Reconstitution of membrane proteins - a GPCR as an example.

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Abstract

Membrane proteins are the gatekeepers to the cell, and are essential to the function of all cells, controlling the flow of molecules and information across the cell membrane. Much effort has been put into the development of systems for studying membrane proteins in simplified environments that nevertheless mimic their native lipid environment. After isolation and production of purified membrane proteins in detergent, it is often necessary to reconstitute them into a lipid structure such as liposome, nanodisc or lipodisq. Each of these has the advantage of returning the protein to a defined lipid environment, and the choice of system depends on the application. Regardless of the system to be used, the fundamental process involves the removal of detergent and incorporation of the protein into a stable lipid system. This chapter details methodologies we have developed, mainly focussed on the model G protein-coupled receptor (GPCR) neurotensin receptor 1, and the GPCR-homologue and model, bacteriorhopdopsin.

1. Introduction

All cells are surrounded by a lipid membrane which modulates the flow of molecules, and information, into and out of the cell. However, these membranes do not consist solely of lipids, but incorporate a wide variety of proteins such as receptors,

transporters and structural proteins which modulate cellular behaviour. The protein content of a membrane can be variable (from 500:1 to 10:1 molar ratio, depending on functional criteria), but usually amounts to 50-90% by mass. Membrane protein copy numbers are also highly variable, ranging from 1-2 per cell to $>10^6$ per cell.

Membrane proteins are notoriously difficult to study due to the requirement for detergents to solubilise them out of the membrane in which they are either endogenously present, or have been expressed. Detergents, whilst stabilising the protein for purification, do not represent a native environment and many proteins will require specific lipids to establish functional activity or stability. In the case of transporters and signalling receptors, it is also often necessary to have a system in which the protein can be orientated in order to check for full activity. As such, the production of lipid systems in which proteins can be reconstituted has become a major avenue of research.

In this chapter, we will focus on some of the more common systems for reconstitution of membrane proteins, including liposomes, nanodiscs and lipodisqs using examples and principles from our own work with the G protein-coupled receptor neurotensin receptor 1 (NTS1) and bacteriorhodopsin (bR). The other GPCR for which significant reconstitution methods have been developed for both functional and biophysical studies, is (frog and bovine) rhodopsin, and details have been reported previously (Albert, Yeagle, Whiteway, Watts, & Epand, 1994; Jackson & Litman, 1982; Spooner et al., 2004).

2. Production of liposomes

Lipids spontaneously form liposomes when rehydrated from a dried film into an aqueous buffer. Liposomes formed by such rehydration will range in size (generally between ~30 nm and 50 μm in diameter) and may well form multilamellar vesicles possessing multiple concentric bilayers. Such a population of heterogeneous and multilamellar liposomes may be less desirable for the study of membrane proteins such as receptors and transporters, only those present in the outer layer will be available for study. Therefore, it is usual to create better-characterised unilamellar liposomes for the creation of proteoliposomes.

2.1. Lipid preparation

Lipids are commercially available as liquid stocks which are generally supplied in chloroform or chloroform:methanol at 25 mg/ml (*e.g.* from Avanti Polar Lipids, Inc.). Alternatively, stocks can be prepared from lyophilized powders at similar concentrations. Lipids can be difficult to weigh accurately (due to the hygroscopic nature of unsaturated lipids) and are prone to oxidation. Therefore, it is most convenient to work with lipids supplied in solvent. To prevent evaporation of the solvent during storage, opened stocks are best transferred to Teflon-capped glass vials and closed under an N2 atmosphere.

Materials for liposome creation:
Lipid stocks in chloroform at 25 mg/ml;
Liposome buffer e.g. 50 mM Tris pH7.4, 100 mM NaCl, 1mM EDTA;
Rotary evaporator (optional);
Temperature-controlled waterbath sonicator (optional).

Note, do not use plastic tubes or pipette tips for transferring solvents.

1. To create the desired lipid composition for the proteoliposomes, appropriate volumes of lipid stocks are mixed, along with sterols such as cholesterol which are poorly soluble in aqueous solvents.
2. To create a lipid film, the lipid mixture (or single lipid) is placed into a round bottomed flask and the solvent is evaporated. This can be achieved by a variety of methods, e.g. by blowing a stream of argon or nitrogen over the chloroform. Air should not be used as lipids are prone to oxidation. This solvent removal method is suitable for small (milliliter) volumes, but can be time consuming and difficult to perform for larger volumes. Thus, for large volume samples, it is often easiest to use a rotary evaporator with appropriate solvent trap.
3. The lipid should be fully dried until a film is generated on the bottom of the flask.
4. Films are then thoroughly dried under vacuum ($< 10^{-5}$ Torr) overnight. Dried lipid films are stable for months if stored under nitrogen at < -20 °C.
5. Lipid films are then rehydrated in aqueous buffer, typically to a final lipid concentration of 5 mg/ml. The choice of aqueous buffer for rehydration is very much dependent on the protein to be reconstituted. Generally, the same buffer can be used as the final purified protein preparation, but lacking detergents. Rehydration may also be performed in a variety of manners, but it is important to note that this should be done above any phase transition temperature of the lipid (or mix of lipids) (Marsh, 2013). The buffer should be pre-warmed above the highest phase transition temperature within the lipids being used, and rehydration should be done at the same temperature.
6. In the case of lipids with a phase transition temperature above ambient, one of the simplest methods is to place the sample on a rotary evaporator (with the vacuum off) and heat the waterbath to an appropriate temperature. Such rehydration should be performed for at least an hour, which should yield an homogenous suspension of liposomes. In the case of lipids with a lower phase transition temperature it is possible to vortex and sonicate the sample in a waterbath for 1-5 min, again above any phase transition temperature.
7. Resuspended lipid dispersion will contain multilamellar liposomes, which can be broken down by repeated freeze thawing in liquid nitrogen 6-8 times.

