



Detergent-Free Membrane Protein Purification Using SMA Polymer

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Abstract

One of the big challenges for the study of structure and function of membrane proteins is the need to extract them from the membrane. Traditionally this was achieved using detergents which disrupt the membrane and form a micelle around the protein, but this can cause issues with protein function and/or stability. In 2009 an alternative approach was reported, using styrene maleic acid (SMA) copolymer to extract small discs of lipid bilayer encapsulated by the polymer and termed SMALPs (SMA lipid particles). Since then this approach has been shown to work for a range of different proteins from many different expression systems. It allows the extraction and purification of a target protein while maintaining a lipid bilayer environment. Recently this has led to several new high-resolution structures and novel insights to function. As with any method there are some limitations and issues to be aware of. Here we describe a standard protocol for preparation of the polymer and its use for membrane protein purification, and also include details of typical challenges that may be encountered and possible ways to address those.

Key words Membrane proteins, Solubilization, Purification, SMALP, Polymer, Nanodisc, Detergent

1 Introduction

Membrane proteins carry out a wide range of important roles including movement of molecules in and out of cells and cellular communication. Consequently, they are important potential drug targets for many conditions. However, the study of their structure and function is more challenging than soluble proteins due to their location within a lipid bilayer. The first step in producing a purified protein sample is to solubilize or extract it from the membrane. Traditionally this has been achieved using detergents which disrupt the lipid bilayer and form a micelle around the regions of the

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protein that would usually be in contact with lipids [1]. Despite the many successes achieved using detergents they do pose several challenges: (1) detergents have a tendency to strip away the lipids that surround membrane proteins, which can be important for protein function; (2) there is a loss of lateral pressure provided by the lipid bilayer which the micelle does not replicate well, often leading to protein instability, and (3) there is a fine balance between efficient extraction and protein denaturation [2–5]. As an alternative approach to detergents for membrane protein extraction, the amphiphilic copolymer styrene maleic acid (SMA) was first reported in 2009 [6]. SMA inserts into the lipid bilayer and forms small discs of bilayer (approx. 10 nm diameter), with the polymer wrapped around the outside [7], termed SMA lipid particle (SMALPs). SMA has been shown to be effective at solubilizing membrane proteins from many different protein families and different expression systems [8–17]. Extracted proteins can be effectively purified by affinity chromatography, have demonstrated ligand binding and activity comparable to detergent solubilized and/or native membranes, and an increased thermostability [9–11, 14, 18–20]. In the 12 years since this approach was first reported, the use of SMA has increased dramatically, and it has recently been used for the high-resolution structure determination of numerous proteins [8, 21–27], as well as developing new assays and gaining novel insights into function [8, 12, 28, 29]. It offers researchers the opportunity to extract and purify their target protein without ever removing it from its lipid bilayer. Several studies have investigated the lipids that co-purify with a membrane protein within SMALPs, and this may be useful in the future for fully understanding the function of membrane proteins [8, 11, 13, 30–33]. As with any new method it does have some limitations, including a sensitivity to low pH or divalent cations, and interference caused from the free SMA polymer. Many researchers are investigating alternative polymers in an effort to overcome the current limitations, but while variations have proved to be beneficial in some aspects they usually have some limitations too [19, 34–37]. Here we present our standard protocol for extracting and purifying a membrane protein using SMA (Fig. 1), alongside notes explaining alternative options and troubleshooting ideas.

2 Materials

2.1 SMA Polymer Preparation

1. SMA2000 (styrene maleic anhydride) powder. This polymer has a 2:1 ratio of styrene:maleic anhydride and a molecular weight of 7.5 kDa (Cray Valley) (*see* **Notes 1** and **2**).
2. 1 M NaOH solution.
3. Concentrated HCl (SG 1.18).

