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A Method for Detergent-free isolation of Membrane Protein with its Local Lipid Environment

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ABSTRACT

Despite the great importance of membrane proteins, structural and functional studies present major challenges. A significant hurdle is the extraction of the functional protein from its natural lipid membrane. Traditionally achieved with detergents, purification procedures can be costly and time consuming. A critical flaw with detergent approaches is the removal of the protein from the native lipid environment required to maintain functionally stable protein. This protocol describes the preparation of Styrene Maleic Anhydride Co-polymer (SMA) to extract membrane proteins from prokaryotic and eukaryotic expression systems. Successful isolation into SMA lipid particles (SMALPs) allows membrane proteins to remain with native lipid, surrounded by SMA. We detail procedures for obtaining 25g of SMA (4 days), explain the preparation of both SMALPs with (2 days) and without (1-2 hours) protein, SMALP protein identification and estimation (4 hours), and detail biophysical methods to undertake initial structural studies to characterise SMALPs (~2 days). Together these methods provide a practical tool-kit for those wanting to use SMALPs to study membrane proteins.
INTRODUCTION
The lack of progress in the study of membrane protein structure and function remains a significant frustration for academics and commercial organisations alike. Membrane proteins themselves represent some of the most important molecules in life sitting as they do either between the cell and the outside world or between cellular compartments. As such they underpin a wide range of fundamental cellular functions from cellular signalling, nutrient uptake, and secretion to communication, motility and adhesion. The combination of these activities with the exposure of many membranes to the extracellular milieu, make membrane proteins important targets for therapeutic development. For instance, more than 40% of pharmaceutical agents interact with a single class of membrane proteins, the G-protein coupled receptors\(^1\). However despite the acknowledged importance of these molecules, and the efforts put into their study, research on them remains a significant challenge.

This challenge centres on the need to disrupt the phospholipid bilayer in order to facilitate the separation of the target membrane protein/protein complex from all others. The disruption of the membrane is not a challenge \textit{per se} as many agents are available (generally surface active agents or detergents) that can fragment membranes. However, it has long been known that while the membrane needs to be fragmented, the lipophilic character of the membrane protein means that complete removal of the membrane from around the protein generally renders it unfolded and inactive. This conundrum has challenged the biochemical world for a significant time, with the choice of what membrane protein to study often not being made on the basis of importance but more on which protein can be extracted from the membrane in the active, folded form. This restriction in a biochemist’s freedom to operate is even starker when it is recognised, that although more than 30% of all transcribed proteins are membrane proteins only 2% of high resolution structures are of membrane proteins\(^2\).

\textbf{Existing approaches to membrane protein solubilisation}

Since the discovery of membrane proteins as a distinct sub-class the majority of approaches to their isolation have relied on the use of surface active agents (more commonly called detergents)\(^3,4,5,6\). Simply, detergents provide an alternative solubilisation environment for membrane proteins that, unlike a phospholipid membrane, is not a continuum. The outcome of
any detergent based method is a solution of micellar particles that contain individual membrane proteins. These protein-micelle complexes can then be separated by virtue of their physico-chemical properties. At first view, this seems like a perfect solution and indeed it has been used to produce pure, active samples on essentially all membrane proteins previously studied or currently investigated. However there are a number of fundamental issues that exist with this approach that make it less than perfect. The use of detergents in many ways neglects to consider the physico-chemical complexity of the membrane environment and its importance in maintaining protein structure and activity. Even a simple phospholipid membrane made of a single lipid contains a number of distinct environments that run within the membrane leaflet. Perhaps the most obvious of these are the hydrophilic outer surface and the hydrophobic interior. This becomes significantly more complex in membranes containing mixed lipids where the interfaces between different lipid types provide yet more new and distinct environments. Membrane proteins have evolved to exist in this complex environment and as such it has become increasingly clear that the structure of the membranes are adapted to provide optimum protein folding and hence activity in a very specific lipidic context.

Given this complexity the use of detergent solubilisation with a single detergent will never effectively replicate the native lipid environment and hence will always be a sub-optimal solution. This issue has been encountered countless times with detergent solubilisation experiments failing to produce active membrane proteins. Even a thermostable protein, such as Thermotoga maritima integral membrane pyrophosphatase, is stable and active in only a few detergents but most of these attempts are never published by virtue of the negative nature of the results. The sad consequence is that multiple groups waste valuable time and resources attempting the same experiments without knowing that the experiments have already been proven to be futile.

Attempts to solubilize using detergent also suffer from the lack of a single solution (that is a single methodology and reagent). This means that experiments to develop a detergent solubilisation method for a particular protein is essentially an “open ended” experiment with an infinite number of combinations of detergents to be tried. The lack of a definitive indication that the project is fruitless also wastes time and resources. Detergent solubilized membrane proteins are generally inherently unstable with very short “shelf lives”. This means that
comparative experiments between samples often suffer from uncertainty in terms of the specific activity of the preparation. The presence of the detergent itself also often affects downstream experimentation. For example the interactions between subunits within a membrane protein complex are often perturbed or abolished by the presence of the detergent\textsuperscript{12,13}. In addition the presence of detergent disrupts spectroscopic techniques making it a difficult task to discriminate between the protein and the detergent signal. More recently scientists have acknowledged the failings of detergents and have begun to develop other moieties aimed at stabilising membrane proteins. Many of these aim to address one or more of the acknowledged issues with the current approaches. A range of other surface active agents including fluorinated detergents\textsuperscript{14} are being trialed, longer polymeric materials (termed amphipols) are also showing some success. These contain a wider range of chemical side chains providing a higher likelihood that compound will more effectively replace lipids surrounding a membrane protein\textsuperscript{15}.

Overall detergent solubilisation, has been the method of choice for the past forty years its significant limitations have placed constraints on the study of membrane proteins. It is possible that these new developments will continue to yield success, but there is now a significant effort to find an entirely new methodology.