2.2. Sizing of liposomes

Multilamellar liposomes are often an undesirable form of proteoliposomes due to the limited accessibility of proteins within the internal bilayers. Thus, it is often useful to create unilamellar liposomes of defined size in advance of protein reconstitution. There are a variety of methods to do this with the simplest being sonication.

Materials for sizing of liposomes:
(Temperature-controlled) waterbath sonicator or probe sonicator;
Extruder, e.g. mini-extruder (Avanti Polar Lipids, Inc.);
Polycarbonate filters of appropriate pore size (e.g. Whatman).

Generating small unilamellar vesicles (SUVs):

1. A temperature-controlled sonicating waterbath set above the phase transition temperature of the lipid can be used and lipid dispersions sonicated for approximately 30-60 minutes, depending on the lipid composition.
2. Alternatively, a probe sonicator can be used although care must be taken to use a plastic container. This is a more rapid method (typically on the order of minutes) although care should be taken to avoid foaming and overheating, by using short (typically 30 s) pulses. Probe sonication is more difficult if the lipid mixture has a phase transition temperature above ambient due to the need for a (controlled) heat source.
3. Either method generates small unilamellar vesicles (SUVs) of 30-50 nm diameter yielding a clarified solution as the size of the liposomes drops below the wavelength of visible light.
4. It should be noted that the high level of curvature and surface tension makes SUVs unstable and they should be used promptly after creation. Stability can be checked through turbidity monitoring (~400-700 nm), since larger structures are formed with time (within hours).

Generating large unilamellar vesicles (LUVs):

1. Extrusion can also be used to create large unilamellar vesicles (LUVs) of defined size. Extrusion involves repeatedly passing the lipid mixture through a polycarbonate filter with defined pore size. An excellent guide to extrusion is provided by Avanti Polar Lipids Inc. (http://www.avantilipids.com).
2. It is important to select an appropriate pore size when creating liposomes. We generally extrude to 100 nm although extrusion to 400 nm is also common and can be performed prior to the 100 nm filter to make extrusion easier.
3. As liposomes of 100 nm diameter are smaller than the wavelength of visible light the solution clarifies.
4. It is vital that extrusion is performed an odd number of times in order to retain any large aggregates in the original syringe, as these are undesirable during reconstitution.
5. Extruded liposomes can be stored at -80 ^{o}C for weeks with no obvious signs of fusion. This can be checked via techniques such a dynamic light scattering or turbidity measurements.
6. One drawback of extrusion can be lipid loss during the process. It is therefore advisable to assay the final lipid concentration before use e.g. via a phosphate assay (Chen Jr, Toribara, & Warner, 1956) as described in section 6.1.1. Thin layer chromatography (Skipski, Peterson, & Barclay, 1964) can be used to ensure that the correct ratio of lipids is present.

3. Reconstitution into liposomes

Detergents are typically required to solubilise integral membrane proteins for isolation and purification whether they are expressed into membranes (Link, Skretas, Strauch, Chari, & Georgiou, 2008; Rigaud & Levy, 2003; Weiss & Grisshammer, 2002), or inclusion bodies in bacteria (Baneres & Parello, 2003; Kiefer, Maier, & Vogel, 1999) or are expressed in cell-free systems (Ishihara et al., 2005; Klammt et al., 2007). There are different methods for the reconstitution of detergent-solubilised membrane proteins and the most common ones, dilution and dialysis methods or hydrophobic adsorption (Figure 1), are described below.

3.1. Rapid dilution

Reconstitution by rapid dilution and dialysis has been applied for many membrane proteins (Ambudkar, Lynn, Maloney, & Rosen, 1986; Jap et al., 1992; Kuhlbrandt, 1992), but this method presents a disadvantage for detergents with low CMC (critical micellar concentration) which are difficult to remove and at best require a long time for dialysis, which can result in loss of protein structure and activity.

Materials for sizing of liposomes:
Dialysis buffer (typically the same as liposome buffer used in lipid preparation);
Dialysis membrane with a molecular weight cut-off of ~7-10 kDa (high permeability cellulose membrane, e.g. SnakeSkin, Pierce).