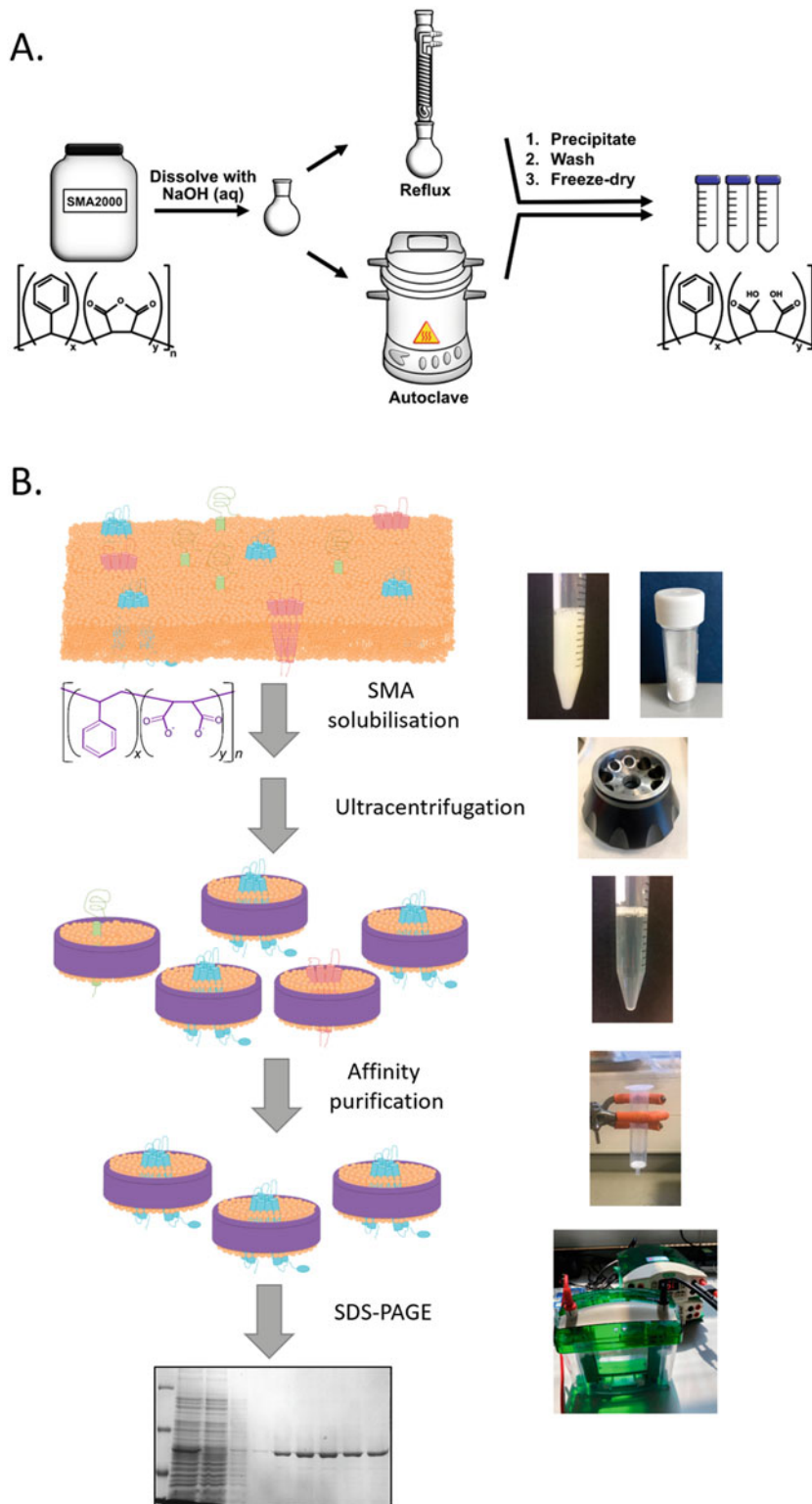


Fig. 1 Schematic of SMA extraction of membrane proteins. (a) The hydrolysis of styrene maleic anhydride to form styrene maleic acid. (b) The use of hydrolyzed SMA for the extraction and affinity purification of membrane proteins, (adapted from [49])

4. 0.6 M NaOH solution.
5. Distilled water.

2.2 Membrane Protein Extraction and Purification

1. Membrane preparations from cells expressing the target protein (*see Note 3*).
2. Buffer 1: 20 mM Tris-HCl, pH 8, 150 mM NaCl (*see Note 4*).
3. Ni²⁺-NTA (Ni²⁺-nitrilotriacetate) agarose resin (*see Note 5*).
4. An empty gravity flow column.
5. 2 M Imidazole.
6. Standard SDS-PAGE.
7. Western blotting equipment and reagents.

