantageous for spectroscopic studies of target proteins (32). As mentioned earlier, membrane proteins in nanodiscs are accessible from both faces and ligand binding studies show the same binding kinetics as in native membranes. Finally, nanodisc composition may be precisely manipulated to enable investigation of membrane protein interactions with surrounding lipids and in an area considerably smaller than is possible with liposomes (33).

2.2.3 Limitations of nanodiscs

Similar to the use of detergent micelles and liposomes, the procedure for reconstitution into nanodiscs requires the use of detergents. As described earlier, there are several *possible disadvantages* in the use of detergents for membrane protein structure and function. Second, the lipid environment generated in nanodiscs, although controlled, is not an exact mimic of the native lipid bilayer and interaction of proteins with exogenous lipids may differ from that with native lipids. Another limitation is that the α-helical structure of MSP can interfere with electron paramagnetic resonance (EPR) and nuclear magnetic resonance (NMR) based assays. Finally, the lack of compartmentalisation prevents vectorial transport measurements on membrane proteins in nanodiscs.

2.2.4 Perspectives in nanodisc-mediated reconstitution

Improvements in this technology have focussed on increasing the yield, homogeneity and stability of membrane proteins in nanodiscs. This has largely been achieved by precisely controlling the lipid

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

composition and by improving genetically engineered MSPs. Despite these efforts, the fundamental knowledge elaborating the exact mechanism and kinetics of spontaneous reconstitution of membrane proteins into nanodiscs and the effect of lipids, temperature and MSPs on nanodisc formation is not yet fully understood.

The systems described in the previous sections have one common disadvantage; the removal of native lipids during extraction which in many cases, renders the membrane proteins unstable and non-functional. Several strategies have been employed to overcome this problem including protein production in cell-free system (34), genetic engineering of membrane proteins (35) or by reducing the exposure time with detergent (36). However, the most promising tool to overcome this problem so far involves the use of styrene maleic acid (SMA) based particles.

2.3 Styrene Maleic Acid Lipid Particles (SMALPs)

Styrene Maleic Acid (SMA) is synthesized by the copolymerization of styrene and maleic acid anhydride monomers. The anhydride form of SMA (SMAnh) has long been used in the plastic industry to increase thermal stability of plastic blends. The hydrolysed form is used as a dispersing agent for water and solvent based inks and coatings to increase heat and water resistance, gloss and colour development. SMA is also used in cancer chemotherapy to form stable drug-conjugates (37,38), which has been adapted as a drug delivery system by encapsulating hydrophobic drugs (39). Co-polymers are available with different ratios of styrene to maleic acid and of different average molecular weight. Even within a single formulation there may be variations in composition and molecular weight caused by varying sequences of styrene and maleic acid monomer incorporation during polymerisation (40).

Knowles and co-workers were the first to report the use of SMA co-polymer for the purification of PagP and bacteriorhodopsin (41). The SMA polymer forms a belt around membrane proteins encapsulating a portion of the lipid bilayer to generate particles of similar structure to nanodiscs but without the need for detergent at any stage of formation. The different terminology used for these particles in the literature includes Styrene Maleic Acid Lipid Particles (SMALPs) (41), Lipodisq™ particles (42), or native nanodiscs (43).

Small angle X-ray scattering (SAXS) and small angle neutron scattering (SANS) measurement studies have shown that SMALPs are disc-shaped with ~10nm diameters and ~4.6nm thickness (44). Transmission electron microscopy (TEM) showed a population with diameters between 11.1±3.3nm and 16.0±3.0nm (44). Lipid composition of the native membranes show no effect on the size of the discs, but it has been suggested that a low polymer to lipid ratio may increase the size of the

ACCEPTED MANUSCRIPT

particles (45,46). Futhermore, the hydrophobic styrene head penetrates into the tails of a lipid bilayer (44,47) to interact with the acyl chains of phospholipids in a manner similar to cholesterol (48). This interaction has been studied by NMR (42) and FTIR spectroscopy (49) and revealed that the SMA phenyl ring intercalates perpendicular to the plane of lipid between acyl chains. An EPR study (42) demonstrates that the movement of phospholipid acyl chains is restricted by this insertion.

There are commercially available variants of SMA polymers based on the styrene to maleic acid ratio. The most hydrophilic SMA is 1.4:1 and the most hydrophobic is 4:1. The potential of these polymers to solubilise different membrane proteins has been assessed with the most promising SMA variant bearing a styrene to maleic acid ratio of 2:1 (50). SMA with a 3:1 ratio has comparable efficiency to insert into membranes and both co-polymers display average M_w in the range of 7.5-10kDa. Thermostability of membrane proteins extracted from *E.coli* using variants of SMA was assessed using DLS and demonstrated equivalent stability, irrespective of the specific formulation of co-polymer (51).

SMA has an amphipathic nature due to hydrophobicity of the styrene units and the two hydrophilic carboxylate groups of maleic acid (52). The carboxylates of maleic acid have pK_a values of ~6 and ~10 (53). At neutral pH or above, SMA is protonated and the charge repulsion between carboxylate groups helps SMA into a coil confirmation which makes it readily soluble in water (40). At low pH on the other hand, it becomes neutral and assumes low solubility (54). This is a major limitation of using SMA as it restricts the pH range that can be used during the solubilisation step. The more hydrophobic the polymer is, the higher the pH it needs to solubilise. For example, a 4:1 ratio of styrene to maleic acid variant of SMA is only soluble at pH 7-9 whereas the most hydrophilic form of SMA (1.4:1 styrene to maleic acid) is soluble within range of pH 4-9 (55). The majority of protein extracting polymer forms are only effective between pH 7 and 8.

2.3.1 Method for preparation of SMA

The first step in purification of membrane proteins is to prepare hydrolysed SMA polymer from its anhydride form. The generic method includes the following steps:

i) Dissolve SMAnh pellets in a round bottom flask at 5% (w/v) in deionized water
ii) Heat and reflux the suspension with base; 1M KOH for 4 hours (56) or 1M NaOH for 2 hours (57,58). This will convert the anhydride units to their acid form containing two deprotonated carboxyl groups to increase the water solubility. The amount of base to be added can be controlled in this step in order to make the pH of the precipitated polymer close to 8.0 (59)

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

iii) Once the solution cools down to room temperature, the pH is lowered to <5 with the addition of concentrated HCl. This causes the polymer to precipitate

iv) The polymer is washed with diluted HCl and then resuspended in 0.6M NaOH/KOH and the pH adjusted to 8.0

v) The polymer is lyophilised in a freeze dryer and stored at room temperature in a sealed bottle

2.3.2 Method for isolation and purification of membrane proteins using SMA

SMA has been used to isolate membrane proteins from membranes of bacterial, yeast or insect cells, or directly from the intact cells (43). The general approach involves:

i) Resuspend the membranes in solubilisation buffer keeping the membrane concentration at 20-40 mg (wet weight)/ml (57). Buffers commonly used for solubilisation include 20-50 mM of MOPS, HEPES, Tris-HCl and 150-500 mM NaCl, with or without the addition of 10% glycerol. The key common feature of all these buffers is to use pH 8.0 (57-61)

ii) Add 2.5% (w/v) SMA to the resuspended membranes. This may be done from a stock solution (5% w/v) or by direct addition of the powder form (57). The concentration of SMA varies according to the target protein and must be empirically optimised. For example, purification of the nucleoside transporter hENT1 with SMA above 0.25% (w/v) renders it non-functional (60)

iii) *Optional* - Exogenous lipids may be added for tightly packed proteins for ease of extraction or to increase the stability of the purified proteins. For example, DMPC (di-myristoyl phosphatidylcholine) was added for extraction of ABCG2 from insect cells (58) and CHS (cholesteryl hemi-succinate) was added for stabilisation of hENT1 nucleotide transporter (60)

iv) Extraction is carried out at ambient temperature for 1-2 hours with gentle shaking. This has been reported for most proteins isolated using SMA except for hENT1 nucleotide transporter, which was incubated at 4˚C for 12-18 hours to prevent degradation at higher temperatures (60)

v) The soluble protein fraction is separated by ultra-centrifugation at 100,000 g for 20-60 minutes, followed by purification using affinity chromatography

vi) Proteins with a poly-histidine tag are incubated with resin bound to either nickel or cobalt (61) at 100 µl resin per ml of solubilised fraction (51). The binding kinetics for different proteins may be slow and it is recommended to undertake this step at 4˚C with gentle rotation overnight. Buffer with high salt concentrations may be used to minimize non-specific binding of SMALPs to the resin (57)

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

vii) Imidazole is used to elute SMALP encapsulated protein from the resin but the concentration needs optimisation as it may vary widely from that used in detergent based applications. Typically, the elution concentration of imidazole is greatly reduced with some proteins eluting at <10mM imidazole (62)

viii) Purified protein may be concentrated using centrifugal filtration for downstream processing after removal of imidazole with a buffer exchange resin (e.g. PD-10)

2.3.3 Advantages of SMALPs

Various methods for purification of membrane proteins have been developed in the past four decades and a major concern has been the inherent instability of these proteins. It has been shown that even mild detergents can disrupt the intramolecular interactions between protein subunits disturbing the stable confirmation of the protein leading to aggregation and loss of function, even occasionally to complete inactivation. In addition, detergent micelles, liposomes and nanodiscs are incompatible with many biophysical techniques (63-66) due to their sizes or light scattering anomalies.

The use of SMALPs for purification of membrane proteins offers a solution to these problems by retaining the native lipid environment that helps maintain protein stability and function (43,49,63). The proteins in SMALPs are accessible from both sides of the bilayer and show comparable ligand binding affinities to the native membranes. SMALPs encapsulated proteins have provided high quality spectra in circular dichroism (CD) with minimal background signal and have been used to study protein dynamics using EPR (65,66). SMA also offers advantages over detergent micelles to study the structures of large membrane proteins using cryo-electron microscopy (cryo-EM) since the proteins are more rigid in SMA and the polymer does not add to the electron density (63) Finally, attempts have also been made to study membrane proteins in SMA using NMR (41,46) providing information on structure of protein. The table below provides an overview of several applications of SMALPs to investigate membrane proteins.

2.3.4 Limitations of SMALPs

Despite being a promising investigative tool, there are limitations associated with SMALPs. The optimum pH for SMALPs is 7-8 and at values below this, the polymer precipitates out of solution rendering the proteins insoluble. This can be a problem for proteins that are stable or active in acidic environments such as lysosomal PfCRT (pH 6.0) which is responsible for resistance against antimalarial drugs (67).

ACCEPTED MANUSCRIPT

Table 1: Overview of some of the experimental techniques used to study proteins in SMALPs

Techniques	Proteins studied	References
Dynamic Light Scattering (DLS)	*E.coli* LeuT,	(51)
	E.coli PagP	(41)
	Bacterial photosynthetic reaction center	(59)
	Bacteriorhodopsin	(65)
	KCNE1	(68)
Transmission and Cryo-Electron Microscopy	*E.coli* AcrB trimer	(61)
	S. cerevisae respiratory complex	(69)
	P-glycoprotein	(63)
	Rhodobacter sphaeroides photosynthetic reaction center	(59)
	Streptomyces lividans KcsA	(43)
	PagP	(41)
	KcsA	(43)
	Staphylococcus aureus PBP & PBP2a	(70)
	KCNE1	(68)
Circular Dichroism	Adenosine 2A receptor ($A_{2A}R$)	(49)
	PagP, Bacteriorhodopsin	(41)
	KcsA	(43)
	P-glycoprotein	(63)
Ligand Binding Assay	MRP1 (Radioligand)	(63)
	P-glycoprotein (Flourescence)	(63)
	ABCG2 (Flourescence)	(63)
	AcrB (Flourescence)	(61)
	hENT1 (Radioligand)	(60)
	BmrA (Flourescence)	(51)
	KcsA (bilayer electrophysiology)	(43)
Spectroscopy	Photoreaction center (charge recombination)	(64)
	KCNE1 (EPR, NMR)	(46)
	SecYEG (FRET)	(71)
	PagP (NMR)	(41)
Crystallography	HwBR	(72)

Similarly SMALPs are sensitive to divalent cations such as calcium and magnesium (51,73). The possible explanation for this is that the carboxyl group of maleic acid chelates these ions inducing a confirmation change subsequently leading to precipitation of the polymer. The sensitivity to divalent cations is an issue for many biochemical assays of protein function (e.g. ATP hydrolysis). Another limitation is the size of the disc as it is too small to encapsulate large protein complexes. The recent work on bacterial light harvesting complex1 demonstrated that SMALPs may not accommodate large

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

proteins or oligomers (59). However, some studies have suggested that the size of the disc is flexible (70) and can be exploited for large proteins by targeting the protein to lipid ratio (45,74) and the ratio of the polymer (75).

Lateral pressure is an important aspect to be taken into consideration as detergents provide little or no pressure. In contrast, the lateral pressure in SMALPs may be too high and thereby limit conformational changes (62). For structural studies involving NMR, SMALPs offer some hindrance due to the heterogeneity of the discs formed, the root cause of which can be the high poly-dispersity of the polymer itself (76). Finally, SMALPs cannot measure vectorial transport *per se*. However, efforts are underway to "transfer" SMALP-encapsulated proteins into proteoliposomes (PLs), which would enable their use in transport assays (72).