**A new approach to membrane protein solubilisation**

In the late 1990s, several research groups became aware that the continued focus on developing better detergents to extract membrane proteins was not yielding success and proposed a new approach. It was reasoned that the common action of detergents to replace lipids from the immediate environment surrounding the protein ignored the importance of these lipids in protein structure and function. In fact, a large number of excellent studies of membrane protein folding had for some time been pointed to the fact that successful membrane protein folding required the physically complex membrane structure\textsuperscript{8,16}. The new approach recognised the importance of the lipid environment that surrounded the membrane protein and proposed a methodological innovation that extracted the protein complete with the surrounding lipid environment: in general these methods extract between 10-100 lipids in close contact with the chosen protein. Early pioneers in this approach were the groups of Sligar and co-workers who showed that amphipathic peptides could be used to stabilise nanoscale disc-like structures that contained a lipid bilayer\textsuperscript{17}. This method offered an alternative approach to allow the stabilization of
membrane proteins, that allowed biophysical analysis of any protein in the encapsulated bilayer, in a near to native protein conformation. Sligar was also showed some generic applicability in protein stabilisation by producing nanoparticles with a range of different membrane proteins including cytochrome P450 enzymes\textsuperscript{18}, the SecYEG channel\textsuperscript{19} and the β2-adrenergic receptor\textsuperscript{20}. These truly pioneering studies have shown that membrane proteins could be isolated complete with an intact lipid bilayer. However, neither provides the perfect solution, as both required that the protein was pre-solubilised in detergent before insertion into the new lipid containing nanoparticle. In 2009, we published data that showed that a simple organic polymer (Styrene Maleic Acid Co-polymer, SMA, \textbf{(Fig. 1a)}) could be used to directly extract proteins from membranes into self-contained styrene maleic acid lipid particles (SMALPs)\textsuperscript{21,22}. This work built on earlier work by Tighe and colleagues on the conformational transitions of SMA and its resultant physical properties\textsuperscript{23,24}. Our work in 2009 provided, perhaps for the first time, a generically applicable method that could extract active membrane proteins without the need for a detergent, whereby the simple observation of the cloudy initial solution clearing gave a clear indication of SMALP formation (\textbf{Fig.1b}). The SMALP contains a central lipid bilayer supported by an outer annulus of the SMA polymer\textsuperscript{25}(\textbf{Fig.1c}). The structure is stabilised by the intercalation of the hydrophobic styrene groups between the acyl chains of the bilayer while the hydrophilic maleic acid groups face the solvent (\textbf{Fig.1d}). We have also shown that the encapsulated bilayer retains many of the physical properties of the parent membrane including the lipid mixture\textsuperscript{26}, structural organisation and phase behaviour\textsuperscript{25}. Since the publication of this work we have worked with a number of collaborators to examine whether the method is widely applicable and if the resulting preparations are appropriate for analysis using a range of biophysical and biochemical methods. Our work so far has shown that the method can be successfully employed to extract proteins with up to 36 transmembrane helices. These studies have included solubilisation of AcrB\textsuperscript{27}, the ABC transporter, Pgp\textsuperscript{28} the potassium channel, KcsA\textsuperscript{29}, the penicillin binding protein, PBP2A\textsuperscript{30}, and the adenosine A\textsubscript{2A} receptor\textsuperscript{31}. The method has also been used successfully by Watts and colleagues to purify the seven transmembrane protein bacteriorhodopsin\textsuperscript{32} alongside the respiratory Complex IV\textsuperscript{33}. Importantly, these studies also showed that the extracted proteins maintained activity, with Pgp and AcrB displaying greater activity within the SMALP scaffold compared to detergent isolation. As part of these studies we have also shown that SMALP encapsulated proteins are amenable to study using a
range of techniques including Circular Dichroism (CD), Analytical Ultracentrifugation (AUC), Differential Scanning Calorimetry (DSC), Negative stain and cryo Transmission Electron Microscopy (TEM), and small angle Neutron (SANS) scattering demonstrating the general utility of the method.

**Limitations of the SMALP method**

The Styrene Maleic Acid Lipid Particle (SMALP) method detailed in this protocol solves a number of issues that have historically afflicted detergent based systems. These include the inability to preserve the native membrane context around the protein, diminished sample stability, poor success in solubilisation, interference with characterisation methods and cost. However like any method there are a number of important considerations that have to be met for the method to be successful. The first is the size of the protein that is being solubilised. The disc shaped nanoparticle that forms the basis of the method has a nominal maximal diameter that is close to 15 nm corresponding to a molecular mass of less than approximately 400 kDa. This means that proteins that are too large to fit within this limit are unlikely to be successfully solubilised which eliminates many large membrane protein complexes. In our own studies we have solubilised more than 30 membrane proteins and have shown that proteins that contain up to 36 transmembrane helical elements can be solubilised. Users should therefore consider carefully whether to attempt the SMALP method if their protein or complex is likely to contain more than 36 transmembrane helices. The second important consideration is the pH at which downstream studies of the protein have to be carried out. The SMA polymer only forms SMALPs above pH 6.5 (below this value SMA is not water soluble), which means that experiments with the protein in the SMALP form have to be carried out above pH 6.5 and preferably above pH 7.0. The third limitation is linked to the pH issue, as the SMA polymer is also an effective chelator of divalent cations (e.g. Mg$^{2+}$ and Ca$^{2+}$) with the chelate also being insoluble. This means that experiments that require high concentrations of divalent cations (e.g. above 5 mM) are likely to lead to disruption of the SMALP. This can present issues with membrane proteins that bind nucleotides such as ABC transporters and ATPases. However, the assays for such proteins often use concentrations of nucleotide that are significantly above what is required. Often the levels can be safely reduced to preserve the SMALP while maintaining native levels of activity and/or allowing enzyme assays to be performed.
Experimental design
Here we describe a comprehensive set of protocols required to prepare the relevant reagents, use these reagents to purify membrane proteins in SMALPs and carry out initial biophysical characterisation of the resulting preparation. We also describe how to prepare the SMA polymer and SMALPs without encapsulated membrane proteins. We demonstrate how this SMALP protocol has been used to prepare a variety of active proteins from various sources including bacteria, insect cells, mammalian cells and the yeast.

Preparation of Styrene Maleic Anhydride Co-polymer from Styrene Maleic Anhydride Co-polymer
The Styrene Maleic Anhydride Co-polymer reagent used in this method uses a styrene to maleic acid ratio of 2:1. This polymer is currently commercially available only as an anhydride precursor. Styrene Maleic Acid Co-polymer with a 3:1 ratio is available commercially but our studies have shown that discs produced using this material contain bilayers with physical properties that differ from the bulk. Therefore a protocol is included that allows the 2:1 Styrene Maleic Anhydride Co-polymer to be hydrolysed to produce the maleic acid form.

The starting point of the synthesis is the anhydride form of the polymer which is available as an inexpensive powder. The anhydride is converted to the acid using a basic hydrolysis protocol. This protocol describes the production of 25 g of dried SMA 2000 Co-Polymer. The Styrene Maleic Anhydride Co-polymer is dissolved in 1 M NaOH and the reaction is carried out while heating and refluxing the solution (Fig. 2a-c). After cooling at room temperature (20 °C), (Fig. 2d), the Styrene Maleic Anhydride Co-polymer is precipitated by reducing the pH to below 5 by the addition of concentrated HCl (Fig. 2e). To ensure the full precipitation of the polymer, it is important the pH is monitored at this stage. Preferentially use pH test strips at this stage rather than a pH meter as the probe can easily be contaminated with residual polymer. The precipitate is washed three times with water followed by separation using centrifugation. At the end of the third wash the precipitate is resuspended in 0.6 M NaOH. The solution is precipitated and washed again, and finally resuspended in 0.6 M NaOH. The pH is then adjusted to pH 8. As the pH adjustment can lead to polymer precipitation, this step can be a lengthy process. Finally, the
polymer is lyophilized using a freeze dryer. The desiccated Styrene Maleic Anhydride Co-polymer powder can be stored indefinitely at room temperature in a sealed vessel.

**Preparation of SMA Lipid Particles using Co-Polymer**

We have found that downstream characterisation of SMALP solubilised membrane proteins often requires control samples that contain both lipid and SMA in the form of a protein free SMALP. For example, in studies of ligand binding, such particles provide a measure of the nonspecific binding of the ligand. In this section we discuss how to produce a protein free SMALP. When the Styrene Maleic Anhydride Co-polymer is added to a suspension of lipid, the solution changes from a cloudy solution to a clear one (Fig. 1b). The SMA interacts with the lipid bilayer, self-assembling into SMALPs (Fig. 1c).

We have prepared SMALPs from a variety of lipids and relevant mixtures. Here, we give a protocol to prepare protein-free SMALPs using *E. coli* polar lipid extract, which provides a relevant control for experiments using SMALPs containing protein from *E. coli* membrane. If required, SMALPs containing other type of lipids can be prepared following the same procedure.

**Membrane Protein Preparations**

This protocol outlines the purification of membrane proteins from *E. coli* membranes; but we have not detailed methods to express membrane proteins, as these methods are independent of the SMALP process. This protocol is intended to be applicable for proteins expressed in many different prokaryotic and eukaryotic systems. The protocol begins with prepared membranes containing the chosen membrane proteins of interest.

**Isolation of Membrane proteins in SMALPs**

Here, we detail the purification of membrane proteins overexpressed in *E. coli* as an example, but the same protocol can be used in other systems such as, insect cells, mammalian cells and yeast. In the example, we show how a SMALP containing a protein with a histidine affinity tag is purified by Nickel affinity chromatography. We have applied the same methodology to the purification of recombinant membrane proteins in SMALP with polyhistidine affinity tags with between 6 and 10 residues at either the N or C terminus of the protein. As with any protein purification, it is critical to add the affinity tag to a sterically accessible part of the protein to
ensure optimum binding of the target to the affinity resin. Therefore, we recommend that affinity
tags should be inserted distal from the predicted membrane spanning region. Upon protein
extraction from membranes and their encapsulation in SMALPs, they can be purified as you
would any globular protein.