3 Methods

3.1 SMA Polymer Preparation

SMA2000 is supplied as a styrene-maleic anhydride copolymer. To be active for membrane solubilization it must be hydrolyzed to form styrene-maleic acid (Fig. 1a) (*see Note 1*). There are two methods which can be used to do this (A) reflux [38] or (B) autoclaving [39] (Fig. 2) (*see Note 6*).

1. Dissolve 25 g SMA2000 powder in 250 mL 1 M NaOH overnight at room temperature using a magnetic stirrer and a round bottomed flask.
2. *For the reflux method:* In a fume hood put the round bottomed flask containing the dissolved SMA2000 on a heating mantle and attach a condenser. Bring the solution to a boil, then reflux the polymer solution for 2–3 h. Allow to cool.
For the autoclave method: Transfer SMA 2000 dissolved in NaOH to a 1 L glass bottle. Autoclave for 16 min at 120 °C. Leave the solution on the bench to cool down (until the bottle is cool enough to touch comfortably). Repeat the same autoclave and cooling steps once or twice (*see Note 6*).
3. Divide the polymer solution between four 250 mL centrifuge tubes, and in a fume hood gradually add concentrated HCl to each one and mix well to precipitate the polymer. Approximately 1 mL HCl per 6 mL polymer solution is required. Then add 100 mL distilled water to each tube and mix well (*see Note 7*).
4. Centrifuge at $10,000 \times g$ for 10 min at room temperature and carefully pour off supernatant.

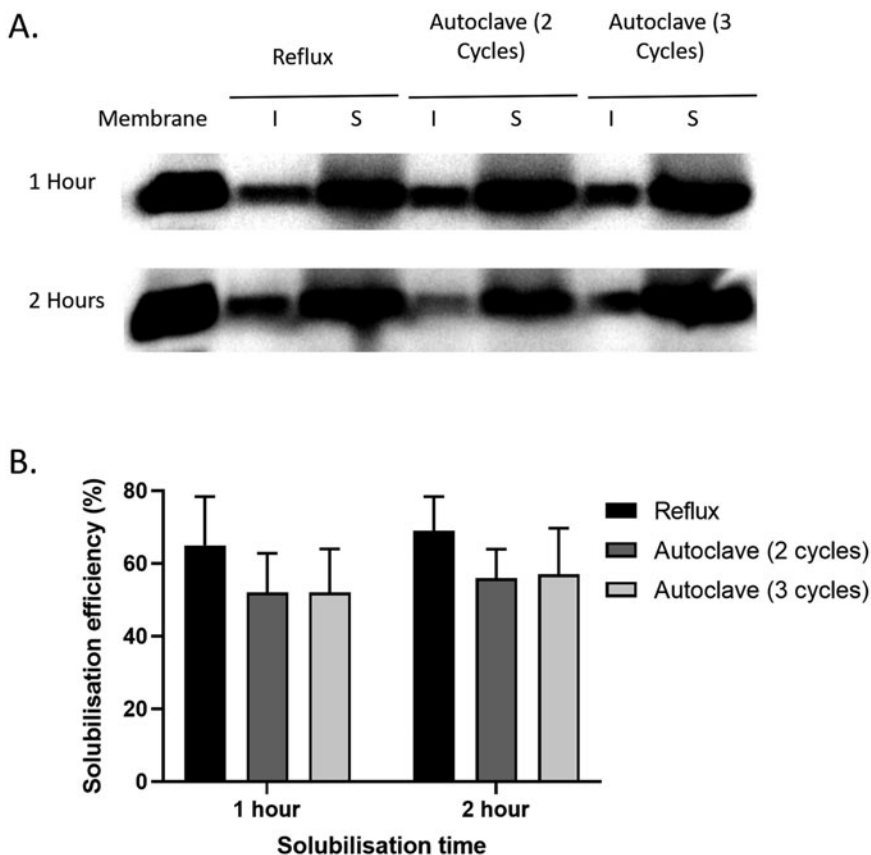


Fig. 2 Comparison of reflux or autoclave for SMA hydrolysis. **(a)** The tetraspanin CD81 was solubilized from *Pichia pastoris* yeast membranes using 2.5% (w/v) SMA hydrolyzed by reflux or autoclave methods, for either 1 h or 2 h at room temperature. Following ultracentrifugation, the soluble and insoluble fractions were analyzed by Western blot with an anti-His antibody. **(b)** Western blots as in **a** were analyzed by densitometry to determine solubilization efficiency. Data are mean \pm SD, $n = 3$. Data were analyzed by an ANOVA with a Tukey's post hoc test, there were no significant differences