2.3.5 Future Directions in SMA Technology

SMALPs have proven to be a versatile tool in studying membrane proteins albeit with some drawbacks. Whilst many of the problems may be overcome by optimizing the conditions of purification, the generation of alternate polymer types offers a considerable benefit. For example, to improve the issue surrounding SMA polydispersity, the more aliphatic di-isobutylene maleic acid (DIBMA) co-polymer has been investigated (72). DIBMA has a considerably lower polydispersity index, which produces less heterogeneity in particle size and has the added advantage of lower UV absorbance that will enable spectroscopic investigation of proteins. Furthermore, a zwitterionic series of SMA polymers (z-SMA) has been developed with the ability to tolerate divalent cations in the low millimolar range (77). The field of biopolymer extraction of membrane proteins should thus be considered in its infancy with considerable potential for incorporation of new features to enhance extraction and stability of encapsulated proteins.

3 COMPARTMENT BASED RECONSTITUTION SYSTEMS

The reconstitution methods in Section 2 provided the means to investigate many aspects of membrane protein function. However, a two compartment system is required to investigate the transport (or translocation) activity of IMPs (78). Classically this involves the use of liposomes, self-closed lipid bilayer vesicles, and their use with IMPs began in the 1970s with the work of Racker and colleagues (79-81). They reconstituted and demonstrated the activity of both, cytochrome oxidase from bovine heart mitochondria, and the Ca^{2+}-dependant ATPase from the sarcoplasmic reticulum. Rigaud *et al*., developed the system further and introduced a number of "guidelines" for the process of protein reconstitution into liposomes. This inspired the use of PLs to investigate the function of a

ACCEPTED MANUSCRIPT

huge range of IMPs including ion channels and receptors (82-91), enzymes (92-96), and transporters (8,97-109).

3.1 Small and Large Unilamellar Vesicles

Different sizes of liposomes can be made and those with diameter ϕ<100nm are loosely defined as small unilamellar vesicles (SUVs), while large unilamellar vesicles (LUVs) have diameters ϕ=100-300 nm (110,111). Both types are made by a variety of techniques including, freeze-thawing, sonication, extrusion, reverse phase evaporation and/or detergent extraction (99,110). The starting material for their production are multilamellar vesicles (MLV), which are made by dispersion of dried lipid films in an aqueous buffer through extensive vortexing (99,112). The simplest method for SUV production is via prolonged sonication of MLVs. LUVs are commonly made by extrusion of MLVs through a polycarbonate filter containing monodispersed pore sizes between 100-400 nm. Factors affecting the LUV size include the pore size of filters used in extrusion, the number of passes through the filter (113), lipid composition (114) and the buffer composition (115). Generally, LUVs made with 200nm filter are more homogeneous and have a lower number of multi-lamellar vesicles in the population compared to filters with 400nm pores (78).

The size distributions of liposome (or PL) populations may be determined by dynamic light scattering (78), and the internal volume of liposomes as described (116). Liposomes made by extrusion through a 200 or 400nm filter produce approximate sizes of ϕ=170±20 nm and ϕ=350±100 nm respectively, with internal volumes of 0.73±0.04 µl/(mg lipid) and 0.9±0.05 µl/(mg lipid) respectively [Knol and B.P., unpublished data as cited in (78)].

A generic method to make LUVs is as follows:

i) A mixture of lipids from a stock solution in organic solvent is dried under vacuum to form a lipid film in a glass tube
ii) The dried lipid film is dispersed in aqueous buffer by repeated rounds of vortexing and sonication until all of the lipid is re-suspended
iii) The lipid suspension then undergoes 10 rounds of freeze-thawing in liquid nitrogen or dry ice and a heated water bath. This will cause coalescence and the formation of MLVs with heterogeneous size
iv) MLVs are extruded through a filter with the desired pore size to generate homogeneously sized unilamellar vesicles (117)

Reconstitution of IMPs following their extraction from cellular membranes by detergent (Section 2.1) into liposomes remains a difficult task that requires optimisation for each protein MP (118). The

ACCEPTED MANUSCRIPT

process of reconstitution begins by mixing detergent-solubilised purified IMPs with lipids; the latter consisting of mixed detergent lipid micelles or detergent-destabilised preformed vesicles. This generates a mix of lipid-detergent and lipid-detergent-protein micelles (117,119,120). The subsequent removal of detergent leads to the incorporation of the IMP into vesicles (3,78,99,117,119). The mechanism of reconstitution by detergent removal is still not fully understood and the current view has not drifted far from that of Rigaud *et al.* (120). During detergent removal, a critical point is reached where the protein cannot be stabilised in detergent containing micells. At this point, the protein may either precipitate or spontaneously associate with the available lipids and form PLs (119). The reconstitution efficiency depends on a number of factors; (i) the initial detergent concentration, (ii) the type of detergent used (121,122), (iii) the membrane protein of interest, (iv) the composition of lipids, (v) the rate of detergent removal, and (vi) the ionic conditions (117). For a more comprehensive review of IMP reconstitution into liposomes, the reader is directed to the review by Rigaud *et al.* (120).

Detergent removal can be achieved by a variety of methods (refer to Table 2 for details) including dilution, gel filtration, dialysis or adsorption to polystyrene beads (3,78,117). Dilution, gel filtration and dialysis techniques work best for detergents with a high CMC (78,111). Gel filtration and dilution techniques require additional steps post detergent removal to concentrate the protein (117). Adsorption with polystyrene beads on the other hand, is effective for detergents with a low CMC such as DDM or TX-100 (78,111). The adsorption capacity of the beads is 0.2-0.45 mmol detergent per gram of wet beads and although lipids and proteins may also be adsorbed, this is typically two orders of magnitude lower than detergent (123). This is related to the size of the mixed micelles, which are larger than the pore size of the beads. Consequently, adsorption of micelles occurs only to the surface of the beads whereas monomeric detergent is also adsorbed into the beads. Binding of lipids may also be kept to a minimum by maintaining a low bead-to-detergent ratio in the first step of reconstitution to promote the higher affinity interaction with detergent (117).

Following reconstitution two populations of liposomes will exist; PLs and empty liposomes, which may be separated by gradient separation as they have different densities (117). PLs (d=1.03-1.05 g/cm^3) are denser than empty liposomes (124) and the two populations may be separated with a discontinuous gradient such as sucrose (83,100). The latter is readily removed by dialysis or repetitive dilution-concentration cycles (117).

ACCEPTED MANUSCRIPT

Table 2: Methods of detergent removal

Method	Method Description	Notes	References
Dilution	Detergent free dilution buffer is added to lower the detergent concentration. PLs form when [detergent]<CMC	*Advantages:* • detergent removal rate may be controlled • may monitor the PL formation *Disadvantages:* • cannot remove all detergent • heterogeneous and dilute samples	(117,125)
Gel filtration	Separates monomer and mixed micelles from PLs based on the difference in size. Elution in a detergent free buffer	*Advantages:* • fast, simple, efficient and reproducible. *Disadvantages:* • dilute sample • may lose lipids in the resin	(83,88,90,126)
Dialysis	The lipid/detergent/protein mix is dialysed against detergent-free buffer in a dialysis bag	*Advantages:* • gentle process suitable for labile or unstable molecules in liposomes • inexpensive and simple *Disadvantages:* • no control over detergent removal rate • poor reproducibility and long time scale	(84,89,90,101)
Adsorption to polystyrene beads	A physical adsorption method. The hydrophobic detergent tails bind to the hydrophobic surface of the beads. Polystyrene beads are removed by filtration, centrifugation or gravity	*Advantages:* • fast (minutes-hours) • control of detergent removal rate *Disadvantages:* • minor loss of lipids	(100,120,123,127-129)

A relatively recent approach to IMP reconstitution uses organic solvents or oil mixed with water to create water-in-oil (W/O) microdroplets that are coated by phospholipids. The hydrophilic heads of the phospholipids reside in the water phase and the tails orientate to the oil/organic phase. This procedure has primarily been used for monolayer and planar lipid bilayer applications; however, it has been adapted for liposomes. The W/O microdroplets may be extended to form LUVs using a droplet-transfer method (130). A major advantage of this method is the ability to produce different lipid compositions in the outer and inner leaflets of the liposome and to orientate the reconstituted IMP within the liposomes (131).

3.1.1 Applications of IMP reconstitution into LUVs/SUVs

ACCEPTED MANUSCRIPT

Reconstitution of IMPs into SUVs and LUVs permits the direct measurement of substrate translocation. Typically this is often done using substrates containing isotopic, radioactive, fluorescent or photoaffinity moieties (78,89,102,108,109,111). For example, fluorescent voltage sensitive dyes were used to monitor function of the Ca^{2+}-ATPase and of Maxi-K potassium channels (83,117) and its intermediate steps including substrate binding and coupling to energy production. Radioisotope assays are highly sensitive and provide the advantage of quantifying substrate accumulation in absolute units, which enables determination of transport stoichiometry and flux capacity. Fluorescent assays enable real-time measurements of transport rates but are typically depicted as “relative transport measurements”.

The process of transmembrane transport involves numerous intermediate steps including substrate association and dissociation, energy provision, bioenergetic coupling and conformational transitions. Proteins reconstitued into LUVs and SUVs are amenable to such measurements. For example, nucleotide hydrolysis is essential for substrate translocation by the ATP Binding Cassette (ABC) transporter family. There have been many assays used to measure the hydolysis of ATP by reconstituted ABC transporters including luciferin-luciferase assays (132), malachite green-molybdate assays (97,133) or variations thereof (8,98,134).

A specific advantage of SUVs and LUVs is their high membrane curvature which can be exploited to study curvature-dependent effects that influence protein-membrane interactions (135-137). Protein function once reconstituted (for example of Rhodopsin, G proteins, pro-apoptotic Bcl-2 proteins and t-Bid, phosphocholine cytidyltransferase (CT) and P protein kinase C (PKC) (92-96,138)), has been found to be sensitive to the membrane curvature of liposomes. Furthermore, different phospholipids may cause curvature stress changes in liposomes (139) and curvature stress has been suggested to affect the free energy and folding of IMPs (140). Unfortunately, this high membrane curvature is a limitation in using SUVs and LUVs to study trans-leaflet lipid transporter IMPs (i.e. flippase and floppase activity). This is due to the fact that vectorial movement of lipids between PL bilayer leaflets increases the surface tension that may inactivate the IMP (141,142).

3.1.2 Disadvantages of reconstitution into LUVs/SUVs

Reconstitution into SUV and LUV-type proteoliposomes has led to a large accumulation of information on the structure and function of IMPs (143). However, there are some limitations to the technique such as the difficulty in controlling the size of PLs formed during reconstitution. Reconstitution into pre-formed liposomes of defined size can help, but the addition of IMPs in detergent micelles to pre-formed liposomes may alter their size. In addition, further treatments

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

during PL formation (e.g. sonication, freeze-thawing) can also change their size (117). It is also difficult to control the amount and orientation of the reconstituted IMPs in the PLs (144), which has been reported to cause aggregation (119). The orientation of reconstituted IMP is difficult to predict and seems to be unique to each protein and specific set of reconstitution conditions. For example, 80% of the reconstituted Ca^{2+}-ATPase was outward facing (100) whereas 70% of the Maxi-K potassium channel (BK), was orientated inside-out (83).

Another limitation in their use to investigate the molecular mechanisms of IMPs, is the small size and internal volume of SUVs and LUVs (118,143). Structural and functional analysis through optical microscopy techniques is not possible in SUVs and LUVs since manipulation of the vesicles (e.g. with pipettes, tweezers, electrodes or atomic force microscopy) is difficult (143). In particular, the small internal volume of SUVs and LUVs is a limiting factor for transport assays investigating the substrate translocation activity of IMPs. The concentration of transported substances accumulating inside the vesicle may rapidly reach high molarity and thereby inhibit transporter efficiency (118).

3.2 Giant Unilamellar Vesicles (GUVs)

With diameters ranging from 1-200µm (111,118,143,145-147), Giant Unilamellar Vesicles (GUVs) are similar in size to eukaryotic cells (148). The large size of GUVs renders techniques such as micromanipulation with optical tweezers or micropipettes, and direct observation by optical/fluorescence microscopy, possible (118,143,147). GUVs containing IMPs allow characterisation by the same techniques as in SUVs and LUVs (without the small size and volume limitation), as well as by confocal microscopy, fluorecence correlation spectroscopy and patch clamping (78). Due to their size, GUVs display lower membrane curvature (110) that is similar to cells and thereby enables measurent of lipid translocase activity (143,149).

3.2.1 Advantages of using GUVs

Like SUVs and LUVs, GUVs provide compartmentation to allow measurement of vectorial transport by IMPs. However, they are more flexible and display an internal volume similar to that of cells (146) and are therefore considered promising cell mimetic system (148,150). The GUV membrane may comprise a single or a mixture of lipid classes (145) allowing their use in investigations into the function, shape, mechanical and chemical properties of complex biological membranes (145). They have been produced to contain IMPs (120,143,151-155), fragments from a natural cell membranes (156,157) and soluble components within their lumen (158-160).

GUVs have been used to investigate many biological functions such as adhesion (143,146,161-164), fusion and fission (143), the effects of local delivery and interactions of biologically active substances

ACCEPTED MANUSCRIPT

(165), DNA-lipid interactions (166), endo and exocytosis (167-169), antimicrobial peptides (170), antibody binding (171), and cell division (172). They may be used to investigate properties of lipid membranes (118), cell functions that involve the lipid membrane (148), biochemical reactions and self-assembly processes that occur on the membrane (146). More specifically, single molecule techniques such as fluorescence correlation microscopy have been employed with the GUV model to investigate lipid mobility (143,173), membrane dynamics and ordered lipid microdomains (156,173,174). Fluorescent imaging has been used to observe lateral membrane organisation (175) and the micropipette aspiration technique has been employed with GUVs as cell membrane models (176,177). The bending stiffness of lipid bilayers and the free enrgies of interactions between membranes as well as membrane deformities (143), lipid membrane phase transitions (178) and membrane flickering (145,179) have also been investigated using GUVs.