The SMALP is compatible with a variety of buffers. We routinely use either a simple 50 mM
TRIS 150 mM, NaCl at pH 8.0, or 50 mM potassium or sodium phosphate 150 mM, NaCl pH
8.0 as a final purification and storage buffer (Tris-buffered or phosphate-buffered saline). As a
precaution the choice of buffers to some extent can be dictated by any downstream use of your
SMALP protein or biophysical characterisation that is being undertaken. However, as previously
stated, buffers should be above pH 7.0 and free of divalent cations (e.g. Mg$^{2+}$ and Ca$^{2+}$) as they
interfere with the formation of the SMALP and lead to SMA precipitation.

As a guide to the amount of SMA needed for membrane protein purification, approximately 1g
of SMA is required for every 10 g of membranes (wet weight). For example, we would calculate
the amount of SMA to use by estimating the membrane wet weight: we generally resuspend
membranes between 20 - 40 mg ml$^{-1}$ of buffer and add polymer at 2.5% wt/vol. We have success
either with adding powdered polymer directly (described here) to membrane solutions, or by
adding a 5% solution to an equal quantity of resuspended membranes. We do not keep the
sample on ice at this point as this reduces membrane fluidity, preventing SMA from excising the
protein from the lipid environment. This is a significant departure from established methods of
membrane proteins purified with detergents.

**Purification of histidine tagged proteins encapsulated in SMALPs**
During the development of a chromatographic separation strategy for a SMALP protein there are
a number of parameters that have to be considered. We have found that the most important of
these are resin choice and binding regime. When purifying SMALP proteins with polyhistidine
tags, resin choice can have a significant effect on protein purification. Of particular importance is
the choice of metal bound to the resin (Nickel or Cobalt). We therefore suggest that an initial
small scale binding trial is carried out to determine which is optimal for the protein being
purified. For simplicity we have detailed here the use of Nickel resin for the purification of
SMALP proteins with polyhistidine affinity tags. However we have also had success with Cobalt resins: indeed a case in point would be with SMALP AcrB where purity and yield were higher using Cobalt resin rather than nickel. The second optimisation choice involves the binding regime used to apply the protein to the resin. Binding of SMALP proteins to resins can be weak and/or slow, thus a slow “batch” method, allowing the SMALP protein to bind overnight or for a minimum of two hours with gentle mixing at 4 °C may be optimal. In some cases we observe very tight binding to resins, meaning that a more conventional column format binding step can be used. We have also found that the relatively high negative charge on the SMALP leads to significant non-specific binding to the resin. This can be mitigated by using an affinity chromatography buffer that contains at least 500 mM NaCl and in some cases up to 1 M of NaCl.

**SDS-PAGE analysis and protein concentration estimations**

After chromatographic separations have been carried out the protein needs to be characterised in terms of purity and amount. Conventionally SDS polyacrylamide gel electrophoresis (SDS-PAGE) has provided the main means to assess purity while a number of methods are available to determine the amount of protein. For proteins in SMALPs SDS-PAGE remains the method of choice with the only change from working with soluble proteins being the presence of a low molecular weight “streak” that stains with Coomassie blue due to the presence of the SMA in the sample. To confirm the identity of proteins, western blotting is recommended or proteins can be excised from SDS-PAGE acrylamide gels for analysis by mass spectrometry.

Solutions containing free SMA polymer and/or lipid can interfere with traditional protein estimation methods. For example free polymer has a small but significant absorbance at 280 nm therefore interfering with UV detection methods for proteins. Similarly SMA and lipid can interfere with dye based assays including the Bradford assay. However when free SMA and lipids are removed from solution for example by dialysis or size exclusion chromatography (SEC), this problem is reduced. Protein estimates using 280 nm UV detection methods can be performed on purified SMALP proteins when free SMA has been removed, and provide a reliable estimate of protein concentration.

**Initial SMALP-protein characterisation**
Once the SMALP encapsulated protein has been made, the process of characterisation can begin. It is outside the scope of the protocol to describe all the downstream characterisation methods that are employed for the study of membrane proteins. However we have found significant utility in performing three analyses with all the proteins that we have produced. Circular Dichroism (CD) spectroscopy provides invaluable information on the secondary structure of the protein in the SMALP allowing a rapid confirmation that the protein is folded within the particle. Ideally samples should be made up in a buffer containing a low chloride ion concentration (<50 mM), the most suitable being a phosphate buffer made using the method of Gomori (after Sørenson)\(^\text{36}\). In these experiments a demountable cuvette is used to hold the sample. The cuvette has two pieces, comprising of two quartz plates, one of which has a sample chamber etched into its surface.

Sedimentation velocity analytical ultracentrifugation (svAUC) allows the determination of the size of the SMALP-protein complex and can be used to answer questions related to the oligomerization states of the protein. A quick method of studying the quality of the SMALP protein sample is provided by negative stain microscopy as exemplified in\(^\text{27}\). This can, in the first instance, give clear indications of global shape, subunit stoichiometry, aggregation and degradation. Subsequent data collection and processing can provide further structural insight to a modest resolution but are outside the scope of this report.

At this stage of the SMALP experiment, it is not necessary to add any further SMA. SMALP protein can be treated like a globular protein and studied in many downstream applications including characterisation using AUC and CD analysis. It is also further possible to continue with activity assays and structural studies as you would with a membrane protein that has been purified with a detergent method.

**MATERIALS**

**REAGENTS**

- Anti-bumping granules (Fisher Scientific cat. no. 10283320)
- \textit{E. coli} polar lipid extract (Avanti Polar Lipids cat. no. 100600C)
- EDTA (Fisher Scientific cat. no. D/0700/53) !CAUTION is an irritant
- Face mask (Fisher Scientific cat.no 12560237)
Glycerol (Fisher Scientific cat. no. G/0650/17)

Hydrochloric acid specific Gravity (SG) 1.18 (Fisher Scientific H/1200/PB17) !CAUTION HCl is corrosive, is very hazardous in contact with skin (corrosive, irritant, permeator), and through ingestion, hazardous in case of eye contact (corrosive), and inhalation (lung corrosive).

HyperPAGE molecular weight marker (Bioline Cat. no. BIO-33066)

Imidazole (Acros Organics cat. no. 301870010) !CAUTION is a corrosive and an irritant

InstantBlue Coomassie stain (Expedeon Cat. no. ISBL)

Membranes prepared from *E.coli* BL21 (DE3) expressing ZipA with a V5 epitope and a C-terminal 6 x His tag.\(^{37}\)

MilliQ water

Na\(_2\)HPO\(_4\) (Fisher cat no. S/4520/53)

NaH\(_2\)PO\(_4\).2H\(_2\)O (Fisher cat.no. S/3760/53)

Novex 12% (wt/vol) Tris Glycine mini gels (Life Technologies, cat. no. EC6005BOX)

Novex NuPAGE LDS buffer (4x) Loading Dye (Life Technologies cat.no NP000)

Novex TRIS/Glycine/SDS 10x running buffer (Life Technologies LC2675)

NuPAGE sample reducing agent (10x) (Life Technologies novel NP0009)

pH indicator strips 1-14 (GE Healthcare Whatman cat. no. 10360005)

Pierce BCA Protein Assay kit (Thermo Scientific Cat.no. 23225)

Protease inhibitor tablets EDTA free (Thermofisher cat. no. 88266 NSH) !CAUTION. Irritating to eyes and skin.