5. Add 150 mL distilled water to each tube and resuspend the polymer by shaking.
6. Centrifuge at $10,000 \times g$ for 10 min at room temperature and carefully pour off supernatant.
7. Repeat **steps 5** and **6** four times.
8. Dissolve the polymer by adding 60 mL 0.6 M NaOH to each tube and either shake or stir for several hours.
9. Check the pH and adjust to pH 8 (*see Note 8*).
10. Freeze-dry the SMA polymer.
11. Store at room temperature.

3.2 Membrane Protein Extraction and Purification

1. Resuspend the membrane preparation in buffer 1 at a concentration of 60 mg/mL wet weight of membrane pellet (*see Note 9*).
2. Mix the membranes with an equal volume of 5%(w/v) hydrolyzed SMA to give final concentrations of 2.5%(w/v) SMA and 30 mg/mL wet pellet weight membranes (*see Notes 10–12*).
3. Incubate at room temperature for 1 h, shaking (*see Notes 13 and 14*).
4. Centrifuge at $100,000 \times g$ for 20–30 min at 4 °C and harvest the supernatant containing the “solubilized” protein.
5. Measure solubilization efficiency by running a Western blot of solubilized sample against protein retained in the insoluble fraction (to do this resuspend the pellet in buffer 1 supplemented with 2% (w/v) SDS) (*see Note 15*).
6. Mix the solubilized protein with Ni²⁺-NTA resin (prewashed in buffer 1), at a ratio of 100 µL resin/mL solubilized protein and mix gently overnight at 4 °C (*see Note 16*).
7. Pour into an empty gravity-flow column and wash the resin five times with 10 bed volumes (bv) of buffer 1 supplemented with 20 mM imidazole (*see Notes 17 and 18*).
8. Wash resin twice with 10 bv of buffer 1 supplemented with 40 mM imidazole.
9. Elute six times with ½ bv of buffer 1 supplemented with 200 mM imidazole (*see Note 18*).
10. Run samples from each step on SDS-PAGE and stain (use either silver stain or Coomassie, depending on the abundance of the protein).
11. Pool elution fractions containing the protein of interest.
12. Purified proteins can be concentrated using centrifugal concentrators and/or further purification can be undertaken by size exclusion chromatography (*see Note 19*).
13. Store sample short term at 4 °C, or long term at –80 °C.

4 Notes

1. We routinely use SMA2000, but alternative polymers with similar properties are available from Orbiscope (<https://www.orbiscope.com/>) [8, 18, 19] (Table 1). If supplied as a styrene maleic anhydride, follow the same method outlined here. However, Orbiscope now sell the polymers (including SMA25010P/SMALP 300 and SMA30010P/SMALP 200) as hydrolyzed liquids, which means the polymer hydrolysis

Table 1
Key features of the SMA and DIBMA polymers

Name	Supplier	Hydrophobic group	Hydrophilic group	Ratio S/DIB: MA	Mw (g/mol)
SMA2000	Cray Valley	Styrene	Maleic acid	2:1	7500
SMA25010P/SMALP 300	Orbisphere	Styrene	Maleic acid	3:1	10,000
SMA30010P/SMALP 200	Orbisphere	Styrene	Maleic acid	2.3:1	6500
DIBMA	BASF	Diisobutylene	Maleic acid	1:1	15,300 ^a 12,000 ^b

^aAs calculated experimentally in Oluwole et al. (2017) *Angew. Chem. Int. Ed.* 56, 1919–1924 [34]

^bAs provided by BASF data sheet

steps can be omitted and you can proceed directly to Subheading 3.2.