The formation of GUVs with soluble proteins or nucleic acids in the lumen is well established (158,180-182). On the other hand, reconstitution of functional IMPs into GUVs is less advanced, mainly due to the harsh methods for GUV formation (refer to section 3.2.2 and 3.2.3) (111,143). However, methods for GUV formation and GUV membranes themselves, can be designed to incorporate MPs (146) and this is currently an active research area (118,148,153,183-185).

3.2.2 Preparation of GUVs

In general, there are four main methods for the formation of GUVs, (i) dehydration-rehydration, (ii) electroformation, (iii) microfluidic injection and (iv) emulsion based. Each of these methods is discussed below and Figure 1 provides a comparative summary.

3.2.2.1 Method of GUV formation by dehydration-rehydration

Introduced in 1969 by Reeves and Dowben (186) then modified in 1996 (187), this was the first method established for GUV formation. Briefly, lipid deposits (from lipids dissolved in solvent, membranes or preformed liposomes) are dehydrated on a support surface, for example glass, forming stacks of lipid bilayers (186,188). GUVs grow by swelling when they are rehydrated by exposure to aqueous solution (146,186,187,189-191). The GUVs form spontaneously via membrane fusion which is facilitated by osmotic pressure, electrostatic interactions and hydrophobic effects as water enters the dried lamellar structures (111,192).

Temperature is an important factor in this method at each step since it is essential that the lipids are in the correct phase (either liquid crystalline or gel) (147). Unfortunately, this method does not produce a high yield of high quality GUVs (147). The yield can be increased by the addition of

ACCEPTED MANUSCRIPT

charged lipids (187) especially 10-25% (w/w) anionic lipids or alternatively, 10mM Mg^{2+} may be added to neutral lipids after pre-wetting (193). The addition of charged lipids has the added advantage of enabling the use of buffers at physiological ionic strength (147) that otherwise impede GUV formation.

Advantages of this method include the ability to prepare GUVs in a large range of buffer compositions, including buffers with high ionic strengths (143). However, there are several *disadvantages* to the method as well. The yield is low compared to other GUV forming methods, and the process has a long time scale in the range of days (194). Additionally, due to the uncontrolled rehydration step, the process forms predominantly multilamellar with very few unilamellar vesicles and the population formed is heterogeneous in size (143,145,188).

3.2.2.2 Method of GUV formation by electroformation

Now the most common method for GUV formation (195,196), electroformation was introduced in 1986 by Angelova and Dimitrov (196). A high yield of GUVs can be achieved if dehydrated lipid deposits are rehydrated in the presence of an alternating current (AC) electric field (155,195,197,198). The method is based on electro-osmosis and the ability of lipids to self-organise in aqueous solution (146). In addition to the high yield and quality of GUVs formed (110), this method also generates more unilamellar GUVs with more homogeneity in size ϕ~20μm (range from ~5-150μm (199)) compared to the dehydration-rehydration method (143,195). It has a reduced timeframe and does not use the high temperatures used in dehydration-rehydration that damage biological components (146). The GUV formation during electroformation may be monitored by fluorescence microscopy (78).

The process of electroformation also requies dehydrated lipid deposits on a solid support, although in this case the support is a homogeneous conductive surface (146). Most commonly used are indium tin oxide (ITO) coated glass electrodes, although platinum (Pt) or titanium electrodes may also be used. The lipid films are rehydrated (usually with a sucrose solution) and an alternating current (AC) electric field is applied to the electrodes (110). Upon addition of the rehydration buffer, the chamber should be sealed and the AC electric field applied immediately in order to avoid any spontaneous, uncontrolled swelling (200). The AC electric field is applied to the electroformation cell with either (i) a function or pulse generator, or, (ii) a generator able to produce sqaure or sinusoidal voltages at different frequencies (200). Formation of larger GUVs is facilitated by rehydration with an AC electric field where the amplitude voltage increases incrementally (143). Lowering the AC

ACCEPTED MANUSCRIPT

frequency (Hz) and raising the voltage helps the GUVs become spherical and separate from the electrodes (143).

Electroformation of GUVs has three stages; a growing, a swelling and a rebounding stage (200,201). In the first *growing stage*, the AC electric field amplitude is increased stepwise to a maximum point while the frequency remains fixed. Generally, GUVs will have started to form within 20-30 minutes. A strong electric field is needed at this stage in order that the growing vesicles develop and maintain a spherical shape (200,201). The *swelling stage* is the point at which the GUVs increase in size while the electric field parameters are kept constant. This stage is optional when making GUVs at low salinity since the amplitude is increased very slowly to its maxumum in the *growing stage*, which allows time for the GUVs to grow in size. In other conditions however, the *swelling stage* is essential to control the final size of the vesicles. If the field amplitude is rapidly increased to maximum in the *growing stage* and the *swelling stage* is omitted, the GUVs formed will be small (5-10µm) (200,201). In the final electroformation step, the *rebounding stage*, the electric field frequency is decreased to promote vesicle closure and to prepare them for future detachment from the electrodes. At the end of electroformation, the formed GUVs can be left on the electrodes or can be detached easily by gentle tapping (200,201).

The two main parameters to consider in electroformation are the electrode material, and the applied electric field parameters (147). The use of high voltages , or the wrong choice of electrode for the specific conditions may lead to degradation of lipids by hydrolysis (147). A disadvantage of the commonly used ITO-coated electrodes is that they can be unstable at the voltages usually used in electroformation (200,202) and the resultant decomposition may cause lipid peroxidation (147). This problem can be circumvented by using cylindrical platinum electrodes (200), or titanium electrodes that decrease lysolipid formation (147). The AC electric field parameters can impede, rather than assist, with GUV formation if compatible preparative conditions are not used (111). In the original electroformation technique (196), the preparative conditions that could be used were limited. Even buffers with low ionic strength, or the presence of charged lipids, inhibited GUV formation (110,147) thereby limiting the addition of physiological salt buffers to the external medium until completion of electroformation (110).

This limitation was overcome by varying the voltage and frequency of the electric field and the method could be optimised to form GUVs under a range of different conditions. Methods have been developed to form GUVs using electroformation in different electrolyte-containing buffers at physiological ionic strengths. Physiological temperatures and a range of lipid mixtures (e.g. anionic, zwitterionic) or native membranes have also been implemented (143,145,157,201,203). In general,

ACCEPTED MANUSCRIPT

the amplitude of AC electric field used ranges between 1.5-5V and the initial frequency used in the electroformation process is ~10-20Hz (110). A lower field amplitude is preferable for charged lipids and a higher frequency helps GUV formation in physiological salt buffers (110,157). For examples of relevant electroformation parameters depending on the medium used for rehydration and more specifically those relevant in low and high salinity buffers at pH 7.4, the reader is directed to tables in references (200,201).

The dehydration-rehydration and electroformation methods described above, can be used to readily incorporate cargo into GUVs. However, the techniques are beset with a significant drawback related to the difficulty in removal of non-encapsulated cargo. The removal is readily achieved with the sturdier SUVs/LUVs by ultrafiltration, size-exclusion chromatography or dialysis. Unfortunately, GUVs are not amenable to these procedures and so they are inefficient at removing the un-encapsulated cargo (110).

3.2.2.3 GUV formation by microfluidic jetting and emulsion-based methods

Although used less frequently, microfluidic jetting and emulsion-based methods provide alternatives to generate GUVs. These two methods are efficient at forming GUVs with encapsulated compounds or asymmetric membranes (158,204,205).

Microfluidic jetting involves controlled jets of fluid directed at a planar lipid bilayer or a droplet interface bilayer. The jet causes the bilayer to deform and a small section to break off and produce a vesicle filled with the solution from the jet (146,206). This method has been demonstrated to form homogeneous populations of GUVs in the range of ϕ=100-200µm (146).

Emulsion-based methods such as double-emulsion templating (207), involve the transfer of lipid stabilised water-in-oil droplets through an oil-water interface. The lipid droplets are pushed through the oil-lipid interface by the flow passing through a glass microcapillary microfluidic device (146)**.** The lipid droplet "picks up" another lipid monolayer during the transfer to form a complete bilayer and leave the GUV in water (110). Vesicles can be collected from the emulsions by evaporating or using dialysis to remove the solvent (146). GUVs with diameters in the range 20-150µm can be made using this method (146,207).

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Figure 1: Overview of main methods for GUV formation

Method	Dehydration-rehydration	Electroformation	Microfluidic jetting	Emulsion-based
Starting material	Aqueous lipids/lipid films dissolved in organic solvent/ SUVs/LUVs/native membranes		Planar lipid bilayer, or, Droplet interface bilayer	Lipid stabilised water-in-oil droplets
Process overview	Lipid deposits ↓ Dried on a support surface by solvent evaporation(vacuum or rotary evaporator) ↓ Rehydrate lipids in desired buffer	Lipid deposits onto electrodes ↓ Dehydrate ↓ Add hydration buffer (carefully not to disturb the lipid film) ↓ Seal the electroformation chamber ↓ Apply AC electric fields in 3 stages: 1) Growing step: Fixed frequency with increasing amplitude 2) Swelling step: Electric fields constant 3) Rebounding step: Frequency decreased ↓ GUVs left on electrodes or removed by gentle tapping	Controlled fluid jet ↓ Deformation of the lipid bilayer ↓ Vesicle forms and detaches from the bilayer	Emulsions made with glass microcapillary microfluidic device ↓ Fluid flow pushes lipid droplets to a lipid bilayer at oil-water interface ↓ Lipid droplet transfers through the interface forming a bilayer ↓ Vesicles removed from emulsions by evaporating or removing the solvent
References/examples	(146,186,187,189-191,193,208-210)	(110,143,145,146,148,155,157,184,185,195-201,211)	(158,206)	(146,204,205,207)

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

3.2.2.2 Perspectives of GUV formation

Since the initial reports of producing GUVs, much progress has been made with methods for their formation and led to improvements in design possibilities. These improvements include controlling GUV sizes and using physiological buffer systems in their formation (146). GUVs have also been generated with passive or active components incorporated within the vesicle lumen and the first report of enzyme encapsulation was reported in 1970 (212). Formation of complex GUV membranes from binary and tertiary lipid mixtures as well as from natural lipid extracts in order to study membrane heterogeneity has been achieved (145,154,157,173,175,213-216). GUVs made from natural lipid extracts (154,157,216) were found to maintain the natural cell membrane asymmetry. This feature has been notoriously difficult to generate when starting from solubilised synthetic lipids due to their propensity to mix homogeneously in artificial systems (146). There are a few reports of asymmetric GUVs produced from synthetic lipids that are stable for 4-24hr (205,217).

3.2.3 Reconstitution of IMPs into GUVs

A major design improvement with GUVs has been the ability to reconstitute IMPs into the vesicle membrane. Initially, the harsh methods for the production of GUVs, rendered their ability to investigate IMPs difficult (143). The main barrier to functional IMP incorporation was the dehydration step in the general methods used for GUV formation (dehydration-rehydration and electroformation) (111,143). A number of novel approaches to the existing methods have been used to generate proteo-GUVs or to insert IMPs into preformed GUVs. The work of Rigaud et al. 1988 outlining detergent-mediated protein insertion into LUVs has been instrumental in devising strategies for reconstitution of IMPs into GUVs (122). In particular, several methods use PLs as the starting material for GUV formation (78,111,146).

3.2.3.1 Variations on existing methods for reconstituting IMPs into GUVs

As mentioned above, the key change to the existing methods of GUV formation to allow for the incorporation of IMPs has been to use reconstituted proteins as the starting material (78,143,185,195,201,210,218). Moreover, using lipid deposits from PL dispersions as the starting material for GUV formation has proven to be advantageous for electroformation (200,201) since the amount of lipid on the electrodes is better defined and due to their propensity to make well-orientated membrane stacks (143,200,201).

The dehydration step in these methods remained a problem. As the PLs are dehydrated, the transition temperature increases by 70-80˚C (219) causing the lipids to go from liquid crystalline to a

ACCEPTED MANUSCRIPT

gel phase, which induces lateral phase seperation. Consequently, the IMPs may denature, aggregate and/or lose activity (111,146). A practical example of the affect of dehydration on IMPs was shown by Girard et al. (143). Their preliminary experiments (prior to their succesful reconstitution of Ca^{2+}-ATPase and bacteriorhodopsin into GUVs) showed that complete dehydration of the PLs under high vacuum caused a decrease in the activity of these proteins. The loss in activity is excacerbated when using single molecule assays (e.g. fluorescence correlation spectroscopy, fluorescence resonance energy transfer and atomic force microscopy) with GUVs to investigate IMP function (111).

A common variation to the method is to use stabilising agents (in particular sucrose) during the dehydration step or in the rehydration buffer. Addition of sucrose (as little as 20-100mg/g lipid may suffice (111)) helps to stabilise the protein and maintain its activity during the sequence of dehydration and rehydration (78). Sucrose prevents lipid phase transitions from liquid crystaline to gel phase (dehydration) and back (hydration) by keeping the transition temperature of the membrane low (219). This reduces protein aggregation, and hydrogen bonding between sucrose and the polar groups of the amino-acid residues stabilise the protein (78,111). Unfortunately, adding sucrose may also inhibit membrane fusion (220). Therefore, the concentration of sucrose must be optimised to maintain protein activity (20-100mg/g lipid) while still allowing membrane fusion (<0.86 g/g lipid) (218). Other dissacharides, such as trehalose, have also been used to stabilise proteins during the dehydration and rehydration steps (111,218). Another strategy to promote retention of protein function is to switch from complete to partial dehydration of the starting material (143).