1. Lipids are added to the protein solution, which is diluted below the CMC of the detergent.
2. The lipid-detergent mixed micelles solution is then placed in a dialysis bag and dialyzed against detergent-free aqueous medium under slow stirring at 4 °C.
3. Optionally, equilibrated polystyrene beads (e.g. Bio-Beads, see section 3.2) can be added to the dialysis buffer (outside the dialysis bag) to absorb detergent.

3.2. Hydophobic adsorption

Reconstitution by hydrophobic adsorption uses polystyrene beads to adsorb and remove detergents (Levy, Bluzat, Seigneuret, & Rigaud, 1990; Richard, Pitard, & Rigaud, 1995; Rigaud, Paternostre, & Bluzat, 1988) and this method has worked better than rapid dilution in our hands (Harding et al., 2009), but this may be protein dependent. Different types of polystyrene beads are commercially available: Bio-Beads SM2 (Bio-Rad, Hercules, CA) or Extracti-Gel D (Pierce, Rockford, IL). Reconstitution by hydrophobic adsorption permits control over the rate of detergent removal and virtually all detergent present can be removed. The adsorptive capacity of Bio-Beads for phospholipids is 100-200 times lower than for detergents and lipid loss can be minimized by using the lowest Bio-Beads-to-detergent ratio needed for complete detergent removal. This method also works for removing detergents with low CMCs in a short time.

Materials for reconstitution of NTS1 using Bio-Beads:
Extruded liposomes at 5mg/mL in liposome buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA);
Purified receptor in storage buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.1% DDM (w/v), 0.01% CHS (w/v), 1 mM EDTA, 10% (v/v) glycerol);
Bio-Beads SM-2 (Bio-Rad);
Reagents for detergent concentration assay (optional);
Reagents for phospholipid concentration determination (optional).

1. Before starting the reconstitution it is advisable to saturate the liposomes with detergent to improve the incorporation the protein into vesicles from solution (Knol, Sjollema, & Poolman, 1998; Paternostre, Roux, & Rigaud, 1988). For each detergent and lipid mixture, there is an effective detergent-to-lipid molar ratio (R_{eff}) at which lipids are saturated but not completely solubilised by detergent, obtained by: R_{eff} = ([detergent] – [CMC]) / [L], where CMC is the critical micellar concentration of the detergent and L is the lipid. A table

listing these parameters for the most common detergents can be found in (Rigaud & Levy, 2003). For DDM, R_{sat} is 1:1 (mol/mol) and R_{sol} is 1:1.6 (mol/mol).

2. Based on the detergent-to-lipid molar ratio, an appropriate amount of detergent is added to lipid solution.
3. If using DDM, incubate the mixture at room temperature for 3 h with stirring to ensure complete equilibration of the detergent with lipids.
4. Following saturation and before the addition of the protein solution, the lipid-detergent mixture is bath sonicated for 10 minutes at room temperature. Bio-Beads are washed several times with methanol following several washes with water and can be stored at 4 ^{o}C in water.
5. Prior to use, Bio-Beads are washed in liposome buffer. The receptor is added to the lipid-detergent solution at an appropriate concentration to achieved the desired lipid-to-protein ratio.
6. The reaction mixture is incubated for ~1 h with gentle mixing before the addition of Bio-Beads. If the detergents used to solubilise the lipids are deleterious to the activity of the protein, this incubation period may be shortened.
7. Washed Bio-Beads are added to the protein-lipid-detergent solution at a ratio of 10:1 (w/w) of wet Bio-Beads to detergent.
8. The sample is incubated above the phase transition temperature of the lipids for 1-2 h and then aspirated into fresh Bio-Beads and incubated overnight with rotation.
9. Turbidity measurements can be used to monitor reconstitution and a detergent assay can be performed to confirm complete detergent removal (within the sensitivity limit of the assay) (Mallya & Pattabiraman, 1997).
10. Proteoliposomes can be recovered by centrifugation (100,000$\times g$, 1-3 h, 4 °C) and resuspended in an appropriate volume of liposome buffer depending on end use.
11. If required, proteoliposomes can be isolated from empty liposomes using sucrose density gradient centrifugation (0-35% in liposome buffer) in 5 % steps for a typical receptor (MW ~40-100 KDa) at medium high lipid-to-protein molar ratios (> 500 to 1). For denser liposomes higher percentages of sucrose may be required.
12. The sample is loaded on top of the gradient and centrifuged (100,000$\times g$, 16 h, 4 °C).
13. The gradient is then fractionated, and fraction can be analysed by SDS-PAGE.
14. Proteoliposomes fractions may be dialyzed or diluted and centrifuged (100,000$\times g$, 1-3 h, 4 °C) to remove sucrose.

