



A comparison of SMA (styrene maleic acid) and DIBMA (di-isobutylene maleic acid) for membrane protein purification

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ARTICLE INFO

Keywords:

SMALP
DIBMALP
Membrane protein
GPCR
ABC transporter

ABSTRACT

The use of styrene maleic acid co-polymer (SMA) for membrane protein extraction and purification has grown in recent years. SMA inserts in the membrane and assembles into small discs of bilayer encircled by polymer, termed SMA lipid particles (SMALPs). This allows purification of membrane proteins whilst maintaining their lipid bilayer environment. SMALPs offer several improvements over conventional detergent approaches, however there are limitations, most notably a sensitivity to low pH and divalent cations. Recently it was shown that the aliphatic diisobutylene-maleic acid (DIBMA) copolymer, was also able to directly solubilise membranes forming DIBMALPs (DIBMA lipid particles), and that this polymer overcame some of the limitations of SMA. In this study the ability of DIBMA to solubilise and purify functional membrane proteins has been compared to SMA. It was found that DIBMA is able to solubilise several different membrane proteins from different expression systems, however for some proteins it gives a lower yield and lower degree of purity than SMA. DIBMA extracted G protein-coupled receptors retain ligand- and G protein-binding. DIBMALPs are larger than SMALPs and display a decreased sensitivity to magnesium. However the stability of DIBMALPs appears to be lower than SMALPs. The lower purity and lower stability are likely linked to the larger size of the DIBMALP particle. However, this also offers a potentially less rigid lipid environment which may be more amenable to protein dynamics. Therefore the optimal choice of polymer will depend on which features of a protein are to be investigated.

1. Introduction

The study of membrane proteins is inherently difficult due to the environment in which they are found. The membrane is too complex to easily study the function or structure of a single protein. To remove a protein from the hydrophobic environment of the lipid bilayer typically requires the use of detergents that can disrupt the bilayer and form micelles around the membrane proteins making them soluble in aqueous solution. However these detergents can often render the proteins unstable and cause a loss in structure and function [1]. This denaturation is likely to be a result of the detergent environment surrounding the protein being too simplistic. Another downside of detergents is that they strip away the native lipid environment of the membrane protein, and the interaction between specific lipids and

proteins can have a big effect on function [2,3].

Over the years a variety of different extraction and/or reconstitution systems have been designed including amphipols, bicelles and membrane scaffold protein nanodiscs, to try to better mimic the natural environment [4–6]. One recent, very promising development has been the use of the amphipathic polymer styrene maleic acid (SMA), which spontaneously inserts into the lipid bilayer without requiring any additional processing. SMA has the ability to form SMALP (SMA lipid particles) nanodiscs which are approximately 9–11 nm in size and contain the membrane protein of interest as well as the native lipid environment surrounding it, with the polymer wrapped around the outside [7,8]. The majority of SMALP encapsulated proteins can be easily purified, are folded and functional, and display increased stability in comparison to detergents [9–15]. In addition, SMALPs once

Abbreviations: ABC, ATP Binding Cassette; A_{2a}R, adenosine A_{2a} receptor; CGRP, calcitonin gene related peptide; CLR, calcitonin receptor-like receptor; DIBMA, diisobutylene maleic acid; DLS, dynamic light scattering; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; FTIR, Fourier transform infrared; GPCR, G protein-coupled receptor; GppNHp, guanosine-5'-[β,γ]-imido]triphosphate; Mn, number average molecular weight; Mw, average molecular weight; PDI, polydispersity index; RAMP, receptor activity-modifying protein; SMA, styrene maleic acid; ZM241385, 4-(2-[7-amino-2-(2-furyl)(1,2,4)triazolo(2,3-a)(1,3,5)triazin-5-yl amino]ethyl)phenol

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<https://doi.org/10.1016/j.bbamem.2020.183281>

Received 15 January 2020; Received in revised form 6 March 2020; Accepted 19 March 2020

Available online 21 March 2020

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formed are stable, and it is not necessary to supplement all buffers as with conventional detergents, making them technically easier to work with. SMALP purified proteins have successfully been used for both functional [9,12,13] and structural [16–19] studies.

However SMA polymers do not solve all challenges associated with membrane proteins. Some proteins do not solubilise as easily as others, and challenges have been reported relating to binding to affinity purification resins [20,21]. SMALPs also have certain limitations that hinder the use of various biophysical and biochemical techniques to study the extracted protein of interest. They are sensitive to low pH and also to divalent cations which are important for the function of many proteins [10,22]. They absorb light at 260 nm making them incompatible with far-UV optical spectroscopy, possibly due to the presence of aromatic styrene groups in the polymer structure [22,23]. Some of these limitations have recently been addressed by the aliphatic diisobutylene-maleic acid (DIBMA) copolymer, which like SMA has shown some capabilities to directly solubilise membrane proteins and form DIBMALPs (DIBMA lipid particles) nanodiscs [23].