With these variations in place, many IMPs have been succesfully inserted and studied in GUVs. These include; Ca^{2+}-ATPase and bacteriorhodopsin (143), a flippase from yeast endoplasmic reticulum (149), glycophorin and a K^+ channel (221,222), and many more (111,143,153,183,185,223). It has also been shown that IMPs and glycosphingolipids retain their natural orientation post electroformation in GUVs produced from native membranes (157).

3.2.3.1 New methods for MP incorporation into GUVs

Although the methods described above have been used succesfully to reconstitue a number of IMPs into GUVs, the techniques still require that the protein is able to withstand partial drying. Some new methods for the incorporation of IMPs into GUVs have also been developed and are summarised in Figure 2. Our focus will be on those using fusion techniques and detergent removal.

3.2.3.3 Fusion techniques

ACCEPTED MANUSCRIPT

The fusion methods that will be presented here include peptide-induced fusion of PLs with preformed GUVs, and the fusion of oppositely charged vesicles. Reconstitution of IMPs into GUVs by peptide-induced fusion involves mixing PLs containing fusogenic peptides (e.g WAE covalently attached to the LUVs) with preformed GUVs. This method has been used to reconstitute bacteriorhodopsin into GUVs in order to investigate the function, dynamics and aggregation states of the protein (184,224). The primary disadvantages to this technique are the laborious process of anchoring the fusion peptide and the presence of fusion peptides in the IMP containing GUVs (111,143,146).

Figure 2: New methods for IMP incorporation into GUVs

Method	Detergent removal	Fusion methods	
		Peptide induced fusion	**Oppositely charged vesicles**
Process overview	GUVs with solubilising concentrations of detergent → Buffer exchange possible here → Add solubilised protein/native vesicles/PLs → Direct incorporation of solubilised protein or fusion of the native vesicles → Detergent removal	LUVs with fusion peptides covalently attached → Fused with preformed GUVs	Positively charged PL → Fused with GUVs containing negatively charged lipids
References	(148)	(184,224)	(118)

Another strategy is to initiate fusion between oppositely charged GUVs (225,226), although these studies used high levels of cationic lipids to achieve fusion. The approach was modified by Biner *et al.* (118), using less harsh conditions to achieve fusion of positively charged SUVs containing IMPs with empty GUVs containing negatively charged lipids. Their technique fused oppositely charged PLs

ACCEPTED MANUSCRIPT

to co-reconstitute bo_3 oxidase and ATP synthase from *E. coli* as well as the ATP synthase and cytochrome c oxidase from *Rhodobacter sphaeroides*, into unilamellar liposomes of varying sizes (100nm-50µm) (118). This method was also used to co-reconstitute bo_3 oxidase and ATP synthase, which were labelled with different fluorophores, into GUVs. Fluorescent microscopy confirmed that both proteins were incorporated into the same GUV membrane and protein function was confirmed by measuring ATP synthesis. Taking the method one step further; SUVs containing fluorescently labelled cytochrome c oxidase, the Na^+/H^+ antiporter NapA and the green light-activated H^+ pump proteorhodopsin, were mixed and then incubated with GUVs. GUVs containing all three proteins were then confirmed by fluorescence microscopy. It was observed that the fusion rate was dependent on the percentage of charged lipids present, positively charged lipids needed to be present in both leaflets for complete vesicle fusion, and that fusion of the inner leaflet of the bilayer provided the rate limiting step. The fusion seems to be a two-step process involving the breakage and then fusion of the inner leaflets driven by an electrostatic interaction.

This method is promising as it is a simple, mild, non-destructive and detergent free way to reconstitute into GUVs, and the controlled fusion of protein containing SUVs with GUVs also ensures the orientation of the proteins are maintained in the GUVs. As in most methods, the ideal conditions (in this case the lipid composition) need to be estimated for each different IMP. A potential drawback is that some target IMPs may not be incompatible with cationic lipids, which do not occur in biological systems. Moreover, the lipid composition of the vesicles cannot be freely chosen so the method is incompatible for experiments where the influence of the lipid composition is being investigated.

3.2.3.4 Detergent based approaches to IMP reconstitution into GUVs

Other new techniques to reconstitute IMPs into GUVs were developed by Dezi et al. (148). Detergents are used to either directly incorporate proteins solubilised in detergent micelles (mimicking detergent reconstitution into preformed SUVs/LUVs), or to fuse PLs or purified native vesicles with pre-formed GUVs.

For the direct incorporation of solubilised proteins, GUVs were formed in the presence of detergent concentrations above the CMC but below levels that would solubilise the vesicles. The purified IMP (in mixed detergent-lipid-protein micelles) are mixed with the detergent treated GUVs. The detergent was then removed with Bio Beads causing the proteins to be inserted by transfer from the mixed micelles into the GUV membrane, without needing holes to form (78,120,227). This strategy

ACCEPTED MANUSCRIPT

avoided protein aggregation as seen in previous studies when trying to incorporate proteins at detergent concentrations below the CMC (228,229).

Several detergents (e.g. Triton X-100) have been shown to induce fusion of lipid LUVs, PLs or native membranes when added at solubilising concentrations (230-232). Mixing GUVs with native vesicles or PLs and increasing the detergent concentration resulted in fusion and insertion of IMPs into GUVs (148). The extent of fusion increased with the detergent concentration until GUV were solubilised by the surfactant (148).

Using these methods, Dezi *et al.* directly incorporated the light-induced proton pump, bacteriorhodopsin, and the *E. coli* outer mem protein, FhuA, into preformed GUVs, and reconstituted the bacterial heterodimeric ABC efflux transporter, BmrC/BmrD, into GUVs by fusion from PLs (148). In all cases they verified activity of the reconstituted protein. These methods have many advantages for the reconstitution of MPs into GUVs. The main advantage is that the GUVs can be made using well-established electroformation techniques, usually too harsh for IMPs, enabling the formation of GUVs with different lipid compositions and with encapsulated materials. Furthermore, making GUVs with solubilising concentrations of detergent present increased the ionic permeability of the GUV. Consequently, GUVs could be formed in a sucrose solution (this forms more GUVs than salt solutions (201)), and a buffer exchange step introduced post-formation to exchange the internal and external contents. The detergent present helps to protect the GUVs from osmotic shocks during buffer exchange, but also allows large encapsulated molecules (proteins or DNA) to remain inside the vesicles. Both the direct incorporation and fusion techniques were detergent dependant and not related to the specific protein or lipids present. Therefore, these methods could be used to co-reconstitute functionally coupled IMPs (233). The fusion of liposomes to GUVs also offers the potential that the internal contents of a liposome could be delivered to GUVs with an IMP already reconstituted within them and expand the list of potential experiment types.

Another new method has been developed which circumvents the use of detergents, fusion peptides and a dehydration step (234). In this technique, GUVs are made by electroformation while PLs are assembled in the commercial Rapid Translation System. This system is a cell-free protein expression system based on T7 RNA polymerase and an optimised *E. coli* lysate (235). Transfer of proteins from the PLs to the GUVs happens spontaneously in this method. One disadvantage however, is that the cell-free protein express system cannot make post translational modifications so the protein quality and relevance for comparison with the natural biological protein is limited (146).

3.2.3.5 Perspectives with GUVs

ACCEPTED MANUSCRIPT

Reconstitution of IMPs into GUVs offer a number of advantages for functional assessment; for example, assays on lipid translocase activity that are not possible in SUV/LUV systems. However, methods to use GUVs are considerably more comnplex or labour intensive than simpler reconstitution approaches. If the experimentor decides on using this syste, it is important to consider the target protein being investigated and the conditions needed for to preserve its function. Different methods for GUV foramation and MP reconstitution have conditions that they are best suited to and therefore, each set of conditions and IMP will need its own specific method (146,157,201,236).

ACKNOWLEDGEMENTS

The work in this manuscript was generously supported by a project grant (APP1085340) from the National Health and Medical Research Council (NHMRC) of Australia.

REFERENCES

1. Seddon, A. M., Curnow, P., and Booth, P. J. (2004) Membrane proteins, lipids and detergents: not just a soap opera. *Biochimica et biophysica acta* **1666**, 105-117
2. Arachea, B. T., Sun, Z., Potente, N., Malik, R., Isailovic, D., and Viola, R. E. (2012) Detergent selection for enhanced extraction of membrane proteins. *Protein Expr Purif* **86**, 12-20
3. Rigaud, J. L., and Levy, D. (2003) Reconstitution of membrane proteins into liposomes. *Methods in enzymology* **372**, 65-86
4. Lund, S., Orlowski, S., de Foresta, B., Champeil, P., le Maire, M., and Moller, J. V. (1989) Detergent structure and associated lipid as determinants in the stabilization of solubilized Ca2+-ATPase from sarcoplasmic reticulum. *The Journal of biological chemistry* **264**, 4907-4915
5. le Maire, M., Champeil, P., and Møller, J. V. (2000) Interaction of membrane proteins and lipids with solubilizing detergents. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1508**, 86-111
6. Reeves, P. J., Hwa, J., and Khorana, H. G. (1999) Structure and function in rhodopsin: kinetic studies of retinal binding to purified opsin mutants in defined phospholipid-detergent mixtures serve as probes of the retinal binding pocket. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 1927-1931
7. Reinsberg, D., Booth, P. J., Jegerschold, C., Khoo, B. J., and Paulsen, H. (2000) Folding, assembly, and stability of the major light-harvesting complex of higher plants, LHCII, in the presence of native lipids. *Biochemistry* **39**, 14305-14313
8. Pollock, N. L., McDevitt, C. A., Collins, R., Niesten, P. H. M., Prince, S., Kerr, I. D., Ford, R. C., and Callaghan, R. (2014) Improving the stability and function of purified ABCB1 and ABCA4: The influence of membrane lipids. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1838**, 134-147
9. Taylor, A. M., Storm, J., Soceneantu, L., Linton, K. J., Gabriel, M., Martin, C., Woodhouse, J., Blott, E., Higgins, C. F., and Callaghan, R. (2001) Detailed characterization of cysteine-less P-

ACCEPTED MANUSCRIPT

glycoprotein reveals subtle pharmacological differences in function from wild-type protein. *Br J Pharmacol* **134**, 1609-1618
10. Gohon, Y., and Popot, J. L. (2003) Membrane protein-surfactant complexes. *Curr Opin Colloid In* **8**, 15-22
11. Helenius, A., and Simons, K. (1975) Solubilization of membranes by detergents. *Biochimica et biophysica acta* **415**, 29-79
12. Prive, G. G. (2007) Detergents for the stabilization and crystallization of membrane proteins. *Methods* **41**, 388-397
13. Popot, J. L., Althoff, T., Bagnard, D., Baneres, J. L., Bazzacco, P., Billon-Denis, E., Catoire, L. J., Champeil, P., Charvolin, D., Cocco, M. J., Cremel, G., Dahmane, T., de la Maza, L. M., Ebel, C., Gabel, F., Giusti, F., Gohon, Y., Goormaghtigh, E., Guittet, E., Kleinschmidt, J. H., Kuhlbrandt, W., Le Bon, C., Martinez, K. L., Picard, M., Pucci, B., Sachs, J. N., Tribet, C., van Heijenoort, C., Wien, F., Zito, F., and Zoonens, M. (2011) Amphipols from A to Z. *Annu Rev Biophys* **40**, 379-408
14. Matar-Merheb, R., Rhimi, M., Leydier, A., Huche, F., Galian, C., Desuzinges-Mandon, E., Ficheux, D., Flot, D., Aghajari, N., Kahn, R., Di Pietro, A., Jault, J. M., Coleman, A. W., and Falson, P. (2011) Structuring detergents for extracting and stabilizing functional membrane proteins. *PloS one* **6**, e18036
15. Schrader, M., Almeida, M., and Grille, S. (2012) Postfixation detergent treatment liberates the membrane modelling protein Pex11beta from peroxisomal membranes. *Histochem Cell Biol* **138**, 541-547
16. McGovern, R. E., Fernandes, H., Khan, A. R., Power, N. P., and Crowley, P. B. (2012) Protein camouflage in cytochrome c-calixarene complexes. *Nat Chem* **4**, 527-533
17. Cho, K. H., Husri, M., Amin, A., Gotfryd, K., Lee, H. J., Go, J., Kim, J. W., Loland, C. J., Guan, L., Byrne, B., and Chae, P. S. (2015) Maltose neopentyl glycol-3 (MNG-3) analogues for membrane protein study. *Analyst* **140**, 3157-3163
18. Frick, A., Eriksson, U. K., de Mattia, F., Oberg, F., Hedfalk, K., Neutze, R., de Grip, W. J., Deen, P. M., and Tornroth-Horsefield, S. (2014) X-ray structure of human aquaporin 2 and its implications for nephrogenic diabetes insipidus and trafficking. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 6305-6310
19. Kellosalo, J., Kajander, T., Kogan, K., Pokharel, K., and Goldman, A. (2012) The structure and catalytic cycle of a sodium-pumping pyrophosphatase. *Science (New York, N.Y.)* **337**, 473-476
20. Bayburt, T. H., Grinkova, Y. V., and Sligar, S. G. (2002) Self-assembly of discoidal phospholipid bilayer nanoparticles with membrane scaffold proteins. *Nano Letters* **2**, 853-856
21. Denisov, I. G., and Sligar, S. G. (2016) Nanodiscs for structural and functional studies of membrane proteins. *Nat Struct Mol Biol* **23**, 481-486
22. Bayburt, T. H., and Sligar, S. G. (2010) Membrane protein assembly into Nanodiscs. *FEBS letters* **584**, 1721-1727
23. Bayburt, T. H., Grinkova, Y. V., and Sligar, S. G. (2006) Assembly of single bacteriorhodopsin trimers in bilayer nanodiscs. *Archives of Biochemistry and Biophysics* **450**, 215-222
24. Ritchie, T. K., Grinkova, Y. V., Bayburt, T. H., Denisov, I. G., Zolnerciks, J. K., Atkins, W. M., and Sligar, S. G. (2009) Reconstitution of Membrane Proteins in Phospholipid Bilayer Nanodiscs. *Methods in enzymology* **464**, 211-231
25. Denisov, I. G., and Sligar, S. G. (2017) Nanodiscs in Membrane Biochemistry and Biophysics. *Chemical Reviews* **117**, 4669-4713
26. Hagn, F., Etzkorn, M., Raschle, T., and Wagner, G. (2013) Optimized phospholipid bilayer nanodiscs facilitate high-resolution structure determination of membrane proteins. *J Am Chem Soc* **135**, 1919-1925
27. Alvarez, F. J. D., Orelle, C., and Davidson, A. L. (2010) Functional Reconstitution of an ABC Transporter in Nanodiscs for use in Electron Paramagnetic Resonance Spectroscopy. *Journal of the American Chemical Society* **132**, 9513-9515