4. Reconstitution into nanodiscs

Nanodiscs consist of a lipid bilayer disc stabilised by an amphiphilic so-called membrane scaffold protein (MSP, an engineered construct of the human apolipoprotein A-1). A dimer of MSP wraps around a lipid bilayer forming a disc with a diameter of 10 nm or larger depending on the length of the MSP construct used; several constructs are available containing multiples of the same repeat domain, allowing formation of larger discs, to suit the incorporation of membrane proteins of varying sizes (Denisov, Grinkova, Lazarides, & Sligar, 2004). Nanodiscs can in

principle be made with any bilayer forming lipids to suit individual protein requirements, although in practice some lipids yield better results than others, and reconstitution conditions need to be determined empirically for each membrane protein and lipid mix (Ritchie et al., 2009).The use of nanodiscs for reconstitution of membrane proteins has become an increasingly popular and useful alternative to liposomes, not least because they are homogenously-sized, soluble while still maintaining the protein in a membrane environment, and relatively rapid to prepare (Bayburt, Grinkova, & Sligar, 2002; Denisov et al., 2004; Shih, Arkhipov, Freddolino, Sligar, & Schulten, 2007). Furthermore, the protein is accessible from both sides of the membrane, making nanodiscs highly suitable for simultaneously studying intracellular and extracellular membrane protein interactions *in vitro* using techniques such as microscale thermophoresis or surface plasmon resonance. In our hands, preparation of NTS1-loaded and empty nanodiscs follows the protocol developed by Sligar and co-workers (Ritchie et al., 2009) with some modifications based on the lipid types used and empirically determined optimal MSP:lipid:NTS1 ratios, with reference to (Bayburt, Grinkova, & Sligar, 2006; Inagaki et al., 2012; Leitz, Bayburt, Barnakov, Springer, & Sligar, 2006). A schematic of the method is illustrated in Figure 2.

4.1. Production of MSP for nanodiscs

The MSP belt protein is commercially available from Sigma-Aldrich, or can be expressed in house as described by Ritchie et al. (Ritchie et al., 2009). The use of a fermenter can be replaced with standard bacterial expression in 2L flasks. If the scaffold protein is to be used without its His-tag, it can be incubated with TEV protease overnight at 4 °C at a 1:10 TEV:MSP molar ratio, and then reverse purified by collecting the flow-through of a Ni^{2+} column.

4.2. NTS1 reconstitution into nanodiscs

Materials:
Purified MSP
Purified receptor in storage buffer (50mM Tris-HCl pH 7.4, 100mM NaCl, 0.1% DDM (w/v), 0.01% CHS(w/v), 1mM EDTA, 10% (v/v) glycerol)
Lipids (purchased from Avanti Polar Lipids, Inc.)
Lipid sodium cholate buffer (50 mM Tris-HCl pH 7.4, 100 mM sodium cholate, 100 mM NaCl, 1 mM EDTA)
Bio-Beads SM-2 (Bio-Rad), prepared as described (section 3.2)

1. A lipid films is prepared made as in section 2.1, using the desired lipid mixture, e.g. 3:1:1 POPC:POPG:POPE with 25 mol % cholesterol; 1:1 POPC:POPG; 3:1 POPC:POPE or 1.07:1.5:1 BPL:POPC:POPG.
2. At least 8-10 μmol lipid per batch of discs is made up to 50 mM in lipid sodium cholate buffer..
3. The lipid suspension is freeze-thawed with liquid nitrogen three times.
4. Detergent-solubilised lipids can be stored overnight at 4 °C, or snap-frozen in liquid nitrogen and stored at -80 °C.
5. The following guidelines are used for successful nanodisc formation: the final [lipid] must be ≥ 4 mM, final [sodium cholate] must be 12-40 mM, final [glycerol], must be less than 4 %.

6. MSP-to-lipid ratios of 1:50 to 1:60 are most effective for NTS1. Empty nanodiscs are reconstituted with 5 additional lipid molecules per MSP, and are prepared simultaneously with loaded nanodiscs. Ratios of between? 80:1 and 50:1 MSP:NTS1 are effective.
7. NTS1 (2-5 nmol at ~0.2-0.5 mg/ml), or NTS1 buffer alone (for empty nanodiscs) is added to sodium cholate-solubilised lipid mixtures.
8. MSP1D1 (4-10 mg, or 0.16-0.25 μmol at ~4.5 mg/ml) is added in a final volume of 2.5-3.5 ml, with final concentrations of receptor at approximately 1 μM, MSP at 1.6-3.5 mg/ml (70-170 μM) and lipid at 7 mM.
9. The solution is incubated for 1 h with rotation at 4-6 rpm at 4 °C.
10. Washed Bio-Beads (0.8-1.0 g/ml) are added to the solution and rotation continues overnight at 4 °C.
11. Bio-Beads are then replaced (0.5 g/ml) and the sample is further incubated for 1 h.
12. Bio-Beads are removed and the sample is concentrated (using e.g. Vivaspin 6, 10,000 MWCO, Satorius) followed by centrifugation (10,000 × *g*, 4 °C, 10 min).
13. The supernatant is aspirated off any pellet that forms and filtered through a Nanosep® MF low-volume 0.2 μm centrifugal filter (PALL Corporation).
14. Nanodiscs are separated from other material on a (calibrated) Superdex 200 10/300 GL column (GE Healthcare) equilibrated in 50 mM Tris HCl pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$.
15. Peak fractions containing NTS1 as well as MSP are pooled and concentrated if required.
16. NTS1 nanodiscs may be enriched by purification using an affinity tag on the receptor, e.g. via FLAG-tag using anti-FLAG M2 resin (Sigma-Aldrich) according to the manufacturer's instructions, or for via a His-tag, in which case the His-tag on MSP1D1 must be cleaved with TEV protease prior to nanodisc formation and loaded nanodiscs may be enriched from the mixed nanodisc population using a nickel column.
17. Nanodiscs can then extensively be dialysed at 4 °C in three changes of dialysis buffer appropriate for end use.