Sodium Chloride (Fisher Scientific cat. no. S/3160/53)

Sodium hydroxide pellets (Fisher Scientific cat. no. S/4920/53) !CAUTION corrosive, causes severe burns.

Styrene maleic anhydride Co-Polymer SMA2000P (Cray Valley, SMA\(^{\text{®}}\) 2000) !CAUTION. SMA may cause eye, skin and respiratory tract irritation. We recommend wearing protective gloves while handling it. Wear safety glasses and a laboratory coat to limit skin exposure.

Super Cobalt NTA Affinity Resin (Generon Super-CoNTA25) !CAUTION flammable harmful sensitiser. Wear gloves when handling.

Super-NiNTA25 Affinity Chromatography resin (Generon Super-NiNTA25) !CAUTION flammable harmful sensitiser. Wear gloves when handling

TRIS Base (Fisher Scientific cat. no. T/9630/53) !CAUTION is an irritant
TRIS HCl (Sigma Aldrich cat. no. T5941) **CAUTION** is an irritant
1% Uranyl acetate solution **CAUTION** Uranium is radioactive, and a heavy metal. Wear gloves when handling and do not swallow.

**Equipment**

Analytical Ultracentrifuge (Beckman Coulter Inc., model XL-1, with an eight-cell An50Ti rotor)
Balance (Mettler Toledo model College B502)
Microbalance (Oxford, cat. no. G2105D)
Bath sonicator (Ultrawave U50, cat. no. F0002202)
Benchtop manifold freeze-dryer (Fisher Scientific, cat. no. 12783075)
Borosilicate glass measuring cylinders 50 ml, 100 ml, 250 ml, 500 ml, 1000 ml
Centrifuge Tube 50ml (Fisher Scientific cat.no.11512303)
Circular Dichroism Spectrometer JASCO model J 715
Clamps x 2
Cork ring (Fisher Scientific, cat. no. 07-835A)
Cressington 208 Carbon coater (Cressington)
FEI T12 Electron Microscope
Carbon Coated grids (Agar Scientific, AGS160)
Dialysis tubing 15.5 mm (Biodesign, cat. no. 511-0723)
Glow Discharge Lamp (UV Products, Low pressure mercury vapour type R51)
Graduated transparent pipette 10 ml (VWR, cat.no. 612-4752)
Heating mantle (Fisher Scientific, cat. no. EMA0500CEB)
High speed centrifuge (Beckman Coulter Inc., Avanti JXN-26 with Fiberlite F10BCI-6 x 500 and JA25.50 rotor)
Microfuge (Beckman Coulter Inc., Microfuge 16)
Liquid Chromatography System (G.E. Healthcare, ÄKTA explorer)
Magnetic stirrer (Stuart, cat. no. CB162)
Microtitre Plate Reader with ADAP 2.0 Basic software (Biochrom Anthos Zenyth 340 rt Cat.no. GF2530001, B032081)
Motorized Pipet Fillers/Dispensers (Fisher Scientific, cat. no. 05100501)
Nitrogen tank and gauge.
pH indicator strips 1-14 (GE Healthcare, Whatman, cat. no. 10360005)
pH meter (Corning, cat. no. 120)
Nalgene Oakridge centrifuge tubes (VWR, cat. no 3119-0050)
Polypropylene centrifuge bottles 500 ml (Thermo Scientific, cat. no. 3141-00500)
Polycarbonate centrifuge bottle and cap (AY, PC ½ x 3 Beckman Coulter Inc., cat. no. 355603)
Polycarbonate ultracentrifuge bottle and cap (Bottle AY, PC 1 x 3.5 Beckman Coulter Inc., cat. no. 355618)
Poly-net® protective netting (Sigma Aldrich, cat. no. Z183652)
PowerPac HC Power Supply (BioRad, cat. no. 1645052)
Quartz Demountable Cuvette (Starna, cat. no. 20/C-Q-1)
Quickfit® (Fisher Scientific, cat. no. FR500/3S, FR1L/3UM)
Quickfit® Condenser coil (Fisher Scientific, cat. no. C3/13/SC)
Retort stand (Fisher cat. no.11715396)
Round Bottom Flasks 500 ml and 1000 ml (VWR, cat. no. 201-11329H and 201-11327H)
Sealing cling film (Fisher Scientific, cat. no. SEL-360-20H)
Sealing film (Fisher Scientific, cat. no. PM992)
Sedfit software (NIH) 38
Solid-glass beads (Sigma Aldrich, cat. no. Z143928)
Superdex 200 Increase 10/300 GL prepacked column (G.E. Healthcare)
Ultracentrifuge (XL90 ultracentrifuge with Ti70 and Ti70.1 Beckman Coulter Inc., rotas)
Vivaspin 20 (Sartorius, cat. no. VS2001)
XCell Surelock mini cell (Life Technologies, cat. no. E10001)

REAGENT SETUP

1 M NaOH
Prepare 1 litre of solution containing 40 g of NaOH in H₂O in a 1 litre conical flask. This can be stored at room temperature for several months.

0.6 M NaOH
Prepare 1 litre of solution containing 24 g of NaOH in H₂O in a 1 litre conical flask. This can be stored at room temperature for several months.

**Buffer A Stock 500 mM TRIS buffer at pH 8.0 at room temperature**
Prepare 1 litre of solution in H₂O containing 44.4 g of TRIS/HCl and 26.5 g TRIS Base (500 mM) in a 1 litre conical flask. Adjust to pH 8.0 before making up to the final volume. Autoclave and store at room temperature. The solution can be stored for 6 months.

**Lipid nanoparticle buffer A (TRIS)**
Prepare 5 ml solution containing 0.5 ml of buffer A (50mM) in H₂O in a 1 litre conical flask. This Buffer should be freshly prepared.

**5% (wt/vol) SMA solution buffer A (TRIS)**
Prepare 1 ml of solution containing 5% wt/vol of SMA in lipid nanoparticle buffer A (TRIS) in a 1.5 ml Eppendorf tube. This buffer should be freshly prepared.

**SMA solubilisation buffer A (TRIS)**
Prepare 1 litre of a solution containing 100 ml of 500 mM TRIS buffer A pH 8.0 (50 mM), containing 29.22 g NaCl (500 mM), 10% (vol/vol) glycerol in H₂O in a 1 litre conical flask. This buffer should be freshly prepared.

**SMALP affinity purification elution buffer A (TRIS)**
Prepare 1 litre of a solution containing 100 ml of 500 mM TRIS buffer A at pH 8.0 (50mM) 29.22 g NaCl (500 mM ) 10% (vol/vol) glycerol and 34 g Imidazole (500 mM) in a 1 litre conical flask. Adjust to pH 8.0 before making up to the final volume. Dilute this buffer with solubilisation buffer to obtain buffer concentrations between 10 mM and 500 mM if necessary. This buffer should be freshly prepared.

**Size exclusion chromatography buffer A (TRIS)**
Prepare 1 litre of solution containing 100 ml of 500 mM TRIS buffer A at pH 8.0 (50 mM), 8.76 g of NaCl (150 mM) pH 8.0 before making up to the final volume in a 1 litre conical flask. Degas and filter sterilise. This buffer should be freshly.
Buffer B
First prepare 200 mM of monobasic and dibasic Sodium phosphate solutions. Prepare 1 litre each of solution containing (I) and (II) containing (I) 28.4 g of Na₂HPO₄ (200 mM) and (II) 1 litre of solution containing 31.2 g NaH₂PO₄.2H₂O (200 mM) both in 1 litre conical flasks. Autoclave and store at room temperature, for up to 6 Months. Then prepare 1 litre of phosphate buffer at pH 8.0 by combining 473.5 ml of solution (I) Na₂PO₄, and 26.5 ml of (II) NaH₂PO₄.2H₂O (200 mM) in a 1 litre conical flask. Check that the is pH 8.0. Autoclave and store at room temperature (phosphate buffer B pH 8.0), for up to 6 months.