ACCEPTED MANUSCRIPT

28. Bao, H., Duong, F., and Chan, C. S. (2012) A Step-by-step Method for the Reconstitution of an ABC Transporter into Nanodisc Lipid Particles. *Journal of visualized experiments : JoVE*, 3910
29. Bayburt, T. H., and Sligar, S. G. (2003) Self-assembly of single integral membrane proteins into soluble nanoscale phospholipid bilayers. *Protein Sci* **12**, 2476-2481
30. Boldog, T., Grimme, S., Li, M., Sligar, S. G., and Hazelbauer, G. L. (2006) Nanodiscs separate chemoreceptor oligomeric states and reveal their signaling properties. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 11509-11514
31. Denisov, I. G., Grinkova, Y. V., Lazarides, A. A., and Sligar, S. G. (2004) Directed self-assembly of monodisperse phospholipid bilayer Nanodiscs with controlled size. *J Am Chem Soc* **126**, 3477-3487
32. Kijac, A. Z., Li, Y., Sligar, S. G., and Rienstra, C. M. (2007) Magic-Angle Spinning Solid-State NMR Spectroscopy of Nanodisc-Embedded Human CYP3A4. *Biochemistry* **46**, 13696-13703
33. Shaw, A. W., Pureza, V. S., Sligar, S. G., and Morrissey, J. H. (2007) The Local Phospholipid Environment Modulates the Activation of Blood Clotting. *Journal of Biological Chemistry* **282**, 6556-6563
34. Roos, C., Zocher, M., Muller, D., Munch, D., Schneider, T., Sahl, H. G., Scholz, F., Wachtveitl, J., Ma, Y., Proverbio, D., Henrich, E., Dotsch, V., and Bernhard, F. (2012) Characterization of co-translationally formed nanodisc complexes with small multidrug transporters, proteorhodopsin and with the E. coli MraY translocase. *Biochimica et biophysica acta* **1818**, 3098-3106
35. Mizrachi, D., Chen, Y., Liu, J., Peng, H. M., Ke, A., Pollack, L., Turner, R. J., Auchus, R. J., and DeLisa, M. P. (2015) Making water-soluble integral membrane proteins in vivo using an amphipathic protein fusion strategy. *Nat Commun* **6**, 6826
36. Shirzad-Wasei, N., van Oostrum, J., Bovee-Geurts, P. H., Kusters, L. J., Bosman, G. J., and DeGrip, W. J. (2015) Rapid transfer of overexpressed integral membrane protein from the host membrane into soluble lipid nanodiscs without previous purification. *Biol Chem* **396**, 903-915
37. Maeda, H. (2001) SMANCS and polymer-conjugated macromolecular drugs: advantages in cancer chemotherapy. *Adv Drug Deliv Rev* **46**, 169-185
38. Maeda, H., Takeshita, J., and Kanamaru, R. (1979) A lipophilic derivative of neocarzinostatin. A polymer conjugation of an antitumor protein antibiotic. *Int J Pept Protein Res* **14**, 81-87
39. Tonge, S. R., and Tighe, B. J. (2001) Responsive hydrophobically associating polymers: a review of structure and properties. *Adv Drug Deliv Rev* **53**, 109-122
40. Klumperman, B. (2010) Mechanistic considerations on styrene-maleic anhydride copolymerization reactions. *Polymer Chemistry* **1**, 558-562
41. Knowles, T. J., Finka, R., Smith, C., Lin, Y.-P., Dafforn, T., and Overduin, M. (2009) Membrane Proteins Solubilized Intact in Lipid Containing Nanoparticles Bounded by Styrene Maleic Acid Copolymer. *Journal of the American Chemical Society* **131**, 7484-7485
42. Orwick, M. C., Judge, P. J., Procek, J., Lindholm, L., Graziadei, A., Engel, A., Grobner, G., and Watts, A. (2012) Detergent-free formation and physicochemical characterization of nanosized lipid-polymer complexes: Lipodisq. *Angew Chem Int Ed Engl* **51**, 4653-4657
43. Dörr, J. M., Koorengevel, M. C., Schäfer, M., Prokofyev, A. V., Scheidelaar, S., van der Cruijsen, E. A. W., Dafforn, T. R., Baldus, M., and Killian, J. A. (2014) Detergent-free isolation, characterization, and functional reconstitution of a tetrameric K+ channel: The power of native nanodiscs. *Proceedings of the National Academy of Sciences* **111**, 18607-18612
44. Jamshad, M., Grimard, V., Idini, I., Knowles, T. J., Dowle, M. R., Schofield, N., Sridhar, P., Lin, Y., Finka, R., Wheatley, M., Thomas, O. R. T., Palmer, R. E., Overduin, M., Govaerts, C., Ruysschaert, J.-M., Edler, K. J., and Dafforn, T. R. (2015) Structural analysis of a nanoparticle containing a lipid bilayer used for detergent-free extraction of membrane proteins. *Nano Research* **8**, 774-789

ACCEPTED MANUSCRIPT

45. Vargas, C., Arenas, R. C., Frotscher, E., and Keller, S. (2015) Nanoparticle self-assembly in mixtures of phospholipids with styrene/maleic acid copolymers or fluorinated surfactants. *Nanoscale* **7**, 20685-20696
46. Zhang, R., Sahu, I. D., Liu, L., Osatuke, A., Comer, R. G., Dabney-Smith, C., and Lorigan, G. A. (2015) Characterizing the structure of lipodisq nanoparticles for membrane protein spectroscopic studies. *Biochimica et biophysica acta* **1848**, 329-333
47. Skar-Gislinge, N., Simonsen, J. B., Mortensen, K., Feidenhans'l, R., Sligar, S. G., Lindberg Møller, B., Bjørnholm, T., and Arleth, L. (2010) Elliptical Structure of Phospholipid Bilayer Nanodiscs Encapsulated by Scaffold Proteins: Casting the Roles of the Lipids and the Protein. *Journal of the American Chemical Society* **132**, 13713-13722
48. Lee, S. C., Khalid, S., Pollock, N. L., Knowles, T. J., Edler, K., Rothnie, A. J., R.T.Thomas, O., and Dafforn, T. R. (2016) Encapsulated membrane proteins: A simplified system for molecular simulation. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1858**, 2549-2557
49. Jamshad, M., Charlton, J., Lin, Y. P., Routledge, S. J., Bawa, Z., Knowles, T. J., Overduin, M., Dekker, N., Dafforn, T. R., Bill, R. M., Poyner, D. R., and Wheatley, M. (2015) G-protein coupled receptor solubilization and purification for biophysical analysis and functional studies, in the total absence of detergent. *Biosci Rep* **35**
50. Grethen, A., Oluwole, A. O., Danielczak, B., Vargas, C., and Keller, S. (2017) Thermodynamics of nanodisc formation mediated by styrene/maleic acid (2:1) copolymer. *Scientific Reports* **7**, 11517
51. Morrison, K. A., Akram, A., Mathews, A., Khan, Z. A., Patel, J. H., Zhou, C., Hardy, D. J., Moore-Kelly, C., Patel, R., Odiba, V., Knowles, T. J., Javed, M.-u.-H., Chmel, N. P., Dafforn, T. R., and Rothnie, A. J. (2016) Membrane protein extraction and purification using styrene–maleic acid (SMA) copolymer: effect of variations in polymer structure. *Biochemical Journal* **473**, 4349
52. Henry, S. M., El-Sayed, M. E., Pirie, C. M., Hoffman, A. S., and Stayton, P. S. (2006) pH-responsive poly(styrene-alt-maleic anhydride) alkylamide copolymers for intracellular drug delivery. *Biomacromolecules* **7**, 2407-2414
53. Banerjee, S., Pal, T. K., and Guha, S. K. (2012) Probing molecular interactions of poly(styrene-co-maleic acid) with lipid matrix models to interpret the therapeutic potential of the co-polymer. *Biochimica et biophysica acta* **1818**, 537-550
54. Sugai, S., Nitta, K., and Ohno, N. (1982) Studies on Conformational Transition of the Maleic-Acid Co-Polymer with Styrene in Aqueous Salt Solution by Derivative Spectroscopy. *Polymer* **23**, 238-242
55. Scheidelaar, S., Koorengevel, Martijn C., van Walree, Cornelius A., Dominguez, Juan J., Dörr, Jonas M., and Killian, J A. (2016) Effect of Polymer Composition and pH on Membrane Solubilization by Styrene-Maleic Acid Copolymers. *Biophysical journal* **111**, 1974-1986
56. Scheidelaar, S., Koorengevel, Martijn C., Pardo, Juan D., Meeldijk, Johannes D., Breukink, E., and Killian, J. A. (2015) Molecular Model for the Solubilization of Membranes into Nanodisks by Styrene Maleic Acid Copolymers. *Biophysical journal* **108**, 279-290
57. Lee, S. C., Knowles, T. J., Postis, V. L. G., Jamshad, M., Parslow, R. A., Lin, Y.-p., Goldman, A., Sridhar, P., Overduin, M., Muench, S. P., and Dafforn, T. R. (2016) A method for detergent-free isolation of membrane proteins in their local lipid environment. *Nature protocols* **11**, 1149
58. Rothnie, A. J. (2016) Detergent-Free Membrane Protein Purification. *Methods Mol Biol* **1432**, 261-267
59. Swainsbury, D. J. K., Scheidelaar, S., Foster, N., van Grondelle, R., Killian, J. A., and Jones, M. R. (2017) The effectiveness of styrene-maleic acid (SMA) copolymers for solubilisation of integral membrane proteins from SMA-accessible and SMA-resistant membranes. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1859**, 2133-2143

ACCEPTED MANUSCRIPT

60. Rehan, S., Paavilainen, V. O., and Jaakola, V.-P. (2017) Functional reconstitution of human equilibrative nucleoside transporter-1 into styrene maleic acid co-polymer lipid particles. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1859**, 1059-1065
61. Postis, V., Rawson, S., Mitchell, J. K., Lee, S. C., Parslow, R. A., Dafforn, T. R., Baldwin, S. A., and Muench, S. P. (2015) The use of SMALPs as a novel membrane protein scaffold for structure study by negative stain electron microscopy. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1848**, 496-501
62. Pollock, N. L., Lee, S. C., Patel, J. H., Gulamhussein, A. A., and Rothnie, A. J. (2017) Structure and function of membrane proteins encapsulated in a polymer-bound lipid bilayer. *Biochimica et Biophysica Acta (BBA) - Biomembranes*
63. Gulati, S., Jamshad, M., Knowles, Timothy J., Morrison, Kerrie A., Downing, R., Cant, N., Collins, R., Koenderink, Jan B., Ford, Robert C., Overduin, M., Kerr, Ian D., Dafforn, Timothy R., and Rothnie, Alice J. (2014) Detergent-free purification of ABC (ATP-binding-cassette) transporters. *Biochemical Journal* **461**, 269
64. Swainsbury, D. J. K., Scheidelaar, S., van Grondelle, R., Killian, J. A., and Jones, M. R. (2014) Bacterial Reaction Centers Purified with Styrene Maleic Acid Copolymer Retain Native Membrane Functional Properties and Display Enhanced Stability. *Angewandte Chemie (International Ed. in English)* **53**, 11803-11807
65. Orwick-Rydmark, M., Lovett, J. E., Graziadei, A., Lindholm, L., Hicks, M. R., and Watts, A. (2012) Detergent-Free Incorporation of a Seven-Transmembrane Receptor Protein into Nanosized Bilayer Lipodisq Particles for Functional and Biophysical Studies. *Nano Letters* **12**, 4687-4692
66. Sahu, I. D., McCarrick, R. M., Troxel, K. R., Zhang, R., Smith, H. J., Dunagan, M. M., Swartz, M. S., Rajan, P. V., Kroncke, B. M., Sanders, C. R., and Lorigan, G. A. (2013) DEER EPR measurements for membrane protein structures via bifunctional spin labels and lipodisq nanoparticles. *Biochemistry* **52**, 6627-6632
67. Martin, R. E., Marchetti, R. V., Cowan, A. I., Howitt, S. M., Broer, S., and Kirk, K. (2009) Chloroquine transport via the malaria parasite's chloroquine resistance transporter. *Science (New York, N.Y.)* **325**, 1680-1682
68. Zhang, R., Sahu, I. D., Bali, A. P., Dabney-Smith, C., and Lorigan, G. A. (2017) Characterization of the structure of lipodisq nanoparticles in the presence of KCNE1 by dynamic light scattering and transmission electron microscopy. *Chemistry and physics of lipids* **203**, 19-23
69. Long, A. R., O'Brien, C. C., Malhotra, K., Schwall, C. T., Albert, A. D., Watts, A., and Alder, N. N. (2013) A detergent-free strategy for the reconstitution of active enzyme complexes from native biological membranes into nanoscale discs. *BMC Biotechnol* **13**, 41
70. Paulin, S., Jamshad, M., Dafforn, T. R., Garcia-Lara, J., Foster, S. J., Galley, N. F., Roper, D. I., Rosado, H., and Taylor, P. W. (2014) Surfactant-free purification of membrane protein complexes from bacteria: application to the staphylococcal penicillin-binding protein complex PBP2/PBP2a. *Nanotechnology* **25**, 285101
71. Prabudiansyah, I., Kusters, I., Caforio, A., and Driessen, A. J. M. (2015) Characterization of the annular lipid shell of the Sec translocon. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1848**, 2050-2056
72. Broecker, J., Eger, B. T., and Ernst, O. P. (2017) Crystallogenesis of Membrane Proteins Mediated by Polymer-Bounded Lipid Nanodiscs. *Structure* **25**, 384-392
73. Oluwole, A. O., Danielczak, B., Meister, A., Babalola, J. O., Vargas, C., and Keller, S. (2017) Solubilization of Membrane Proteins into Functional Lipid-Bilayer Nanodiscs Using a Diisobutylene/Maleic Acid Copolymer. *Angewandte Chemie International Edition* **56**, 1919-1924
74. Cuevas Arenas, R., Klingler, J., Vargas, C., and Keller, S. (2016) Influence of lipid bilayer properties on nanodisc formation mediated by styrene/maleic acid copolymers. *Nanoscale* **8**, 15016-15026