5. Reconstitution into lipodisqs

Lipodisqs are another discoidal nanoscale lipid bilayer system, similar to nanodiscs, forming particles in which both sides of the inserted membrane protein remain accessible (Orwick et al., 2012). However instead of using a protein belt, lipodisqs are formed using a styrene-maleic acid polymer (SMA). This aspect makes them attractive for techniques such as protein NMR where additional signals arising from the MSP belt would be undesirable.

SMA polymers are sensitive to pH; in its charged form the polymer is extended, but as pH is lowered and charge is lost it collapses into a compact coil. At the critical collapsing pH lipodisqs will form most efficiently. SMA is available in a variety of styrene-to-maleic acid ratios, influencing the charge distribution in the molecule and thus optimal pH range at which it can be used. As the polymer length is not exactly fixed, unlike the length of a particular MSP construct used in nanodisc formation, the range of particle sizes obtained in lipodisq formation can be more variable (depending on the protein and lipids used) and experimental conditions may need to be adjusted to obtain a more homogenous sample size, in combination with careful selection of

sample fractions after gel filtration. The final sample size and dispersion thereof can be verified by dynamic light-scattering experiments and/or electron microscopy. Lipodisqs have been used in our laboratory to reconstitute bacteriorhodopsin (bR, a 7TM receptor) from its native membrane (Orwick-Rydmark et al., 2012), and single TM proteins (unpublished). Others have used SMA to solubilise for example intact mitochondrial complexes directly from mitochondrial membranes (Long et al., 2013), bacterial cell division machinery directly from staphylococcal cells (Paulin et al., 2014), or to reconstituted labelled, purified membrane proteins for spectroscopy studies (Sahu et al., 2013). Here, we provide a protocol for lipodisq formation of bR.

5.1. Preparation of bR proteoliposomes and bR-proteolipodisqs

Materials:
Pre-hydrolysed SMA at a 3:1 ratio of styrene-to-maleic acid was used, which is optimal for working around neutral pH (Malvern Cosmeceutics Ltd, can be purchased from Sigma Aldrich);
400 nm extruded DMPC unilamellar vesicles in 50 mM Tris-HCl, pH 8 at 20 mg/ml (prepared as described in 2.2.);
bR expressed and purified in its native membrane as previously described (Oesterhelt & Stoeckenius, 1974);
Reconstitution buffer (50 mM Tris-HCl, pH 8).

1. Native bR membranes (containing ~4 mg of protein) are resuspended in 1 ml of the DMPC-liposomes (20 mg) at a lipid-to-protein molar ratio of 172:1 and sonicated for 30 minutes in a bath sonicator.
2. A 2.5% (w/v) SMA-polymer solution is prepared in reconstitution buffer, and added drop-wise to the bR-DMPC suspension.
3. Lipodisq formation and kinetics thereof can be followed by monitoring light scattering at 540 nm as a function of time (Jasco V-630 UV spectrophotometer).
4. After incubation for 1 h at room temperature, the resulting bR lipodisq solution is centrifuged for 30 minutes at $40{,}000 \times g$ to remove any unsolubilised bR.
5. Lipodisqs are further purified from non-incorporated bR by gel filtration (Superdex 200, X16/100 column in 50 mM Tris, 300 mM NaCl, pH 8.0), and the resulting purple fractions with an absorbance at 555 nm are pooled.
6. Analysis of the main gel filtration peaks by DLS (Viscotek 801 particle sizer, Malvern Instruments) shows resulting bR lipodisqs to be between 10-20 nm in diameter.

6. Determination of lipid-to-protein ratio

The lipid-to-protein (L:P) ratio of a sample can be used for estimating the receptor density in proteoliposomes. This knowledge can be useful for assessing how experimental conditions compare to the conditions found in vivo, or how they affect biophysical experiments such as energy transfer in FRET, where very high densities might lead to bystander FRET, and is essential in for example protein-to-lipid stoichiometry experiments with spin-labelled lipids.

6.1. Liposome/lipid concentration determination

A number of ways can be used to estimate the L:P ratio of proteoliposmes. If the protein concentration can be determined reliably by absorbance measurements at 280

nm, this can be compared with the lipid concentration determined from a colorimetric phosphate assay (Chen Jr et al., 1956). This assay determines the total phosphorus content of the sample. Although it cannot directly account for cholesterol, which does not have a phosphate headgroup, the cholesterol concentration can be calculated from the molar ratio once the phosphorus concentration is known.