- An alternative polymer which is increasingly being used is DIBMA (diisobutylene maleic acid) (Table 1) which has an aliphatic group in place of styrene. It is commercially available from several suppliers (including Anatrace, Cube Biotech and Glycon Biochemicals), or can be prepared from Sokalan CP9 (BASF) [16, 34, 37].
- Membrane preparations are ideal, but whole cells can also be used [11, 40], which will require addition of DNase. Membranes from all common expression systems (bacteria, insect cells, yeast, and mammalian cells) have been shown to work effectively [8, 9]. However, for plant thylakoid membranes, modified polymers with a partial esterification of the maleic acid group, appear to work better [41].
- The composition of this buffer is generally quite flexible, common alternatives include phosphate buffers or HEPES, and sometimes the addition of glycerol can be beneficial [12, 13]. Also increasing the concentration of NaCl up to 500 mM can improve solubilization efficiency [42, 43]. However, divalent cations must be avoided and the pH is important; a low pH or the presence of divalent cations will cause precipitation of the polymer and prevent efficient extraction [42, 43]. A pH of 8 is ideal, but at slightly lower pH (pH 7–8) extraction is possible but is less efficient. This can be overcome by increasing the time of the solubilization (Fig. 3a), or the amount of polymer [43].
- This protocol details the method for polyhistidine-tagged proteins, but other affinity chromatography methods can be used

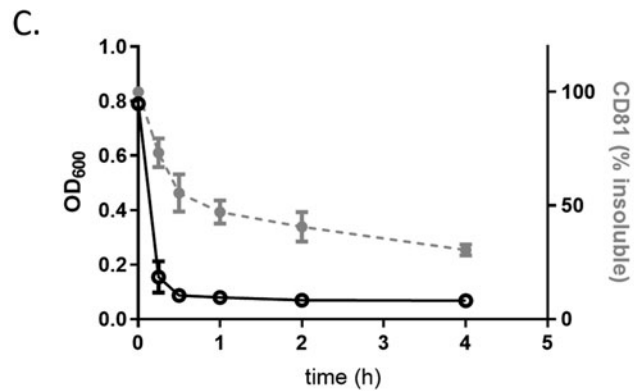
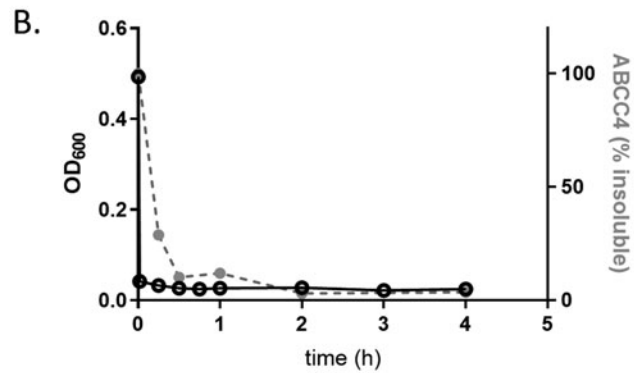
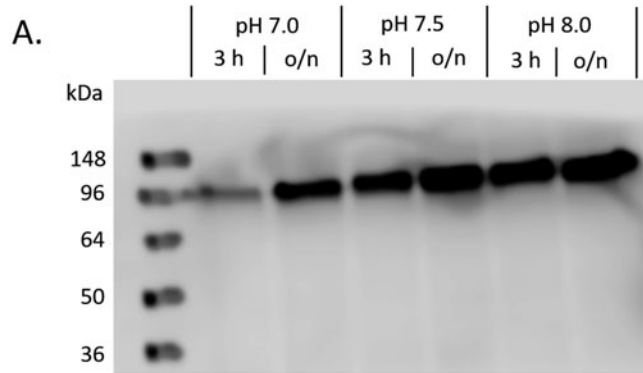


Fig. 3 Solubilization of target proteins using SMA. **(a)** Solubilization of KcsA1.3 ion channel from *E. coli* membranes with 2.5% (w/v) SMA in pH 7–8 buffers (150 mM KCl, 100 mM sodium phosphate) at room temperature for 3 h or overnight. Solubilized protein was harvested following ultracentrifugation and analyzed by Western blot with an anti-His antibody. **(b)** and **(c)** Solubilization of ABCC4 from Sf9 insect cell membranes **(b)** or CD81 from *Pichia pastoris* membranes **(c)** with 2.5% (w/v) SMA at room temperature was monitored by turbidity at 600 nm (open symbols) or protein-specific Western blots (closed symbols) over time (reproduced from Hardy et al 2019 SLAS Discovery and Ayub et al 2020 BBA Biomembranes [13, 47]). Although ABCC4 solubilizes rapidly and is not too dissimilar to changes in turbidity, the solubilization of CD81 is much slower than the changes in turbidity measured

[9, 40, 44–46]. Some studies have used cobalt resins in place of nickel [14, 29]; however for the proteins we have tested, cobalt has not worked well so it appears to be protein specific [3].