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

75. Craig, A. F., Clark, E. E., Sahu, I. D., Zhang, R., Frantz, N. D., Al-Abdul-Wahid, M. S., Dabney-Smith, C., Konkolewicz, D., and Lorigan, G. A. (2016) Tuning the size of styrene-maleic acid copolymer-lipid nanoparticles (SMALPs) using RAFT polymerization for biophysical studies. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1858**, 2931-2939
76. Esmaili, M., and Overduin, M. Membrane biology visualized in nanometer-sized discs formed by styrene maleic acid polymers. *Biochimica et Biophysica Acta (BBA) - Biomembranes*
77. Fiori, M. C., Jiang, Y., Altenberg, G. A., and Liang, H. (2017) Polymer-encased nanodiscs with improved buffer compatibility. *Scientific Reports* **7**, 7432
78. Geertsma, E. R., Nik Mahmood, N. A., Schuurman-Wolters, G. K., and Poolman, B. (2008) Membrane reconstitution of ABC transporters and assays of translocator function. *Nature protocols* **3**, 256-266
79. Hinkle, P. C., Kim, J. J., and Racker, E. (1972) Ion transport and respiratory control in vesicles formed from cytochrome oxidase and phospholipids. *The Journal of biological chemistry* **247**, 1338-1339
80. Racker, E. (1972) Reconstitution of a calcium pump with phospholipids and a purified Ca ++ -adenosine triphosphatase from sacroplasmic reticulum. *The Journal of biological chemistry* **247**, 8198-8200
81. Kagawa, Y., and Racker, E. (1971) Partial Resolution of the Enzymes Catalyzing Oxidative Phosphorylation: XXV. RECONSTITUTION OF VESICLES CATALYZING 32Pi—ADENOSINE TRIPHOSPHATE EXCHANGE. *Journal of Biological Chemistry* **246**, 5477-5487
82. Brohawn, S. G., del Marmol, J., and MacKinnon, R. (2012) Crystal structure of the human K2P TRAAK, a lipid- and mechano-sensitive K+ ion channel. *Science (New York, N.Y.)* **335**, 436-441
83. Wang, L., and Sigworth, F. J. (2009) Structure of the BK potassium channel in a lipid membrane from electron cryomicroscopy. *Nature* **461**, 292-295
84. Nakao, S., Ebata, H., Hamamoto, T., Kagawa, Y., and Hirata, H. (1988) Solubilization and reconstitution of voltage-dependent calcium channel from bovine cardiac muscle. Ca2+ influx assay using the fluorescent dye Quin2. *Biochimica et biophysica acta* **944**, 337-343
85. Ramos-Franco, J., Bare, D., Caenepeel, S., Nani, A., Fill, M., and Mignery, G. (2000) Single-channel function of recombinant type 2 inositol 1,4, 5-trisphosphate receptor. *Biophysical journal* **79**, 1388-1399
86. Tanaka, J. C., Eccleston, J. F., and Barchi, R. L. (1983) Cation selectivity characteristics of the reconstituted voltage-dependent sodium channel purified from rat skeletal muscle sarcolemma. *Journal of Biological Chemistry* **258**, 7519-7526
87. Mignery, G. A., Johnston, P. A., and Sudhof, T. C. (1992) Mechanism of Ca2+ inhibition of inositol 1,4,5-trisphosphate (InsP3) binding to the cerebellar InsP3 receptor. *The Journal of biological chemistry* **267**, 7450-7455
88. Kameyama, A., Shearman, M. S., Sekiguchi, K., and Kameyama, M. (1996) Cyclic AMP-dependent protein kinase but not protein kinase C regulates the cardiac Ca2+ channel through phosphorylation of its alpha 1 subunit. *Journal of biochemistry* **120**, 170-176
89. Epstein, M., and Racker, E. (1978) Reconstitution of carbamylcholine-dependent sodium ion flux and desensitization of the acetylcholine receptor from Torpedo californica. *The Journal of biological chemistry* **253**, 6660-6662
90. Schiebler, W., and Hucho, F. (1978) Membranes rich in acetylcholine receptor: characterization and reconstitution to excitable membranes from exogenous lipids. *European journal of biochemistry* **85**, 55-63
91. Ramos, J., Jung, W., Ramos-Franco, J., Mignery, G. A., and Fill, M. (2003) Single channel function of inositol 1,4,5-trisphosphate receptor type-1 and -2 isoform domain-swap chimeras. *The Journal of general physiology* **121**, 399-411
92. Brown, M. F. (1994) Modulation of rhodopsin function by properties of the membrane bilayer. *Chemistry and physics of lipids* **73**, 159-180

ACCEPTED MANUSCRIPT

93. Escribá, P. V., Ozaita, A., Ribas, C., Miralles, A., Fodor, E., Farkas, T., and García-Sevilla, J. A. (1997) Role of lipid polymorphism in G protein-membrane interactions: Nonlamellar-prone phospholipids and peripheral protein binding to membranes. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 11375-11380
94. Yang, Q., Alemany, R., Casas, J., Kitajka, K., Lanier, S. M., and Escriba, P. V. (2005) Influence of the membrane lipid structure on signal processing via G protein-coupled receptors. *Mol Pharmacol* **68**, 210-217
95. Attard, G. S., Templer, R. H., Smith, W. S., Hunt, A. N., and Jackowski, S. (2000) Modulation of CTP:phosphocholine cytidylyltransferase by membrane curvature elastic stress. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 9032-9036
96. Epand, R. M., and Lester, D. S. (1990) The role of membrane biophysical properties in the regulation of protein kinase C activity. *Trends in pharmacological sciences* **11**, 317-320
97. Hurlimann, L. M., Corradi, V., Hohl, M., Bloemberg, G. V., Tieleman, D. P., and Seeger, M. A. (2016) The Heterodimeric ABC Transporter EfrCD Mediates Multidrug Efflux in Enterococcus faecalis. *Antimicrobial agents and chemotherapy* **60**, 5400-5411
98. Kunert, B., Gardiennet, C., Lacabanne, D., Calles-Garcia, D., Falson, P., Jault, J. M., Meier, B. H., Penin, F., and Bockmann, A. (2014) Efficient and stable reconstitution of the ABC transporter BmrA for solid-state NMR studies. *Frontiers in molecular biosciences* **1**, 5
99. Trépout, S., Taveau, J.-C., Benabdelhak, H., Granier, T., Ducruix, A., Frangakis, A. S., and Lambert, O. (2010) Structure of reconstituted bacterial membrane efflux pump by cryo-electron tomography. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1798**, 1953-1960
100. Young, H. S., Rigaud, J. L., Lacapere, J. J., Reddy, L. G., and Stokes, D. L. (1997) How to make tubular crystals by reconstitution of detergent-solubilized Ca2(+)-ATPase. *Biophysical journal* **72**, 2545-2558
101. Cheng, K. H., Lepock, J. R., Hui, S. W., and Yeagle, P. L. (1986) The role of cholesterol in the activity of reconstituted Ca-ATPase vesicles containing unsaturated phosphatidylethanolamine. *The Journal of biological chemistry* **261**, 5081-5087
102. Wakabayashi, S., and Shigekawa, M. (1985) Rapid reconstitution and characterization of highly-efficient sarcoplasmic reticulum Ca pump. *Biochimica et biophysica acta* **813**, 266-276
103. Tokuda, H., and Konisky, J. (1979) Effect of colicins Ia and E1 on ion permeability of liposomes. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 6167-6171
104. Uratani, Y., and Cramer, W. A. (1981) Reconstitution of colicin E1 into dimyristoylphosphatidylcholine membrane vesicles. *The Journal of biological chemistry* **256**, 4017-4023
105. Phutrakul, S., and Jones, M. N. (1979) The permeability of bilayer lipid membranes on the incorporation of erythrocyte membrane extracts and the identification of the monosaccharide transport proteins. *Biochimica et biophysica acta* **550**, 188-200
106. Sogin, D. C., and Hinkle, P. C. (1980) Binding of cytochalasin B to human erythrocyte glucose transporter. *Biochemistry* **19**, 5417-5420
107. Geibel, S., Zimmermann, D., Zifarelli, G., Becker, A., Koenderink, J. B., Hu, Y. K., Kaplan, J. H., Friedrich, T., and Bamberg, E. (2003) Conformational dynamics of Na+/K+- and H+/K+-ATPase probed by voltage clamp fluorometry. *Annals of the New York Academy of Sciences* **986**, 31-38
108. Lugo, M. R., and Sharom, F. J. (2005) Interaction of LDS-751 with P-glycoprotein and mapping of the location of the R drug binding site. *Biochemistry* **44**, 643-655
109. Alqawi, O., Poelarends, G., Konings, W. N., and Georges, E. (2003) Photoaffinity labeling under non-energized conditions of a specific drug-binding site of the ABC multidrug transporter LmrA from Lactococcus lactis. *Biochemical and biophysical research communications* **311**, 696-701

ACCEPTED MANUSCRIPT

110. Beales, P. A., Ciani, B., and Cleasby, A. J. (2015) Nature's lessons in design: nanomachines to scaffold, remodel and shape membrane compartments. *Physical chemistry chemical physics : PCCP* **17**, 15489-15507
111. Poolman, B., Doeven, M. K., Geertsma, E. R., Biemans-Oldehinkel, E., Konings, W. N., and Rees, D. C. (2005) Functional analysis of detergent-solubilized and membrane-reconstituted ATP-binding cassette transporters. *Methods in enzymology* **400**, 429-459
112. Szoka, F., Jr., and Papahadjopoulos, D. (1980) Comparative properties and methods of preparation of lipid vesicles (liposomes). *Annual review of biophysics and bioengineering* **9**, 467-508
113. Mayer, L. D., Hope, M. J., and Cullis, P. R. (1986) Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochimica et biophysica acta* **858**, 161-168
114. MacDonald, R. C., MacDonald, R. I., Menco, B. P., Takeshita, K., Subbarao, N. K., and Hu, L. R. (1991) Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochimica et biophysica acta* **1061**, 297-303
115. White, G. F., Racher, K. I., Lipski, A., Hallett, F. R., and Wood, J. M. (2000) Physical properties of liposomes and proteoliposomes prepared from Escherichia coli polar lipids. *Biochimica et biophysica acta* **1468**, 175-186
116. Oku, N., Kendall, D., and C. MacDonald, R. (1982) *A simple procedure for the determination of the trapped volume of liposomes*,
117. Wang, L., and Tonggu, L. (2015) Membrane protein reconstitution for functional and structural studies. *Science China. Life sciences* **58**, 66-74
118. Biner, O., Schick, T., Muller, Y., and von Ballmoos, C. (2016) Delivery of membrane proteins into small and giant unilamellar vesicles by charge-mediated fusion. *FEBS letters* **590**, 2051-2062
119. Shen, H. H., Lithgow, T., and Martin, L. (2013) Reconstitution of membrane proteins into model membranes: seeking better ways to retain protein activities. *International journal of molecular sciences* **14**, 1589-1607
120. Rigaud, J. L., Pitard, B., and Levy, D. (1995) Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins. *Biochimica et biophysica acta* **1231**, 223-246
121. Knol, J., Sjollema, K., and Poolman, B. (1998) Detergent-mediated reconstitution of membrane proteins. *Biochemistry* **37**, 16410-16415
122. Rigaud, J. L., Paternostre, M. T., and Bluzat, A. (1988) Mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. 2. Incorporation of the light-driven proton pump bacteriorhodopsin. *Biochemistry* **27**, 2677-2688
123. Levy, D., Bluzat, A., Seigneuret, M., and Rigaud, J. L. (1990) A systematic study of liposome and proteoliposome reconstitution involving Bio-Bead-mediated Triton X-100 removal. *Biochimica et biophysica acta* **1025**, 179-190
124. Bucher, K., Belli, S., Wunderli-Allenspach, H., and Kramer, S. D. (2007) P-glycoprotein in proteoliposomes with low residual detergent: the effects of cholesterol. *Pharmaceutical research* **24**, 1993-2004
125. Dalziel, J. E., Wong, S. S., Phung, T., Zhang, Y. L., and Dunlop, J. (2007) Expression of human BK ion channels in Sf9 cells, their purification using metal affinity chromatography, and functional reconstitution into planar lipid bilayers. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences* **857**, 315-321
126. Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, C., and Reynolds, J. A. (1981) Phospholipid vesicle formation and transmembrane protein incorporation using octyl glucoside. *Biochemistry* **20**, 833-840
127. Holloway, P. W. (1973) A simple procedure for removal of Triton X-100 from protein samples. *Analytical biochemistry* **53**, 304-308