In this study we systematically compare DIBMA with the established SMA2000 polymer for membrane protein solubilisation, purification, function and stability. The membrane proteins used in this study include the membrane tether protein ZipA and the ATP Binding Cassette (ABC) transporter BmrA, both expressed in *Escherichia coli*. In addition the G protein-coupled receptors (GPCRs) adenosine A_{2a} receptor (A_{2a}R) and the calcitonin gene related peptide (CGRP) receptor, expressed in *Pichia Pastoris* and Cos7 cells respectively, were studied.

2. Materials & methods

2.1. Polymer preparation

SMA2000 polymer was a kind gift from Cray Valley, and was supplied as styrene maleic anhydride copolymer. This was hydrolysed to styrene maleic acid copolymer using NaOH as described previously [22,24]. A solution of 10% (w/v) styrene maleic anhydride powder was stirred and dissolved in 1 M NaOH overnight. The solution was then refluxed for 2 h and allowed to cool to room temperature. The resulting polymer solution was then precipitated with the addition of excess concentrated hydrochloric acid, washed vigorously with distilled water and centrifuged at 10,000g for 10 min at room temperature. The wash and centrifugation steps were repeated 4 times and a pH of 3–4 was obtained. The resulting polymer was dissolved in 0.6 M NaOH to give a pH of 8 and freeze dried. The resultant SMA powder could be stored at room temperature for extended periods of time.

DIBMA polymer was a kind gift from BASF and was supplied as Sokolan CP9, an impure sodium salt, 25% (w/v) solution of polymer with a pH of approx. 11, and appeared as a thick viscous liquid. Therefore dialysis was used to prepare DIBMA [23], to remove smaller molecular weight contaminants and to decrease the pH. Sokolan CP9 (85 ml) was placed inside snake skin dialysis tubing with a molecular weight cut off of 3.5 kDa, and dialysed against 1 l buffer 2 (20 mM Tris pH 8, 150 mM NaCl) for 12–15 h at 4 °C, stirring continuously. Following dialysis the polymer had been diluted approximately 3-fold, and the pH was decreased to pH 8–9. This was harvested and freeze dried for long term storage.

2.2. Spectroscopic characterisation of DIBMA

Prepared DIBMA polymer was analysed using a bench attenuated total reflectance (ATR) FTIR machine. The ATR sampling accessory crystal was cleaned with methanol and dried completely. A background scan was conducted in order to determine how much of the original IR intensity in percent transmittance was left after passing through the sample. A small quantity of freeze-dried polymer was placed onto the crystal and a force was applied using the ATR force arm to ensure good contact between the sample and the crystal surface. The samples were

then scanned to acquire data with a scan range of 4000–650 cm⁻¹. The ATR crystal was cleaned with methanol between each sample analysis and a contamination check was conducted to ensure the crystal was properly cleaned.

DIBMA was also characterized by both ¹H and ¹³C NMR on a Bruker Avance-300 spectrometer at room temperature overnight and recorded using a 5 mm normal dual detection probe. 60 µl of DIBMA sample was dissolved in 0.6–0.8 ml of deuterated water D₂O. The ¹H NMR spectra were recorded at 300.13 MHz using a high-resolution dual (¹H ¹³C) gradients probe. Spectra were recorded using the zg30 pulse program with 16 or 128 scans and referenced to the DMSO peak at 2.50 ppm. ¹³C NMR spectra were obtained at 75 MHz for carbon. The pendant pulse program was used with waltz16 decoupling during acquisition with 6 k scans and phased for CH₃/CH positive and quaternary carbons and CH₂ negative with spectra referenced to DMSO peak at 40.0 ppm.

2.3. BmrA & ZipA expression & membrane preparation

Overnight (5 ml) cultures containing LB supplemented with 100 µg/ml ampicillin (LB-amp), were started from glycerol stocks of *E. coli* bacteria transformed with either the pET101-ZipA or pET23b-BmrA plasmid, and were used to inoculate 1 l flasks of LB-amp incubated at 37 °C at a speed of 200 rpm. When OD₆₀₀ reached 0.6, 0.5 mM IPTG was added to induce protein synthesis and the culture incubated overnight at 25 °C, 200 rpm.

The bacterial culture was centrifuged for 10 min at 6000g, 4 °C, and the *E. coli* cell pellets resuspended in buffer 1 (50 mM Tris pH 7.4, 250 mM sucrose, 0.25 mM CaCl₂, 1 µM pepstatin, 1.3 µM benzamide and 1.8 µM leupeptin). The cells were placed on ice and disrupted using sonication (6 x 30s with 30s intervals, at 70% power). Debris and unbroken cells were removed via centrifugation for 20 min at 750g, 4 °C. The cell membranes were further harvested by ultra-centrifugation at 100,000g for 20 min at 4 °C. The membranes were resuspended in buffer 2 (20 mM Tris pH 8, 150 mM NaCl) and aliquoted at a final concentration of 60 mg/ml wet membrane weight and stored at –80 °C.