Materials for phosphate assay:
2.5 % (w/v) ammonium molybdate (VI) tetrahydrate solution;
8.9 N H_2SO_4;
10 % (w/v) ascorbic acid;
phosphorus standard solution;
H_2O_2.

1. A standard curve is constructed from triplicate samples of 0-0.228 µmol phosphorus made up from 0.65 mM stock phosphorus solution (Sigma-Aldrich 661-9).
2. The standard samples and triplicate samples of prepared liposomes, nanodiscs or lipid stocks are acid-hydrolysed by addition of 0.45 ml 8.9 N H_2SO_4 to each tube with heating in an heat block at 200-215 °C for 25 min.
3. The tubes are removed from the heating block and allowed to cool for five minutes before 150 µl H_2O_2 is added to each tube.
4. Heating is continued above 200 °C for a further 30 min. De-ionised water (3.9 ml) is added to each tube, followed by 0.5 ml 2.5 % (w/v) ammonium molybdate (VI) tetrahydrate solution.
5. All tubes are vortexed five times, 0.5 ml 10 % (w/v) ascorbic acid added, and vortexed five times each again.
6. Tubes are covered with glass marbles to prevent evaporation and heated at 100 °C for 7 min.
7. Once cooled to room temperature the absorbance at 820 nm of all standards and samples is determined. The 0 µmol phosphorus standard is used for blank-correction and triplicate averages plotted as a function of phosphorus amount.
8. The phosphorus concentration, and thus the phospholipid concentration of the samples are determined from the equation of a regression line fitted to the data.

6.2. Liposome scatter in UV-Vis: Labelled lipid marker

Unfortunately, the scatter from lipid vesicles often hampers accurate determination of the protein absorbance signal at 280 nm, especially at high sample concentrations and/or high L:P ratios. In such a case one can include a small amount of fluorescently labelled lipid, e.g. rhodamine-PE (Rho-PE) in the reconstituted samples (~0.8:1 Rho-PE:protein) depending on the molar extinction coefficient of the protein, as well as making a protein-free lipid-only background sample containing Rho-PE. The rhodamine signal at 573 nm in both samples can then be used to correct for the lipid scatter: the rhodamine peak from the background sample is normalised to that in the proteoliposome sample, and the scaled background spectrum is then subtracted. The protein concentration can be calculated from the scatter-corrected signal at 280 nm and then used together with a phosphate assay as described above to determine the lipid-to-protein ratio. The protein concentration can also be determined by protein assays such as the BCA assay, or other comparisons with bovine serum albumin, but these methods are less accurate.

Instead of using a phosphate assay to determine the lipid concentration, when employing the Rho-PE method one can also simply use the Rho-PE absorption to calculate the total lipid content by using the Rho-PE-to-total-lipid ratio employed in the reconstitution. This of course relies on the assumption that reconstitution efficiency of Rho-PE does not differ from the other lipids used.

6.3. Sucrose density gradient

Alternatively, a sucrose density gradient can be used to determine the L:P ratio. This method relies on the different densities that proteoliposomes with varying protein content will have; low L:P proteoliposomes will be heavier and thus run further down a sucrose gradient than samples lower L:P.

1. A sucrose density gradient with appropriate concentrations of sucrose added to liposome buffer is prepared (for NTS1 reconstituted in BPL at initial L:P ratios of >500:1 a step gradient of 35-5% with 5% steps is typically employed).
2. The reconstituted sample is layered on top of the gradient, and the gradients are centrifuged overnight in a swing-out rotor (e.g. Beckmann SW28 for large (>1.5 mL) and SW41 for smaller samples) at ~100,000$\times g$.
3. The sucrose gradient is then fractionated and the presence of reconstituted receptor can be verified by SDS-PAGE analysis.
4. The position of the proteoliposome band on the gradient can be used to estimate the density and thus the L:P ratio of the samples using the equation derived by Barber (Barber, 1966):

$$\rho(T) = \left(B_1 + B_2 T + B_3 T^2\right) + \left(B_4 + B_5 T + B_6 T^2\right) Y + \left(B_7 + B_8 T + B_9 T^2\right) Y^2$$

where ρ is the density (kg/dm^3), T the temperature (°C), Y the fraction of sucrose in solution and $B_{1\text{–}9}$ are constants($B_1 = 1.00037$, $B_2 = 3.96805\times10^{-5}$, $B_3 = -5.85133\times10^{-6}$, $B_4 = 0.389824$, $B_5 = -1.05789\times10^{-3}$, $B_6 = 1.23928\times10^{-5}$, $B_7 = 0.1700976$, $B_8 = 4.75301\times10^{-4}$, and $B_9 = -8.92397\times10^{-6}$).