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

128. Zhou, X., and Graham, T. R. (2009) Reconstitution of phospholipid translocase activity with purified Drs2p, a type-IV P-type ATPase from budding yeast. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 16586-16591
129. Kim, M., and Song, E. (2010) Iron transport by proteoliposomes containing mitochondrial F(1)F(0) ATP synthase isolated from rat heart. *Biochimie* **92**, 333-342
130. Hamada, T., and Yoshikawa, K. (2012) *Cell-Sized Liposomes and Droplets: Real-World Modeling of Living Cells*,
131. Yanagisawa, M., Iwamoto, M., Kato, A., Yoshikawa, K., and Oiki, S. (2011) Oriented reconstitution of a membrane protein in a giant unilamellar vesicle: experimental verification with the potassium channel KcsA. *J Am Chem Soc* **133**, 11774-11779
132. McElroy, W. D., and DeLuca, M. A. (1983) Firefly and bacterial luminescence: basic science and applications. *Journal of applied biochemistry* **5**, 197-209
133. Hess, H. H., and Derr, J. E. (1975) Assay of inorganic and organic phosphorus in the 0.1-5 nanomole range. *Analytical biochemistry* **63**, 607-613
134. Chifflet, S., Torriglia, A., Chiesa, R., and Tolosa, S. (1988) A method for the determination of inorganic phosphate in the presence of labile organic phosphate and high concentrations of protein: application to lens ATPases. *Analytical biochemistry* **168**, 1-4
135. Wetterau, J. R., and Jonas, A. (1982) Effect of dipalmitoylphosphatidylcholine vesicle curvature on the reaction with human apolipoprotein A-I. *The Journal of biological chemistry* **257**, 10961-10966
136. Wieprecht, T., Beyermann, M., and Seelig, J. (2002) Thermodynamics of the coil-alpha-helix transition of amphipathic peptides in a membrane environment: the role of vesicle curvature. *Biophysical chemistry* **96**, 191-201
137. Tonnesen, A., Christensen, S. M., Tkach, V., and Stamou, D. (2014) Geometrical membrane curvature as an allosteric regulator of membrane protein structure and function. *Biophysical journal* **106**, 201-209
138. Epand, R. F., Martinou, J. C., Fornallaz-Mulhauser, M., Hughes, D. W., and Epand, R. M. (2002) The apoptotic protein tBid promotes leakage by altering membrane curvature. *The Journal of biological chemistry* **277**, 32632-32639
139. Epand, R. M. (2007) Membrane lipid polymorphism: relationship to bilayer properties and protein function. *Methods Mol Biol* **400**, 15-26
140. Hong, H., and Tamm, L. K. (2004) Elastic coupling of integral membrane protein stability to lipid bilayer forces. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 4065-4070
141. Rothnie, A., Theron, D., Soceneantu, L., Martin, C., Traikia, M., Berridge, G., Higgins, C. F., Devaux, P. F., and Callaghan, R. (2001) The importance of cholesterol in maintenance of P-glycoprotein activity and its membrane perturbing influence. *European biophysics journal : EBJ* **30**, 430-442
142. Pohl, A., Devaux, P. F., and Herrmann, A. (2005) Function of prokaryotic and eukaryotic ABC proteins in lipid transport. *Biochimica et biophysica acta* **1733**, 29-52
143. Girard, P., Pecreaux, J., Lenoir, G., Falson, P., Rigaud, J. L., and Bassereau, P. (2004) A new method for the reconstitution of membrane proteins into giant unilamellar vesicles. *Biophysical journal* **87**, 419-429
144. Iwahashi, Y., and Nakamura, T. (1989) Orientation and reactivity of NADH kinase in proteoliposomes. *Journal of biochemistry* **105**, 922-926
145. Bhatia, T., Husen, P., Brewer, J., Bagatolli, L. A., Hansen, P. L., Ipsen, J. H., and Mouritsen, O. G. (2015) Preparing giant unilamellar vesicles (GUVs) of complex lipid mixtures on demand: Mixing small unilamellar vesicles of compositionally heterogeneous mixtures. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1848**, 3175-3180
146. Fenz, S. F., and Sengupta, K. (2012) Giant vesicles as cell models. *Integrative biology : quantitative biosciences from nano to macro* **4**, 982-995

ACCEPTED MANUSCRIPT

147. Morales-Penningston, N. F., Wu, J., Farkas, E. R., Goh, S. L., Konyakhina, T. M., Zheng, J. Y., Webb, W. W., and Feigenson, G. W. (2010) GUV preparation and imaging: minimizing artifacts. *Biochimica et biophysica acta* **1798**, 1324-1332
148. Dezi, M., Di Cicco, A., Bassereau, P., and Levy, D. (2013) Detergent-mediated incorporation of transmembrane proteins in giant unilamellar vesicles with controlled physiological contents. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 7276-7281
149. Papadopulos, A., Vehring, S., Lopez-Montero, I., Kutschenko, L., Stockl, M., Devaux, P. F., Kozlov, M., Pomorski, T., and Herrmann, A. (2007) Flippase activity detected with unlabeled lipids by shape changes of giant unilamellar vesicles. *The Journal of biological chemistry* **282**, 15559-15568
150. Schwille, P. (2011) Bottom-up synthetic biology: engineering in a tinkerer's world. *Science (New York, N.Y.)* **333**, 1252-1254
151. Bouvrais, H., Meleard, P., Pott, T., Jensen, K. J., Brask, J., and Ipsen, J. H. (2008) Softening of POPC membranes by magainin. *Biophysical chemistry* **137**, 7-12
152. Bouvrais, H., Cornelius, F., Ipsen, J. H., and Mouritsen, O. G. (2012) Intrinsic reaction-cycle time scale of Na+,K+-ATPase manifests itself in the lipid-protein interactions of nonequilibrium membranes. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 18442-18446
153. Aimon, S., Manzi, J., Schmidt, D., Poveda Larrosa, J. A., Bassereau, P., and Toombes, G. E. (2011) Functional reconstitution of a voltage-gated potassium channel in giant unilamellar vesicles. *PloS one* **6**, e25529
154. Plasencia, I., Norlen, L., and Bagatolli, L. A. (2007) Direct visualization of lipid domains in human skin stratum corneum's lipid membranes: effect of pH and temperature. *Biophysical journal* **93**, 3142-3155
155. Bernardino de la Serna, J., Perez-Gil, J., Simonsen, A. C., and Bagatolli, L. A. (2004) Cholesterol rules: direct observation of the coexistence of two fluid phases in native pulmonary surfactant membranes at physiological temperatures. *The Journal of biological chemistry* **279**, 40715-40722
156. Baumgart, T., Hammond, A. T., Sengupta, P., Hess, S. T., Holowka, D. A., Baird, B. A., and Webb, W. W. (2007) Large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 3165-3170
157. Montes, L. R., Alonso, A., Goni, F. M., and Bagatolli, L. A. (2007) Giant unilamellar vesicles electroformed from native membranes and organic lipid mixtures under physiological conditions. *Biophysical journal* **93**, 3548-3554
158. Richmond, D. L., Schmid, E. M., Martens, S., Stachowiak, J. C., Liska, N., and Fletcher, D. A. (2011) Forming giant vesicles with controlled membrane composition, asymmetry, and contents. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 9431-9436
159. Noireaux, V., Maeda, Y. T., and Libchaber, A. (2011) Development of an artificial cell, from self-organization to computation and self-reproduction. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 3473-3480
160. Noireaux, V., and Libchaber, A. (2004) A vesicle bioreactor as a step toward an artificial cell assembly. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 17669-17674
161. Helfrich, W. (1989) Spontaneous and Induced Adhesion of Fluid Membranes. in *Phase Transitions in Soft Condensed Matter* (Riste, T., and Sherrington, D. eds.), Springer US, Boston, MA. pp 271-281
162. Evans, E. (1991) Entropy-driven tension in vesicle membranes and unbinding of adherent vesicles. *Langmuir : the ACS journal of surfaces and colloids* **7**, 1900-1908

ACCEPTED MANUSCRIPT

163. Sackmann, E., and Bruinsma, R. F. (2002) Cell adhesion as wetting transition? *Chemphyschem : a European journal of chemical physics and physical chemistry* **3**, 262-269
164. Smith, A. S., and Sackmann, E. (2009) Progress in mimetic studies of cell adhesion and the mechanosensing. *Chemphyschem : a European journal of chemical physics and physical chemistry* **10**, 66-78
165. Bucher, P., Fischer, A., Luisi, P. L., Oberholzer, T., and Walde, P. (1998) Giant Vesicles as Biochemical Compartments: The Use of Microinjection Techniques. *Langmuir : the ACS journal of surfaces and colloids* **14**, 2712-2721
166. Angelova, M. I., Hristova, N., and Tsoneva, I. (1999) DNA-induced endocytosis upon local microinjection to giant unilamellar cationic vesicles. *European biophysics journal : EBJ* **28**, 142-150
167. Cans, A. S., Wittenberg, N., Karlsson, R., Sombers, L., Karlsson, M., Orwar, O., and Ewing, A. (2003) Artificial cells: unique insights into exocytosis using liposomes and lipid nanotubes. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 400-404
168. Tareste, D., Shen, J., Melia, T. J., and Rothman, J. E. (2008) SNAREpin/Munc18 promotes adhesion and fusion of large vesicles to giant membranes. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 2380-2385
169. Dietrich, C., Angelova, M., and Pouligny, B. (1997) Adhesion of Latex Spheres to Giant Phospholipid Vesicles: Statics and Dynamics. *J. Phys. II France* **7**, 1651-1682
170. Cirac, A. D., Moiset, G., Mika, J. T., Kocer, A., Salvador, P., Poolman, B., Marrink, S. J., and Sengupta, D. (2011) The molecular basis for antimicrobial activity of pore-forming cyclic peptides. *Biophysical journal* **100**, 2422-2431
171. Apellaniz, B., Garcia-Saez, A. J., Huarte, N., Kunert, R., Vorauer-Uhl, K., Katinger, H., Schwille, P., and Nieva, J. L. (2010) Confocal microscopy of giant vesicles supports the absence of HIV-1 neutralizing 2F5 antibody reactivity to plasma membrane phospholipids. *FEBS letters* **584**, 1591-1596
172. Schwille, P., and Diez, S. (2009) Synthetic biology of minimal systems. *Critical reviews in biochemistry and molecular biology* **44**, 223-242
173. Kahya, N., Scherfeld, D., Bacia, K., Poolman, B., and Schwille, P. (2003) Probing lipid mobility of raft-exhibiting model membranes by fluorescence correlation spectroscopy. *The Journal of biological chemistry* **278**, 28109-28115
174. Roux, A., Koster, G., Lenz, M., Sorre, B., Manneville, J. B., Nassoy, P., and Bassereau, P. (2010) Membrane curvature controls dynamin polymerization. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 4141-4146
175. Dietrich, C., Bagatolli, L. A., Volovyk, Z. N., Thompson, N. L., Levi, M., Jacobson, K., and Gratton, E. (2001) Lipid rafts reconstituted in model membranes. *Biophysical journal* **80**, 1417-1428
176. Henriksen, J. R., and Ipsen, J. H. (2004) Measurement of membrane elasticity by micro-pipette aspiration. *The European physical journal. E, Soft matter* **14**, 149-167
177. Kwok, R., and Evans, E. (1981) Thermoelasticity of large lecithin bilayer vesicles. *Biophysical journal* **35**, 637-652
178. Chen, C., Lubensky, T. C., and MacKintosh, F. C. (1995) Phase transitions and modulated phases in lipid bilayers. *Physical review. E, Statistical physics, plasmas, fluids, and related interdisciplinary topics* **51**, 504-513
179. Meleard, P., Pott, T., Bouvrais, H., and Ipsen, J. H. (2011) Advantages of statistical analysis of giant vesicle flickering for bending elasticity measurements. *The European physical journal. E, Soft matter* **34**, 116
180. Pontani, L. L., van der Gucht, J., Salbreux, G., Heuvingh, J., Joanny, J. F., and Sykes, C. (2009) Reconstitution of an actin cortex inside a liposome. *Biophysical journal* **96**, 192-198