Lipid nanoparticle buffer B (Phosphate)
Prepare 5 ml solution containing 1.25 ml of Buffer B (II) (50mM) in a 10 ml Falcon Tube. This buffer should be freshly prepared.

5% (wt/vol) SMA solution buffer B (Phosphate)
Prepare 1 ml of solution containing 5% wt/vol of SMA in lipid nanoparticle buffer B (phosphate) in a 1.5 ml Eppendorf tube. This buffer should be freshly prepared.

SMA solubilisation buffer B (Phosphate)
Prepare 1 litre of solution containing 250 ml phosphate buffer B pH 8.0 (50 mM), 29.22 g NaCl (500 mM), 10% (vol/vol) glycerol in a 1 litre conical flask. This buffer should be freshly prepared.

Nickel or Cobalt agarose beads
Prepare the beads as indicated by the bead manufacturer. Briefly before use, wash the agarose beads in MilliQ and allow to equilibrate in SMA solubilisation buffer for 30 minutes before use.

SMALP affinity purification elution buffer B (Phosphate)
Prepare 1 litre of a solution containing 50 ml phosphate buffer B pH 8.0 (50 mM), 29.22 g NaCl (500 mM ), 10% (vol/vol) glycerol, 34 g imidazole (500 mM). Adjust to pH 8.0 in a 1 litre conical flask. Dilute this buffer with solubilisation buffer to obtain buffer concentrations between 10 mM and 500 mM if necessary. This buffer should be freshly prepared.
**Size exclusion chromatography buffer B (Phosphate)**

Prepare 1 litre of a solution containing 50 ml of phosphate buffer B pH 8.0 (50 mM) containing 8.77 g NaCl (150 mM) in a 1 litre conical flask. Degas and filter sterilise. This buffer should be freshly prepared.

**EQUIPMENT SET UP**

**Reflux equipment for SMA**

When preparing to reflux the Styrene Maleic Anhydride Co-polymer and the 1M NaOH, rest the round bottom flask on a cork ring (Fig. 2a). In fume hood, position the heating mantle, (Fig. 2b) and set up the reflux apparatus with the condenser coil on a retort stand and attach the apparatus to a water supply (Fig. 2c). Ensure that the water is flowing through the condenser.

**Circular Dichroism Spectroscopy**

CD spectra are measured in the far UV (190-260 nm) using a JASCO J-715 spectrophotometer and a 1 mm path length quartz cuvette containing 0.05 mg ml\(^{-1}\) of SMALP protein. Collect with a data pitch of 0.5 nm and sixteen scans per measurement. In addition measure the relevant buffer using the same parameters to allow subtraction of the buffer contribution to the spectra.

**Analytical Ultracentrifuge(AUC)**

Prepare twin channel AUC cells with 400 µl of SMALP protein at a concentration between 0.1 and 0.5 mg ml\(^{-1}\) in one channel and 420 µl of relevant buffer blank in the second channel. Load the cells into the An50Ti rotor in a Beckman Coulter XL-I analytical ultracentrifuge (Beckman 693 Coulter) and operate the centrifuge at 129,000 \(g\) and at a temperature of 20°C until the sample has fully sedimented. The protein within the cell is monitored by absorbance at 280 nm. Analyse data using the program SEDFIT\(^{38}\) using the c(S) and c(M) routines to provide estimations sedimentation coefficient and mass of the particle. Parameters for SMALP protein partial specific volume, solvent density and viscosity were calculated using SEDNTERP.

**Electron Microscopy**

For negative stain microscopy a modest Electron Microscope setup can be used, such as FEI T12 operating at 120 kV accelerating voltage. The negative stain limits the resolution to \(\geq 12\) Å so it
is best to optimise the field of view working at around 4 Å/pixel, resulting in a magnification of ~ 35,000 times based on a GATAN CCD camera. Low dose mode is not required for the initial screening of grids but is recommended for data collection.

PROCEDURE

Part 1: Preparation of Styrene Maleic Anhydride Co-polymer from Styrene Maleic Anhydride Co-polymer

**TIMING: 4 - 5 Days**

*CAUTION* Wear a protective dust mask when dealing with Styrene Maleic Anhydride Co-polymer. The following steps must be carried out in the fume hood. Ensure fume hood is working and the shutter is down. The equipment and protocol described here is for the production of 25 g of Styrene Maleic Acid Co-polymer.

1. **Heating with reflux** In the fume hood, weigh 25 g Styrene Maleic Anhydride Co-polymer in a weighing boat and transfer to a 500 ml round bottom flask.

   ▲**CRITICAL STEP** Ensure that round bottom flasks are completely intact and free of any flaws or ‘starburst’ cracks. Do not use if these are present.

2. Using a glass measuring cylinder, add 250 ml of 1M NaOH to the round bottom flask (**Fig. 2a**).

3. Weigh out 0.5 g of anti-bumping granules and add to the round bottom flask. Place the round bottom flask on the heating mantle with the condenser coil attached (**Fig. 2b**). Ensure the condenser is connected to the water supply and there is water flowing through (**Fig. 2c**). Apply heat and allow the Styrene Maleic Anhydride Co-polymer suspension in NaOH to gently reach boiling point. Once the solution is boiling, turn down the heat level and allow the solution to maintain a steady boil, continuing to reflux for 2 hours.

   ▲**CRITICAL STEP** Do not omit anti-bumping granules. It is very important that these are present during reflux as they prevent violent surges of the solution during reflux.

**TROUBLESHOOTING**

4. Allow the refluxed solution to cool down to room temperature with the condenser still connected and water flowing through (**Fig. 2d**).
The polymer needs to cool to room temperature, before you proceed to the next step.

**PAUSE POINT:** The solution can be stored at 4 °C at this point for up to 4 days.

### 5. Polymer precipitation and washing

Check the refluxed polymer volume (it will be approximately 270 ml) and divide into two equal aliquots in 500 ml centrifuge bottles. Do not transfer more than 150 ml starter volume of polymer into a 500 ml bottle. The suggested volume is 135 ml.

**▲CRITICAL STEP** Use polypropylene centrifuge bottles because of the corrosive nature of the solutions.

6. Using a 10 ml graduated pipette, gradually add concentrated HCl to the polymer. Start initially by adding 10 ml to each bottle and mix well. The polymer will start to precipitate (**Fig 2e**).

7. Continue to add HCl until the pH of the solution surrounding the precipitate is below pH 5. Periodically test the pH of the solution using a pH indicator strip.

**?TROUBLESHOOTING**

8. To the precipitated polymer, add MilliQ water and fill the centrifuge bottles to the maximum permitted volume (approx 250 ml). Balance bottles by further addition of water if necessary.

!CAUTION Do not overfill the centrifuge bottle.

9. Centrifuge the polymer suspensions at 11,000 g for 15 minutes.

10. Carefully pour off the remaining supernatant without disturbing the pellet and thoroughly resuspend the polymer in MilliQ water to close to the maximum permitted volume (approx 250 ml) per 135 ml refluxed polymer.

11. Mix well by vigorous shaking to completely resuspend the precipitate. Balance bottles by further addition of water if necessary.

!CAUTION Do not overfill the centrifuge bottle.
12. Centrifuge the polymer suspensions at 11,000 g for 15 minutes.

13. Repeat steps 10 to 12 two further times.

14. Carefully pour off the supernatant without disturbing the precipitate.

15. **SMA Co-Polymer second precipitation and wash cycle** Leave the pellets in the centrifuge bottles and add 125 ml of 0.6 M NaOH per bottle. Either place on a magnetic stirrer or in an orbital shaker at 37° C, 180 rpm until the pellet has completely dissolved.