5. The partial specific volume ($\bar{v}$ in mL/g) follows from that (as the reciprocal of the density), and can be used to estimate the L:P ratio (w/w) by solving

$$\bar{v}_{proteoliposomes} = x\bar{v}_{lipid} + (1-x)\bar{v}_{protein}$$

for x (fraction of lipid), where $\bar{v}_{proteoliposomes}$ is the partial specific volume of the sample determined from the sucrose gradient, $\bar{v}_{lipid}$ is the contribution of the lipid and can be determined by running lipid-only sample on a sucrose gradient (~0.986 mL/g for BPL was determined in our laboratory), and $\bar{v}_{protein}$ is the contribution of the protein and can be calculated from its amino acid composition using the relation derived by Cohn and Edsall (Cohn & Edsall, 1943) modified for the effect of temperature (Durchschlag, 1986)

$$\bar{v}_T = \bar{v}_{25} + 4.25\times10^{-4}\left(T-25\right) = \frac{\sum n_i M_i \bar{v}_i}{\sum n_i M_i} + 4.25\times10^{-4}\left(T-25\right)$$

where $\bar{v}_T$ is the partial specific volume at temperature T, and $\bar{v}_{25}$ that at 25 °C, calculated from the number of moles (n_i) for each particular amino acid (i) with molecular weight M_i and partial specific volume $\bar{v}_i$.

6. The fraction of lipid x in w/w can then be used to calculate a molar L:P ratio using the molecular weight of the lipid and protein.

7. Concentration methods

Different experimental methods require more or less concentrated protein samples. Proteoliposome samples can be concentrated very easily to high protein concentrations by pelleting the liposomes via high-speed centrifugation (~100,000 × *g*) followed by resuspension in small volumes of liposome buffer to yield the final concentration desired.

If high concentration/small sample volume are critical (e.g. for NMR or EPR application) or high sample homogeneity is required, it is advisable to run the samples on a preparative sucrose density gradient (as described in section 6.3), to separate loaded proteoliposomes from empty liposomes which may be present to varying extent, depending on the method of reconstitution and initial L:P ratio used, which can also be used to estimate the final L:P ratio as discussed in section 6.

Discoidal reconstitutions (nanodiscs or lipodisqs) can be concentrated by the same methods available for membrane proteins solubilised in detergents, such as centrifugal concentrators (e.g. Vivaspin concentrators, Satorius) or by affinity chromatography with small resin volumes.

Summary

For many studies of membrane proteins it is essential that they are present in a lipid bilayer. In this chapter we have detailed methods for incorporating membrane proteins into liposomes and also discoidal lipid structures such as nanodsics and liposdisqs. Whilst the choice of lipid environment is largely dependent upon the downstream application, the importance of investigating membrane proteins in their native environments is clear.

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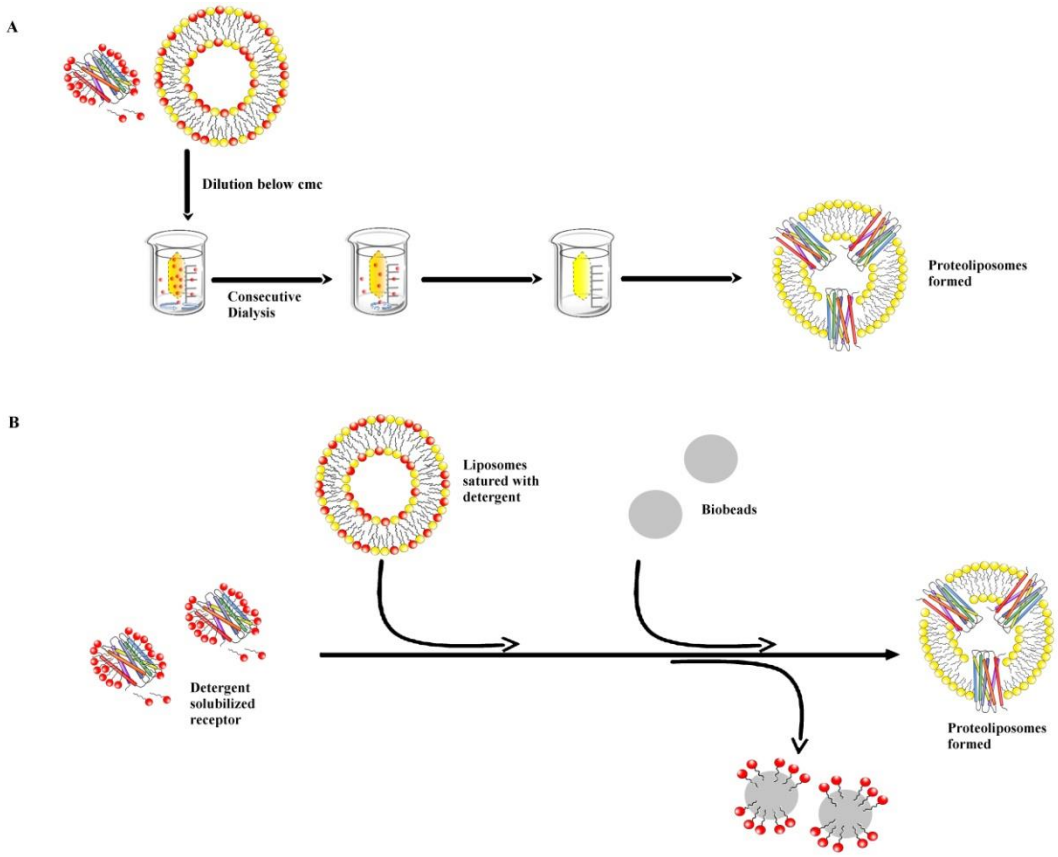