6. Kopf et al previously demonstrated the use of autoclaving for hydrolysis of SMA. They showed no differences in solubilization of lipids [39]. We have compared the solubilization of membrane proteins using polymer produced by both the reflux method and the autoclave method and found no difference in protein solubilization efficiency (Fig. 2). Furthermore, there was no difference with SMA subjected to two rounds of autoclaving compared to three rounds.
7. An alternative method to the acid precipitation and washing in Subheading 3.2, steps 3–9, is to dialyze the hydrolyzed polymer against water or buffer (use a molecular weight cutoff of 3.5 kDa or smaller), until the pH reaches 8, and then proceed with freeze-drying as in step 10. While this seems simpler, it can be time consuming, the dialysis tubing can be prone to bursting, and the polymer gets diluted meaning a larger volume has to be freeze-dried. We prefer the acid precipitation process.
8. Adjust the pH using drops of HCl or NaOH. Note that on addition of HCl localized precipitation of the polymer is likely to occur, but if you keep the solution stirring this will redissolve in time. Therefore, it may take a while to adjust the pH.
9. Many methods using detergents use a specific concentration of total membrane protein; however, we measure the wet pellet weight of the membrane rather than protein concentration, because this includes the lipids and it is the lipids the SMA interacts with. To measure wet pellet weight, weigh an empty ultracentrifuge tube, then add your membrane, spin at $100,000 \times g$ to pellet your membranes, carefully remove all of the supernatant, and weigh the tube again. The difference in weight from the empty tube gives you the wet pellet weight.
10. Although a concentration of 30 mg/mL wet pellet weight is the standard membrane concentration we use during solubilization, this can be varied. Especially in cases where you either have a very low expression of your target protein (Fig. 4a) or a very low solubilization efficiency (Fig. 4b), increasing the membrane concentration to 120–180 mg/mL during solubilization is effective, and enables you to essentially concentrate your purified protein. However, increasing too much over 180 mg/mL may not be beneficial, as higher concentrations of SMA may be required for solubilization, and increased levels of SMA can be problematic (*see* Notes 15 and 16).
11. An alternative to adding SMA solution is to directly add powder to the membrane suspension.