   **PAUSE POINT:** The solution can be left overnight at 37° C to dissolve.

16. Repeat steps 6 through to 14.

17. **SMA Co-Polymer solubilisation in 0.6 M NaOH** Once the polymer has re-dissolved in 0.6 M NaOH, check the pH using a pH meter and adjust to ~ pH 8.0 using concentrated HCl or NaOH.

   ▲**CRITICAL STEP** Add only a few drops of NaOH at a time as localised precipitation will occur. Allow the polymer to completely re-dissolve before adding more drops.

   **PAUSE POINT:** Polymer can be stored in the freezer at - 20° C before freeze drying.

18. **Freeze drying the SMA Co-Polymer** Transfer the solution to a clean 1 litre round bottom flask and freeze at - 20° C. This usually takes at least 18 hours.

   ▲**CRITICAL STEP** Check for imperfections or star bursts in the glass, and ensure that the polymer is thoroughly frozen before proceeding to freeze drying.

19. Cover the flask with Poly-net then place the flask of frozen polymer in the freeze dryer according to the manufacturer's instructions and allow the polymer to dry to a powder.

20. Store the dried polymer at room temperature in a sealed vessel.

   !**CAUTION** Although unlikely, the bottle may crack or split during the freeze drying process. As a precaution, before placing the flask in the freezer, wrap the the flask in sealing film.
PAUSE POINT Dried polymer can be stored at room temperature for up to 12 months.

**Part 2: Preparation of protein-free lipid nanoparticles using SMA Co-Polymer**

- **TIMING:** ~1 to 2 hours.

  !CAUTION Wear gloves and laboratory coat. This step should be carried out in the fume hood as Chloroform is toxic.

21. Transfer 20 mg *E. coli* polar lipid extract (which is supplied in a chloroform solution) to a round-bottomed glass test tube. *NB.* The concentration of lipid in the extract should be provided by the supplier. Dry the lipid to a thin film under a gentle nitrogen gas flow.

  ▲CRITICAL STEP As polymers can leach from plastic in the presence of chloroform, use glass tubes and pipettes for this step.

22. Remove residual traces of chloroform by drying the pellet under continuous vacuum in a desiccator for 30 minutes.

  ▲CRITICAL STEP It is important to remove all the chloroform from the lipid.

PAUSE POINT: The pellet can be left to dry overnight.

23. Rehydrate the lipids with 1 ml of either Lipid nanodisc buffer A or B, depending upon downstream application, to form a homogeneous suspension. For example buffer B which uses a phosphate buffer is appropriate for circular dichroism experiments. Use the same buffer throughout. The addition of 3 mm glass beads and vortexing will help the lipid resuspension. It may be necessary to warm to 50°C to get complete resuspension.

24. Sonicate in a bath sonicator until a translucent milky white suspension of small unilamellar vesicle has been generated.

  ▲CRITICAL STEP When preparing lipid discs the bath temperature should be above the phase transition temperature of the lipid being used. In the case of *E. coli* polar lipid extract this is approximately 3°C.

25. Prepare 5% (wt/vol) SMA solution from powder produced in step 20 (choose A or B dictated by the downstream application). Gradually add 1 ml of 5% (wt/vol) SMA solution at a
temperature above the phase transition temperature of the lipid. During the addition of polymer to the *E. coli* polar lipid suspension, the milky suspension will become a clear solution (**Fig. 1b**)

**PAUSE POINT:** Protein free SMALPs can be stored at 4° C for up to two weeks.

### Part 3: Isolation of SMALP membrane proteins

**TIMING** 2 days.

26. **Membrane solubilization** Isolate membranes from *E. coli* by following steps described in Fotiadis 39 (steps 1-5). If using a different host organism, methods for isolating membranes from heterologously expressed proteins have been previously described for *Saccharomycetes cerevisiae* 40, *Pichia pastoris* 41, Human Embryonic Kidney cells (HEK293), Chinese hamster Ovary (CHO) and A431 cells 42,43,44 and baculovirus expression in Insect High Five *(Trichoplusia ni)* and *Sf9* cells 45. Measure the total mass of the membrane requiring solubilisation. As a guide, 10 g of *E. coli* cell pellet generally yields 1-1.5 g of membrane.

27. On ice, transfer the membranes to a hand homogenizer cooled in an ice bucket. Resuspend the membranes to a final concentration of 40 mg.ml⁻¹ in either SMA solubilisation buffer A (TRIS) or B (Phosphate). Use the same buffer throughout. Homogenize the membranes until they are fully resuspended, ensuring that there are no lumps. When the membranes are fully in solution, the suspension will look turbid.

28. Weigh out sufficient SMA Co-Polymer from step 20 and add this to the suspension to give a final concentration of 2.5% wt/vol (**Fig. 3a**). Allow the polymer to dissolve by gentle inversion of the tube. (**Fig. 3b**). As the SMA polymer solubilizes the membranes, the suspension should become less opaque. Alternatively, add the SMA pre-dissolved in the SMA solubilisation buffer from step 25 (5% wt/vol) and add the solution at 1:1 (vol/vol) ratio to the membranes resuspended at 80 mg ml⁻¹.

29. Allow the sample to incubate at room temperature for 2 hours with gentle agitation.

**PAUSE POINT:** The suspension can then be left overnight at 4° C if required.

??TROUBLESHOOTING
30. Remove the insoluble fraction by centrifugation at 100,000 g using the ultracentrifuge for 45 mins.

31. Immobilized metal affinity chromatography (IMAC) agarose beads can be prepared during the centrifugation step. Transfer 1 ml of washed IMAC agarose beads into a 50 ml centrifuge tube, according to the manufacturer's instructions. 1 ml of bed volume of resin should be sufficient to purify protein solubilized from 1 g of purified membranes. In cases where protein expression levels are high, the amount of beads may need to be increased. The resulting protein is then equilibrated in SMA solubilization buffer A or B.

32. Transfer the supernatant to the pre-washed IMAC resin, and incubate overnight at 4°C with gentle agitation, on a rotary mixer.

**CAUTION** Do not agitate to the extent of causing froth formation.

33. Decant the resin into an empty gravity flow column and collect the flow-through for analysis by SDS-PAGE.

34. Wash the resin with 10 column volumes of SMA solubilisation buffer A or B. Collect the wash for analysis by SDS-PAGE.

35. Elute the SMALPs with 10 column volumes of SMALP elution buffer in 1 ml fractions. **PAUSE POINT** Fractions can be stored at 4°C safely for up to a week.

**Part 4: Purification of SMALP proteins and estimation of concentration **

**TIMING**

36. Identify the location and purity of the protein in each sample using established SDS-PAGE methods. To do this, prepare samples from step 34 and 35 by adding NuPAGE LDS sample buffer and NuPAGE reducing agent according to the manufacturer's instructions.

37. Prepare the XCell surelock mini-cell and fill it with 1 x SDS running buffer.
38. Load the samples from step 36 on the Novex 12 % wt/vol Tris Glycine mini gel, along with 10 µl HyperPAGE molecular weight marker in another well for analysis.

39. Attach the tank to a power pack, and run the gel for 35 minutes at 200 v.

40. Stain the gel with InstantBlue coomassie stain for 1 hour or overnight.

41. Destain the gel with ddH₂O with gentle shaking at room temperature for 2-5 hours, change the ddH₂O a few times during destaining to aid the development of the stain. 

**PAUSE POINT**

42. Identify the SMALP protein bands on the gel with reference to the molecular marker. The SMA polymer will be identifiable as a diffuse band at approximately 8 kDa. In the solubilisation fraction this will dominate the signal due to the high concentration of SMA in the sample.