ACCEPTED MANUSCRIPT

181. Romer, W., Pontani, L. L., Sorre, B., Rentero, C., Berland, L., Chambon, V., Lamaze, C., Bassereau, P., Sykes, C., Gaus, K., and Johannes, L. (2010) Actin dynamics drive membrane reorganization and scission in clathrin-independent endocytosis. *Cell* **140**, 540-553
182. Abkarian, M., Loiseau, E., and Massiera, G. (2011) Continuous droplet interface crossing encapsulation (cDICE) for high throughput monodisperse vesicle design. *Soft Matter* **7**, 4610-4614
183. Gaul, V., Lopez, S. G., Lentz, B. R., Moran, N., Forster, R. J., and Keyes, T. E. (2015) The lateral diffusion and fibrinogen induced clustering of platelet integrin alphaIIbbeta3 reconstituted into physiologically mimetic GUVs. *Integrative biology : quantitative biosciences from nano to macro* **7**, 402-411
184. Kahya, N., Pecheur, E. I., de Boeij, W. P., Wiersma, D. A., and Hoekstra, D. (2001) Reconstitution of membrane proteins into giant unilamellar vesicles via peptide-induced fusion. *Biophysical journal* **81**, 1464-1474
185. Manneville, J. B., Bassereau, P., Ramaswamy, S., and Prost, J. (2001) Active membrane fluctuations studied by micropipet aspiration. *Physical review. E, Statistical, nonlinear, and soft matter physics* **64**, 021908
186. Reeves, J. P., and Dowben, R. M. (1969) Formation and properties of thin-walled phospholipid vesicles. *Journal of cellular physiology* **73**, 49-60
187. Akashi, K., Miyata, H., Itoh, H., and Kinosita, K. (1996) Preparation of giant liposomes in physiological conditions and their characterization under an optical microscope. *Biophysical journal* **71**, 3242-3250
188. Stein, H., Spindler, S., Bonakdar, N., Wang, C., and Sandoghdar, V. (2017) Production of Isolated Giant Unilamellar Vesicles under High Salt Concentrations. *Frontiers in Physiology* **8**
189. Horger, K. S., Estes, D. J., Capone, R., and Mayer, M. (2009) Films of agarose enable rapid formation of giant liposomes in solutions of physiologic ionic strength. *J Am Chem Soc* **131**, 1810-1819
190. Walde, P., Cosentino, K., Engel, H., and Stano, P. (2010) Giant vesicles: preparations and applications. *Chembiochem : a European journal of chemical biology* **11**, 848-865
191. Hishida, M., Seto, H., and Yoshikawa, K. (2005) Smooth/rough layering in liquid-crystalline/gel state of dry phospholipid film, in relation to its ability to generate giant vesicles. *Chemical Physics Letters* **411**, 267-272
192. Tsumoto, K., Matsuo, H., Tomita, M., and Yoshimura, T. (2009) Efficient formation of giant liposomes through the gentle hydration of phosphatidylcholine films doped with sugar. *Colloids and surfaces. B, Biointerfaces* **68**, 98-105
193. Akashi, K., Miyata, H., Itoh, H., and Kinosita, K., Jr. (1998) Formation of giant liposomes promoted by divalent cations: critical role of electrostatic repulsion. *Biophysical journal* **74**, 2973-2982
194. Rodriguez, N., Pincet, F., and Cribier, S. (2005) Giant vesicles formed by gentle hydration and electroformation: a comparison by fluorescence microscopy. *Colloids and surfaces. B, Biointerfaces* **42**, 125-130
195. Angelova, M. I., Soléau, S., Méléard, P., Faucon, F., and Bothorel, P. (1992) Preparation of giant vesicles by external AC electric fields. Kinetics and applications. in *Trends in Colloid and Interface Science VI* (Helm, C., Lösche, M., and Möhwald, H. eds.), Steinkopff, Darmstadt. pp 127-131
196. Angelova, M. I., and Dimitrov, D. S. (1986) Liposome electroformation. *Faraday Discussions of the Chemical Society* **81**, 303-311
197. Husen, P., Arriaga, L. R., Monroy, F., Ipsen, J. H., and Bagatolli, L. A. (2012) Morphometric image analysis of giant vesicles: a new tool for quantitative thermodynamics studies of phase separation in lipid membranes. *Biophysical journal* **103**, 2304-2310
198. Estes, D. J., and Mayer, M. (2005) Giant liposomes in physiological buffer using electroformation in a flow chamber. *Biochimica et biophysica acta* **1712**, 152-160

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

199. Diguet, A., Le Berre, M., Chen, Y., and Baigl, D. (2009) Preparation of phospholipid multilayer patterns of controlled size and thickness by capillary assembly on a microstructured substrate. *Small (Weinheim an der Bergstrasse, Germany)* **5**, 1661-1666
200. Méléard, P., Bagatolli, L. A., and Pott, T. (2009) Chapter 9 - Giant Unilamellar Vesicle Electroformation: From Lipid Mixtures to Native Membranes Under Physiological Conditions. in *Methods in enzymology*, Academic Press. pp 161-176
201. Pott, T., Bouvrais, H., and Meleard, P. (2008) Giant unilamellar vesicle formation under physiologically relevant conditions. *Chemistry and physics of lipids* **154**, 115-119
202. Ayuyan, A. G., and Cohen, F. S. (2006) Lipid peroxides promote large rafts: effects of excitation of probes in fluorescence microscopy and electrochemical reactions during vesicle formation. *Biophysical journal* **91**, 2172-2183
203. Li, Q., Wang, X., Ma, S., Zhang, Y., and Han, X. (2016) Electroformation of giant unilamellar vesicles in saline solution. *Colloids and Surfaces B: Biointerfaces* **147**, 368-375
204. Pautot, S., Frisken, B. J., and Weitz, D. A. (2003) Production of Unilamellar Vesicles Using an Inverted Emulsion. *Langmuir : the ACS journal of surfaces and colloids* **19**, 2870-2879
205. Pautot, S., Frisken, B. J., and Weitz, D. A. (2003) Engineering asymmetric vesicles. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 10718-10721
206. Stachowiak, J. C., Richmond, D. L., Li, T. H., Liu, A. P., Parekh, S. H., and Fletcher, D. A. (2008) Unilamellar vesicle formation and encapsulation by microfluidic jetting. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 4697-4702
207. Shum, H. C., Lee, D., Yoon, I., Kodger, T., and Weitz, D. A. (2008) Double emulsion templated monodisperse phospholipid vesicles. *Langmuir : the ACS journal of surfaces and colloids* **24**, 7651-7653
208. Criado, M., and Keller, B. U. (1987) A membrane fusion strategy for single-channel recordings of membranes usually non-accessible to patch-clamp pipette electrodes. *FEBS letters* **224**, 172-176
209. Ajouz, B., Berrier, C., Besnard, M., Martinac, B., and Ghazi, A. (2000) Contributions of the different extramembranous domains of the mechanosensitive ion channel MscL to its response to membrane tension. *The Journal of biological chemistry* **275**, 1015-1022
210. Darszon, A., Vandenberg, C. A., Schonfeld, M., Ellisman, M. H., Spitzer, N. C., and Montal, M. (1980) Reassembly of protein-lipid complexes into large bilayer vesicles: perspectives for membrane reconstitution. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 239-243
211. Kang, Y. J., Wostein, H. S., and Majd, S. (2013) A Simple and Versatile Method for the Formation of Arrays of Giant Vesicles with Controlled Size and Composition. *Advanced Materials* **25**, 6834-6838
212. Sessa, G., and Weissmann, G. (1970) Incorporation of lysozyme into liposomes. A model for structure-linked latency. *The Journal of biological chemistry* **245**, 3295-3301
213. Bagatolli, L. A., and Gratton, E. (2000) Two photon fluorescence microscopy of coexisting lipid domains in giant unilamellar vesicles of binary phospholipid mixtures. *Biophysical journal* **78**, 290-305
214. Baumgart, T., Hess, S. T., and Webb, W. W. (2003) Imaging coexisting fluid domains in biomembrane models coupling curvature and line tension. *Nature* **425**, 821-824
215. Semrau, S., Idema, T., Holtzer, L., Schmidt, T., and Storm, C. (2008) Accurate determination of elastic parameters for multicomponent membranes. *Physical review letters* **100**, 088101
216. Veatch, S. L., and Keller, S. L. (2005) Miscibility phase diagrams of giant vesicles containing sphingomyelin. *Physical review letters* **94**, 148101
217. Chiantia, S., Schwille, P., Klymchenko, A. S., and London, E. (2011) Asymmetric GUVs prepared by MbetaCD-mediated lipid exchange: an FCS study. *Biophysical journal* **100**, L1-3

ACCEPTED MANUSCRIPT

218. Doeven, M. K., Folgering, J. H., Krasnikov, V., Geertsma, E. R., van den Bogaart, G., and Poolman, B. (2005) Distribution, lateral mobility and function of membrane proteins incorporated into giant unilamellar vesicles. *Biophysical journal* **88**, 1134-1142
219. Ricker, J. V., Tsvetkova, N. M., Wolkers, W. F., Leidy, C., Tablin, F., Longo, M., and Crowe, J. H. (2003) Trehalose maintains phase separation in an air-dried binary lipid mixture. *Biophysical journal* **84**, 3045-3051
220. Hincha, D. K., Zuther, E., and Heyer, A. G. (2003) The preservation of liposomes by raffinose family oligosaccharides during drying is mediated by effects on fusion and lipid phase transitions. *Biochimica et biophysica acta* **1612**, 172-177
221. Vaz, W. L., Kapitza, H. G., Stumpel, J., Sackmann, E., and Jovin, T. M. (1981) Translational mobility of glycophorin in bilayer membranes of dimyristoylphosphatidylcholine. *Biochemistry* **20**, 1392-1396
222. Keller, B. U., Hedrich, R., Vaz, W. L., and Criado, M. (1988) Single channel recordings of reconstituted ion channel proteins: an improved technique. *Pflugers Archiv : European journal of physiology* **411**, 94-100
223. Folgering, J. H., Kuiper, J. M., de Vries, A. H., Engberts, J. B., and Poolman, B. (2004) Lipid-mediated light activation of a mechanosensitive channel of large conductance. *Langmuir : the ACS journal of surfaces and colloids* **20**, 6985-6987
224. Kahya, N., Wiersma, D. A., Poolman, B., and Hoekstra, D. (2002) Spatial organization of bacteriorhodopsin in model membranes. Light-induced mobility changes. *The Journal of biological chemistry* **277**, 39304-39311
225. Pantazatos, S. P., and MacDonald, R. C. (2003) Real-time observation of lipoplex formation and interaction with anionic bilayer vesicles. *The Journal of membrane biology* **191**, 99-112
226. Pantazatos, D. P., and MacDonald, R. C. (1999) Directly observed membrane fusion between oppositely charged phospholipid bilayers. *The Journal of membrane biology* **170**, 27-38
227. Lambert, O., Levy, D., Ranck, J. L., Leblanc, G., and Rigaud, J. L. (1998) A new "gel-like" phase in dodecyl maltoside-lipid mixtures: implications in solubilization and reconstitution studies. *Biophysical journal* **74**, 918-930
228. Battle, A. R., Petrov, E., Pal, P., and Martinac, B. (2009) Rapid and improved reconstitution of bacterial mechanosensitive ion channel proteins MscS and MscL into liposomes using a modified sucrose method. *FEBS letters* **583**, 407-412
229. Kreir, M., Farre, C., Beckler, M., George, M., and Fertig, N. (2008) Rapid screening of membrane protein activity: electrophysiological analysis of OmpF reconstituted in proteoliposomes. *Lab on a chip* **8**, 587-595
230. Kragh-Hansen, U., le Maire, M., and Moller, J. V. (1998) The mechanism of detergent solubilization of liposomes and protein-containing membranes. *Biophysical journal* **75**, 2932-2946
231. Urbaneja, M. A., Goni, F. M., and Alonso, A. (1988) Structural changes induced by Triton X-100 on sonicated phosphatidylcholine liposomes. *European journal of biochemistry* **173**, 585-588
232. Paternostre, M. T., Roux, M., and Rigaud, J. L. (1988) Mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. 1. Solubilization of large unilamellar liposomes (prepared by reverse-phase evaporation) by triton X-100, octyl glucoside, and sodium cholate. *Biochemistry* **27**, 2668-2677
233. Bald, D., Amano, T., Muneyuki, E., Pitard, B., Rigaud, J. L., Kruip, J., Hisabori, T., Yoshida, M., and Shibata, M. (1998) ATP synthesis by F0F1-ATP synthase independent of noncatalytic nucleotide binding sites and insensitive to azide inhibition. *The Journal of biological chemistry* **273**, 865-870
234. Varnier, A., Kermarrec, F., Blesneac, I., Moreau, C., Liguori, L., Lenormand, J. L., and Picollet-D'hahan, N. (2010) A simple method for the reconstitution of membrane proteins into giant unilamellar vesicles. *The Journal of membrane biology* **233**, 85-92

ACCEPTED MANUSCRIPT

235. Liguori, L., Marques, B., and Lenormand, J. L. (2008) A bacterial cell-free expression system to produce membrane proteins and proteoliposomes: from cDNA to functional assay. *Current protocols in protein science* **Chapter 5**, Unit 5.22
236. Shaklee, P. M., Semrau, S., Malkus, M., Kubick, S., Dogterom, M., and Schmidt, T. (2010) Protein incorporation in giant lipid vesicles under physiological conditions. *Chembiochem : a European journal of chemical biology* **11**, 175-179

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

TITLE: Methods of reconstitution to investigate membrane protein function

HIGHLIGHTS:

- Integral membrane proteins require a lipid environment for full function
- Membrane proteins may be reconstituted into a variety of lipid systems
- Symmetric reconstitution systems provide access to both sides of a membrane protein
- Compartment based reconstitution systems enable measurement of vectorial substrate movement
- The type of reconstitution system will depend on the nature of the functional activity and will require considerable optimisation

ACCEPTED MANUSCRIPT