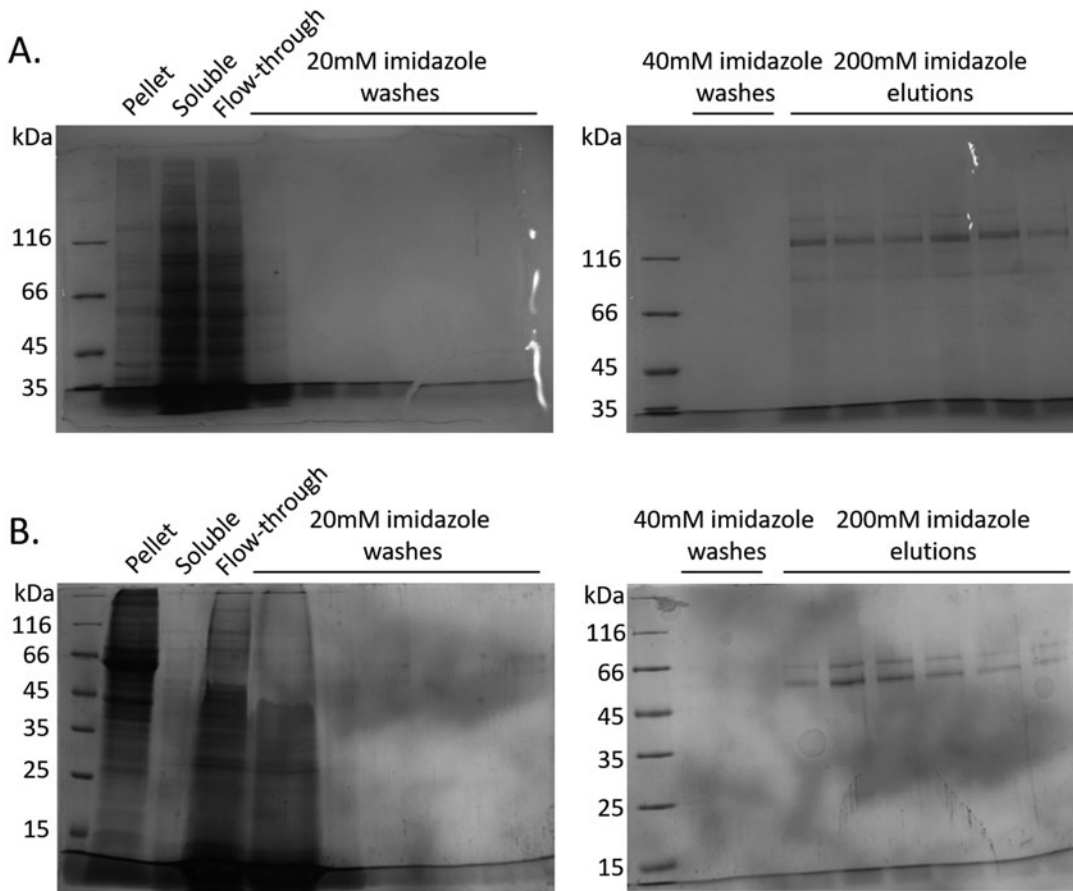


Fig. 4 More concentrated membranes can be solubilized when expression level or solubilization efficiency is low. (a) Purification of ABCC4 from Sf9 insect cell membranes. Expression level of ABCC4 is low, so membranes at a concentration of 120 mg/mL (wet pellet weight) were solubilized with 2.5% (w/v) SMA and then purified as in the standard protocol. (b) Purification of a fungal membrane protein from *Pichia pastoris* yeast membranes. The solubilization efficiency for this protein is low (approx. 10–15%), so membranes at a concentration of 180 mg/mL (wet pellet weight) were solubilized with 2.5% (w/v) SMA and then purified as in the standard protocol. Samples of all steps were analyzed by SDS-PAGE and stained with InstantBlue. In both cases the solubilization efficiency was unaltered by using more concentrated membrane preparations, but volumes of all subsequent wash steps were decreased, and the concentration of eluted protein was increased

12. Although we use 2.5%(w/v) SMA as a standard, successful solubilization can be achieved with lower concentrations [43, 44]. In fact, using a lower concentration of SMA may be beneficial, as free excess SMA is known to interfere with binding to affinity resins or antibodies [13, 44] (see Notes 15 and 16).
13. Although the solution will appear noticeably clearer almost instantly, we have found that it often takes longer to achieve a good extraction of your membrane protein of interest, and highly recommend you monitor the solubilization of your

target protein specifically (by Western blotting for example) rather than the turbidity of the suspension (Fig. 3b, c). While 1 h at room temperature appears sufficient for most proteins we have tested [9], some proteins are quicker (Fig. 3b) [47] and some may require longer (Fig. 3c) [10, 13, 14].

14. The temperature can be important because of the phase-transition of the lipids. Since the SMA interacts with the lipids rather than the protein, whether the temperature is above the phase-transition temperature of the lipids can make a difference [43, 48]. While this may worry many membrane protein researchers who are used to maintaining everything at 4 °C during detergent solubilization, we have generally not found this to be a problem, presumably because the SMALP maintains stability of membrane proteins much better than detergents [9, 10, 18, 19, 47]. In fact, for proteins that prove difficult to solubilize, increasing the temperature to 37 °C for solubilization has been done [9, 10], as has sonication of the membrane to aid disruption [46]. However, it is possible to solubilize proteins at 4 °C if you prefer, it may just take longer at lower temperatures [43].
15. Sometimes the excess SMA in the solubilized sample can interfere with antibody binding in the Western blot [13, 37]. For this reason, we often measure the solubilization efficiency by comparing what remains insoluble in the pellet sample with the total membrane or the pellet obtained after mixing membranes with buffer only.
16. Binding of polyhistidine-tagged SMALP-encapsulated proteins to Ni²⁺-NTA resin can be inefficient (as can binding to other affinity resins). For this reason, we bind overnight in a batch binding process to try to maximize binding, and do not include any imidazole in the binding buffer. We have found that a dodeca-histidine tag is much better than a hexa-histidine tag for efficient binding. Similarly, binding is better if the target membrane protein is oligomeric (as each subunit has a tag), than for monomeric proteins. Sometimes increasing the concentration of NaCl in the buffer, decreasing the concentration of SMA used for solubilization, or the addition of 200 mM arginine to the buffer can help binding. We have also found that resins from different suppliers can affect binding efficiency in a protein dependent manner, for example HisPur Ni²⁺-NTA (ThermoFisher) is better for some proteins, whereas Ni²⁺-NTA agarose (Qiagen) is better for others. Possible reasons might include interactions between the polyhistidine-tag and the polymer or steric hindrance from the polymer. However, we have found that column spoiling by excess free SMA is the major issue. One way to overcome this is to dialyze your solubilized protein sample against buffer to remove the excess