43. Select fractions from step 34, remove imidazole and SMA from the samples containing SMALP protein by dialysis or by using a vivaspin concentrator. For the former, prepare dialysis tubing in accordance with the manufacturer's instructions and dialyzed against 1 litre of size exclusion chromatography buffer A or B, overnight at 4°C. Change the buffer during this time to ensure the complete exchange of buffer. Alternatively for the latter, concentrate using a vivaspin concentrator and then add size exclusion chromatography buffer A or B. Repeat this process 3 times to ensure that residual imidazole has been diluted. Once the imidazole has been removed the SMALP encapsulated protein can undergo size exclusion chromatography using a suitable column and FPLC system (e.g. Superdex 200 Increase 10/300 and ÄKTA purification system) in accordance with the manufacturer's instructions. The resulting fractions can be analysed using the methods described in steps 36-42.

44. Determine the concentration of your SMALP protein using a BCA protein assay kit. Prepare a set of protein standards with a range of concentrations between 25 µg and 2,000 µg/ml by diluting the contents of one Albumin Standard (BSA) ampule into several clean tubes, using the same buffer as the SMALP protein sample(s).
45. Prepare the quantification reagent for the assay by adding 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1 Reagent A:B). Pipette 25 μl of each standard and unknown sample in replicate into a microtitre plate. Add 200 μl of the prepared quantification reagent to each well and mix well for 30 seconds. Incubate the plate at 37° C for 30 minutes. Cool the microtitre plate to room temperature, and measure the absorbance at 562 nm on a plate reader.

**PAUSE POINT** Samples can be stored safely at 4° C for up to a week.

**Part 5: Protein folding and secondary structure determination.**  

**TIMING** 2 days.

46. **Sedimentation Velocity Analytical ultracentrifugation (AUC)** Transfer purified SMALP protein from step 43 at a concentration of 0.5 mg mL⁻¹ into one of the double-sector cells, and protein sample buffer or the remaining dialysis buffer into the other cell. We typically use 400 μl of sample and 420 μl of buffer.

47. Centrifuge at 110,000 g for 20 hours at 20° C and with scanning detection at 280 nm.

48. Analyse the data with the continuous c(s) analysis method to determine sedimentation coefficients and molecular masses using the SEDFIT software using the method of Schuck³⁸.

49. **Protein folding and secondary structure determination using Circular Dichroism spectroscopy (CD)** Prepare SMALP protein sample at a concentration of 0.05 mg.ml⁻¹, determined in step 45 and transfer into a 1 mm cuvette.

50. 150 μl of both the SMALP sample and a buffer blank should be prepared and equilibrated at room temperature.

51. Separate the two quartz plates and pipette 60 μl of buffer into the sample chamber.

52. Place the second quartz plate on top of the sample.

53. Dry any sample that leaks from the cuvette with Kimwipe tissue.
54. Place the sample in the CD Spectropolarimeter, close the lid and allow the machine to purge for 3 minutes.

55. Record the CD spectrum using parameters that suit the purpose of the experiment. A discussion of how parameters may be optimised can be found in\textsuperscript{46}.

56. Remove the cuvette and clean with 3 washes of MilliQ water followed by ethanol and dry in a flow of dry nitrogen.

57. Repeat step 45-50 for the SMALP sample.

58. Subtract the spectrum for the buffer from the spectrum obtained for the SMALP sample. \textbf{\textit{\textbf{\textsuperscript{\textcircled{C}}}RITICAL STEP}} Samples should be made up in a buffer containing a low chloride ion concentration (<50 mM).

59. \textbf{Transmission Electron Microscopy Negative Stain Analysis} Take pre carbon coated grids which can be made manually\textsuperscript{47} or purchased directly, for example from Agar Scientific (AGS160) and charge. This can be achieved through 40 seconds of glow discharge, we typically use a Cressington glow discharge unit or place the grids under a UV lamp for 40 minutes.

60. Using fine point tweezers hold the grid at the edge being careful not to damage the carbon and apply 3 µl of purified protein from step 43 at \textasciitilde 10 µg.ml\textsuperscript{-1} concentration to the carbon coated side. Leave for 30 seconds.

61. Remove the excess liquid using filter paper and apply an additional 3 µl of 1% uranyl acetate solution. Leave for 1 minute.

62. Repeat step 61.

63. Remove excess liquid and air dry the grid. \textbf{\textit{\textsuperscript{\textcircled{P}}}AUSE POINT} The grid can now be stored until required for TEM analysis.
64. Observe the grids within a suitable electron microscope. Detailed protocols from grid preparation, data collection and processing are beyond the scope of this article however we would like to make the reader aware of the following research papers which provide more in depth protocols and advice; Negative stain grid preparation and initial data collection\textsuperscript{48}, cryo-EM sample preparation and data collection\textsuperscript{49,50}, data processing\textsuperscript{47,51,52}.

\textbf{\textsuperscript{\textbullet}CRITICAL STEP} Air drying the grids under a desktop lamp can produces a more consistent stain depth.

\textbf{TIMING}

\textbf{Part 1: Preparation of Styrene Maleic Co-Polymer from Styrene Maleic Anhydride Resin} 4 days.
Steps 1-4 5 h
Steps 5-14 3-4 h
Steps 15-16 3-4 h
Steps 17-20 48-60 h

\textbf{Part 2: Preparation of Lipid nanoparticles using SMA Co-Polymer} 1-2 h
Steps 21-25 1-2 h

\textbf{Part 3: Isolation of SMALP Membrane proteins} \textasciitilde 2 days
Step 26-35 \textasciitilde 2 days

\textbf{Part 4: Identification of SMALP proteins and estimation concentration}
Step 36 - 41 \textasciitilde 4-5 h
Step 42 - 43 \textasciitilde 4-5 h
Step 44 - 45 \textasciitilde 1-3 h

\textbf{Part 5: Biophysical Characterisation} \textasciitilde 2-3 days
Step 46-48 \textasciitilde 24- h
Step 49 - 58 3h
Step 59 - 64 12-24 h

\textbf{TROUBLESHOOTING}
Troubleshooting advice can be found in Table 1
## Table 1 – Troubleshooting table

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>If the volume in the round bottom flask decreases during the refluxing</td>
<td>The condenser is not working efficiently.</td>
<td>Mark the flask with the level of the solution at the beginning of the experiment to allow any loss of solution to be detected. Ensure water is flowing through the condenser.</td>
</tr>
<tr>
<td></td>
<td>reaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Violent over boiling</td>
<td>The heat setting is too high.</td>
<td>Turn heat down.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No anti bumping granules.</td>
<td>Turn off the heat, allow to cool down. Then add anti bumping granules.</td>
</tr>
<tr>
<td>7</td>
<td>The amount of HCl to be added may vary.</td>
<td>Batch variability.</td>
<td>Suggested amount of HCl required to add to 135 ml solution is approximately 20 ml. When all the SMA Co-Polymer is precipitated there will be very little liquid remaining and the pH will be 5 or less.</td>
</tr>
<tr>
<td>29</td>
<td>If the level of Possible problem with</td>
<td>Increasing the</td>
<td></td>
</tr>
</tbody>
</table>
solubilisation of the membrane preparation is low, and solution does not appear to clear.

SMA batch, incubation temperature to 37°C. Furthermore, sonication using a probe sonicator (6 x 20 second cycles) or high pressure homogenisation can also aid in solubilisation during this step.