Figure 1: Reconstitution by dilution and dialysis (A) and by using Bio-Beads for detergent removal (B). **(A):** Lipids, detergent, and protein are mixed at the desired ratios and incubated for 1 h, after which the reconstitution mixture is rapidly diluted below the CMC of the detergent and the detergent monomers are removed by extensive dialysis. **(B):** Detergent-solubilised receptor is mixed with liposomes desestabilised by detergent at the required concentration (incubation of mixture for 1 h), after which detergent is removed with Bio-Beads at a 10:1 Bio-Beads-to-detergent ratio (w/w).

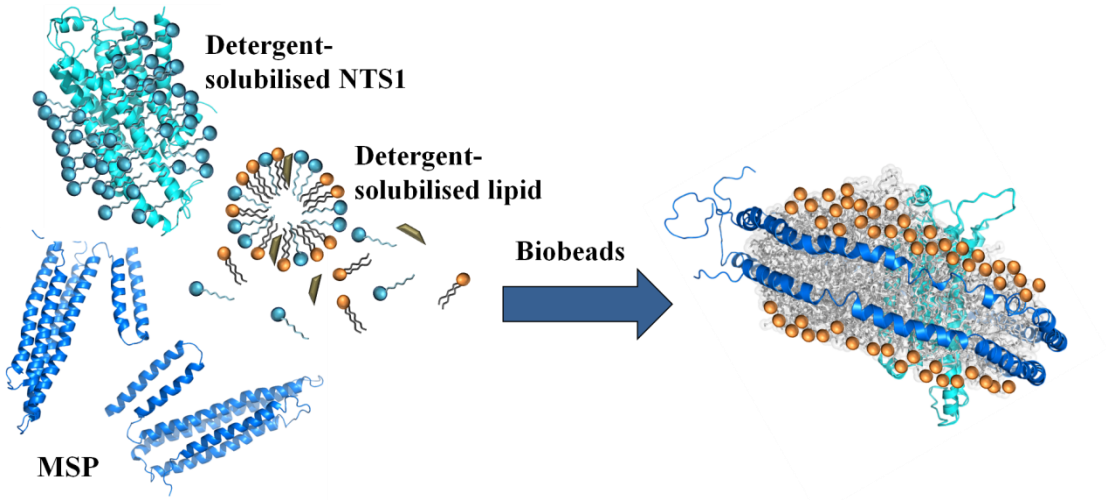

Figure 2: Schematic diagram illustrating nanodisc formation. A lipid film is solubilised in 100 mM sodium cholate to a final concentration of 50 mM lipid. Detergent-solubilised, purified NTS1 (cyan ribbon structure surrounded by blue detergent molecules) is added to the lipid-detergent mixture at the target concentration (lipid molecules are orange with gold rectangles representing cholesterol). Membrane scaffold protein is added to the reaction mixture at the target MSP:lipid and MSP:NTS1 ratio, and the sample is incubated with rotation at 4 °C for 1 h. Bio-Beads (1g/ml) are added to remove the detergent overnight, and nanodiscs spontaneously form.

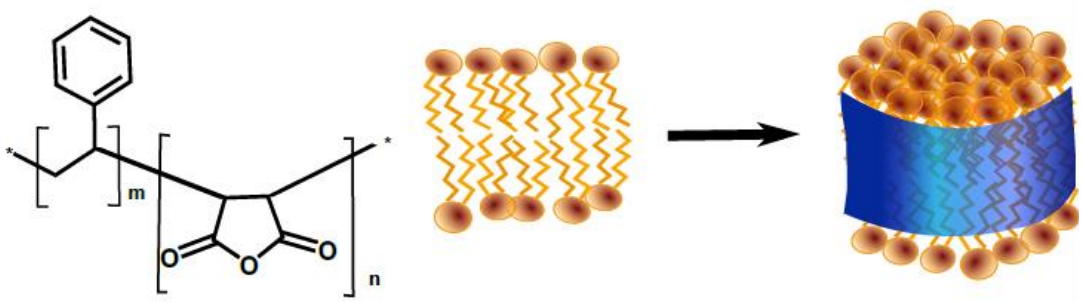

Figure 3: Schematic of a lipodisq. Lipodisqs are a discoidal nanoscale lipid bilayer system, formed using a styrene-maleic acid polymer (SMA, left). If SMA is incubated with lipids at a critical collapsing pH, lipodisqs will form spontaneously. SMA is available in a variety of styrene-to-maleic acid ratios, influencing the charge distribution in the molecule and thus optimal pH range at which it can be used.