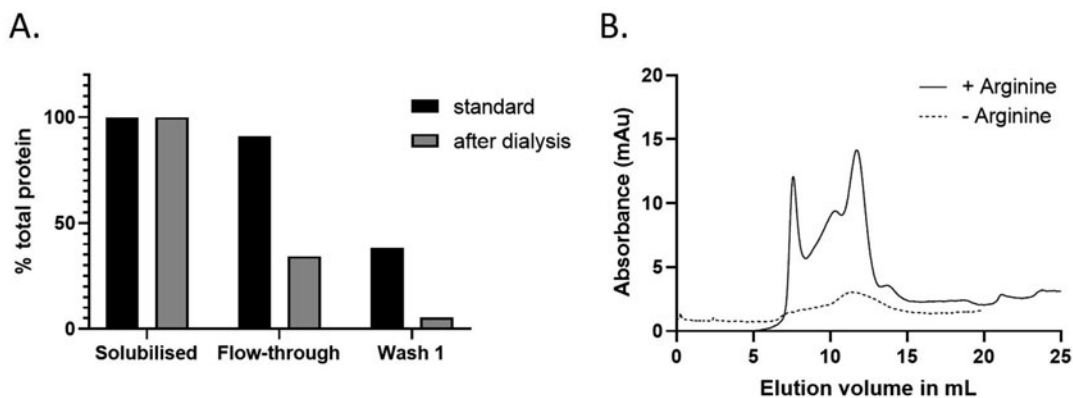


Fig. 5 Interactions of SMALP-encapsulated proteins with chromatography resins. (a) Strep-tagged ABCG2 solubilized from HEK cells with 2.5% SMA does not bind to the Strep-Tactin affinity resin (and is almost all in the flow-through and wash 1). However, following dialysis of the soluble sample against 100× the volume of purification buffer, overnight at 4 °C, with one change of buffer, to remove excess SMA, the ABCG2 binds effectively to the resin and can be purified using the standard protocol for Strep-Tactin XT (IBA Life Sciences). Samples run on Western blot with an anti-ABCG2 (Bxp-21) primary antibody, and analyzed by densitometry using Image J. (b) Size exclusion chromatography traces (Superdex 200 10/30 column) of affinity purified KcsA1.3 channel within SMALPs in the presence or absence of 200 mM arginine

SMA polymer prior to binding to the affinity resin (Fig. 5a), removal of SMA using centrifugal concentrators [44] or size exclusion chromatography [12].

17. One of the biggest advantages of this method is that once formed the SMALPs are stable, and, unlike with detergent, it is not necessary to supplement the purification buffers or assay buffers with further SMA.
18. The decreased affinity for Ni-NTA resin means that elution often occurs at lower imidazole concentrations. So, we only use low concentrations of imidazole for the washing steps and would recommend trying a gradient of different imidazole concentrations initially to see where your protein elutes.
19. For size exclusion chromatography some proteins work fine [9, 10, 13, 38, 44], but others stick to the column medium. In these cases, supplementing the equilibration buffer with 200 mM arginine can help (Fig. 5b) [16].

Acknowledgements

We are grateful for funding from the European Union's Horizon 2020 research and innovation programme under Marie Skłodowska-Curie grant agreement No. 847,419 (MemTrain) as well as the ERACoBioTech MeMBrane project and BBSRC (BB/R02152X/1) to A.D.G, A.J.R and R.M.B. Also, BBSRC

CASE studentship (BB/L015846/1) and BBSRC grant (BB/S008160/1) to A.J.R., and BBSRC grant (BB/S001611/1) to I.D.K.

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