ANTICIPATED RESULTS

Preparation of Styrene Maleic Anhydride Co-polymer will yield approx 25 g of hydrolysed white powder that can be used to prepare SMALP membrane proteins. SMA can be added directly to resuspended membranes or added as a solution at a recommended final concentration of 2.5% wt/vol. When SMA Co-Polymer is added to a membrane preparation the cloudy solution should begin to clear and after 2 hours will be translucent (Fig 1b). At this point it can be assumed that the protein will be in the SMALP. Purification of SMALP proteins using an attached affinity tag such as a 6 Histidine tag allows the isolation of the protein with surrounding lipids in the form of a SMALP protein. When isolating SMALPs containing membrane proteins, size exclusion chromatography as a secondary procedure after elution from IMAC resin, significantly improves yield and purity. To demonstrate the SMALP method, we describe here the purification of the bacterial divisome protein ZipA from *E. coli* ⁵³. ZipA is a 36.5 kDa protein which has one transmembrane helix. ZipA purified here contains a C-terminal 6 Histidine tag and a V5 epitope, increasing the total size of the protein to 39.5 kDa. ZipA was expressed and extracted from *E. coli* membranes using Styrene Maleic Anhydride Co-polymer, and bound to IMAC resin. Resin was washed with low concentrations of imidazole, and eluted with buffer containing 500 mM imidazole. Fractions were analysed by SDS-PAGE and protein containing SMALP-ZipA were pooled and concentrated and applied to a Superdex 200 Increase column (Fig. 4a). Pooled fractions eluted from SEC were analysed using SDS-PAGE, to show the protein purity after a
two step purification approach (Fig. 4a). Purification of ZipA from membranes, through the purification process can be seen in (Fig. 4b). The band from SDS-PAGE was confirmed by FT-ICR mass spectrometry to be ZipA. The protein is seen here at 52 kDa (Fig. 4b). This protein is known to run aberrantly on an SDS-PAGE, a property that is unrelated to ZipA being within a SMALP and membranes containing ZipA, show a protein band of comparative size to purified SMALP protein (Fig.4b).

SMALP-ZipA is active and it is able to interact with its functional partner FtsZ. A sedimentation assay for the polymerisation of FtsZ in the presence or absence of SMALP-ZipA confirms biological activity, with SMALP-ZipA increasing the amount of FtsZ polymers which are found to be isolated in the pellet fraction (Fig.4c).

Sedimentation velocity AUC of a fraction taken from SEC chromatography revealed that SMALP-ZipA is present as a single species with a sedimentation coefficient of 4.0 S. This is consistent with a molecular mass of ~70 kDa. of SMA and lipid present in the SMALP contribute 30 - 35 kDa to the total molecular mass of the protein-SMALP particle (Fig. 4d). However not all fractions of purified SMALP-ZipA analysed by AUC were shown to be monomeric. Samples of pure SMALP-ZipA analysed from the beginning of the SEC elution peak were consistent with aggregated SMALPs, dimers and tetramers. This is evidence that proteins are either a) sampled as they exist in the membrane (i.e together) or b) they are associating with each other as demonstrated with SMALP AcrB.

CD spectroscopy spectrum of purified SMALP-ZipA showed purified SMALP-ZipA consists of α helices, β sheets and unstructured regions, which is consistent with the predicted structure of ZipA. It suggests purified SMALP-ZipA maintained its native secondary structure after solubilisation and purification steps. CD data were collected using a JASCO J-715 and CD spectra were collected using a 1 mm path length cuvette and averaged over 8 scans in the far-UV domain. Spectra acquired were corrected for the buffer signal. The protein concentration of purified SMALP-ZipA was 0.05 mg ml⁻¹ (Fig. 4e).

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an MRC Career Development Award (G100567). We dedicate this work to the late Professor Stephen A. Baldwin.

**Competing Financial Interests** The authors declare no competing financial interests.

**Author Contribution**
2. Devised the SMALP method. T.J.K., T.R.D., M.O.

**Figure legends**

**Figure 1.**
SMA production and SMALP formation from lipids. (a) The chemistry of the conversion of Styrene maleic anhydride Co-Polymer to the Styrene Maleic Anhydride Co-polymer (SMA) (b) Tube (i) contains a cloudy lipid suspension (ii) The solution clears when the SMA lipid particles form (c) Negative stain electron micrograph shows 10 nm disc formed by the addition of SMA to lipid suspension (scale bar represents 10 nm). The SMALP particles are represented as a cartoon with the polymer belt in blue and the lipids as CPK models (d) Illustrates how the styrene group of the SMA Co-Polymer is expected to intercalate with the tail group of lipids. (c and d published in 25)

**Figure 2.**
Styrene Maleic Anhydride Co-polymer preparation (a) 25 g of Styrene maleic anhydride Co-Polymer is added to 250 ml of 1M NaOH and 0.5 g of anti bumping granules in a round bottom flask, that rests on a cork ring in the fume hood. (b) The round bottom flask containing the reaction mixture is transferred to the heating mantel. (c) Illustrates how the reflux apparatus is assembled with the water flowing through it to enable condensed water to return to the solution without losing the solution by evaporation. (d) After the solution has been heated for 2 hours it is allowed to cool to room temperature. (e) When the solution has cooled to room temperature the
Styrene Maleic Anhydride Co-polymer appears as a light white precipitate at the beginning of the addition of HCl.

**Figure 3.** Forming SMALP from membrane preparations using SMA (a) Membranes are suspended in buffer at a concentration of 20-40 mg.ml\(^{-1}\) prior to the addition of SMA. (b) The membrane solution with SMA at first appears cloudy (c) After 2 hours the suspension has become clear.

**Figure 4.** Purification and biophysical characterisation of SMALP protein. (a) Typical elution profile of size exclusion chromatography using Superdex 200 Increase 10/300 column. 250 µl of sample was loaded at a flow rate of 0.5 ml.min\(^{-1}\). The protein elution was monitored by absorbance at 280 nm and 254 nm, enabling the detection of both protein in SMALP and free SMA, which is detected by the latter wavelength. The trace shows that SMALP ZipA is effectively separated from free SMA. (b) SDS-PAGE stained with Coomassie blue shows from left to right, protein standards marker (Marker), membrane fraction containing overexpressed ZipA (Membrane), SMALP ZipA protein from the IMAC step (IMAC), SMALP ZipA from SEC step (SEC), 2.5 % wt/vol SMA alone. (SMA) The SMA polymer can be identified as a diffuse band at approximately 8 kDa, and is also present in the IMAC and SEC lanes, where it has separated from the SMALP ZipA during SDS PAGE (c) Sedimentation assay for the polymerisation of FtsZ in the presence or absence of SMALP ZipA. SDS-PAGE stained coomassie blue shows SMALP ZipA activity. Expressed and purified FtsZ, Supernatant (S) and pellet (P). Lane 1 and 2, negative control, ZipA in polymerisation buffer (50 mM MES pH 6.5, 50 mM KCl, and 2.5 mM MgCl\(_2\)) and 2 mM GTP. Lane 3 and 4, positive control, FtsZ was in polymerisation buffer and 2 mM GTP. Lane 5 and 6, experimental, represent ZipA and FtsZ at 3.3 μM and 11μM, as SMALP ZipA promotes FtsZ polymerisation, an increased amount of FtsZ is isolated in the pellet fraction. (d) Sedimentation velocity AUC of a fraction taken from SEC chromatography show a single species with a sedimentation coefficient of ~4.0 S. this is consistent with a molecular mass of ~70 kDa. Corresponding to about 40 kDa of protein and 35 kDa of SMA and lipid present in the SMALP particle. (e) CD data shows purified SMALP ZipA to consist of alpha helices, beta sheets and unstructured regions, consistent with the predicted structure of ZipA. It suggests purified SMALP-ZipA maintained its native secondary structure after solubilisation and purification.
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Figure 1.

(a) Chemical reactions and polymers.

(b) Images of solutions.

(c) Image of a material.

(d) Molecular structures.
Figure 2.
Figure 3.

a) Add SMA copolymer at 2.5 % wt/vol

b) Incubate for 2 hours

c) SMA acts on membranes to form SMALP

Membrane protein suspension in buffer
Figure 4.

(a) Size Exclusion elution profile

(b) Purified SMALP ZipA

(c) AUC shows single peak

(d) CD

- Sedimentation coefficient (S)
- Wavelength (nm)
- Elipticity (mdeg)