2.4. A_{2a}R expression & membrane preparation

A_{2a}R was expressed in *Pichia pastoris* as previously described [13]. 50 g of frozen *Pichia pastoris* cells were suspended in a 1:1 ratio of ice-cold buffer 3 (5% glycerol, 2 mM EDTA, 50 mM Tris-HCl, pH 7.4, 0.2% protease inhibitor cocktail tablets), the solution was poured into an Avestin-C3 cell disrupter. The cells were fragmented by 2 or more cycles through the chilled cell with homogenizing pressures of ~30,000 psi. Any cell debris and unbroken cells were removed from the membrane suspension by centrifugation at 10,000g for 30 min. The membranes were collected by ultracentrifugation (100,000g in a Beckman type Ti 70 rotor for 45 min). The membrane pellets were collected and resuspended in buffer 2 (20 mM Tris and 150 mM NaCl at pH 8) and aliquoted at a final concentration of 60 mg/ml wet membrane weight and rapidly stored at –80 °C.

2.5. Solubilisation & affinity purification

Solubilisation and purification was carried out as described previously [10,24]. Membrane aliquots containing the protein of interest were mixed with SMA or DIBMA and buffer 2 to give final concentrations of 30 mg/ml membrane (wet pellet weight) and 2.5% (w/v) polymer. This was incubated for 1 h at room temperature with gentle shaking. Samples were then ultracentrifuged (100,000g, 20 min, 4 °C), the supernatant containing the solubilised protein was retained, whilst the pellet containing insoluble material was resuspended in buffer 2 supplemented with 2% (w/v) SDS. Samples of the soluble and insoluble material were analysed by western blotting, using an anti-his primary antibody (R&D Systems) and an anti-mouse HRP secondary antibody (Cell Signalling). Blots were developed using chemiluminescence

(Pierce Supersignal) and imaged using a LI-COR C-digit scanner.

Solubilised protein was combined with HisPur Ni²⁺-NTA resin (ThermoFisher) at 100 µl packed resin bed volume (BV) per ml of solubilised protein, overnight with gentle rotation at 4 °C. The sample mix was transferred to a gravity flow column and the flow-through containing any unbound material was collected. The resin was washed five times with 10 BVs of buffer 2 supplemented with 20 mM imidazole, twice with 10 BVs of buffer 2 containing 40 mM imidazole and once with 1 BV of buffer 2 supplemented with 60 mM imidazole. Proteins were eluted with buffer 2 supplemented with 200 mM imidazole, and six fractions of half a BV were collected. The flow through, wash and elution fractions were analysed on a 10% SDS-PAGE and stained with InstantBlue (Expedeon). The elution fractions containing the protein of interest were combined and stored at 4 °C. Proteins were quantified using densitometric analysis (Image Studio Lite, LI-COR) of SDS-PAGE with BSA as a standard [25].

2.6. Magnesium sensitivity

Purified protein (100 µl) was mixed with various concentrations of MgCl₂ ranging from 0 to 10 mM. Samples were centrifuged (100,000g, 20 min, 4 °C), the supernatant containing soluble protein was harvested and the pellet containing insoluble material was resuspended in the same volume of buffer 2. Samples of both soluble and insoluble proteins were run on 10% SDS-PAGE, stained with InstantBlue and the percentage of protein remaining in solution was calculated by densitometry (Image Studio Lite).

2.7. A_{2A}R radioligand binding assays

ZM214385 at varying concentrations (0.001 µM – 1 mM) was prepared in DMSO. A blank control comprising of 100% DMSO was also used to identify maximum A_{2A}R binding with radioligand. The radioligand ³H-ZM214385 was prepared at a concentration of 100 nM. Adenosine deaminase was added to the A_{2A}R sample preceding the radio ligand reaction in order to remove adenosine from the A_{2A}R binding site.

Varying concentrations of ZM214385 and DMSO in a volume of 5 µl were pipetted into assay tubes along with 5 µl of ³H-ZM214385. 500 µl of SMA/DIBMA solubilised A_{2A}R membranes were added to each tube, vortexed to mix well, and incubated at 30 °C for half an hour to allow ligands to bind with A_{2A}R. P-30 desalting columns with a molecular weight cut off of 30,000Da were used to separate the unbound ligands from the protein. 1 ml of scintillant was then added to each assay tube and the solution was mixed. Each assay tube was then analysed in a scintillation counter.

2.8. Dynamic light scattering (DLS)

DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) lipids were dissolved in 2:1 chloroform: methanol and dried down under nitrogen. The lipid film was resuspended in buffer 2 to form a 2% (w/v) suspension. The suspension was mixed at a 1:1 ratio with 2.5% (w/v) polymer, to form lipid-only SMALPs/DIBMALPs. 100 µl of lipid-only SMALPs, lipid-only DIBMALPs or 2% (w/v) DMPC lipid suspension was added to 1900 µl of buffer 2. Dynamic light scattering (DLS) data were recorded using a Brookhaven NanoBrook 90plus Zeta instrument (640 nm) and 1.0 cm path length disposable cuvette (Brand BMBH, Germany) Measurements were taken at a temperature of 25 °C with 30 s equilibration time. Automated instrument parameters were used. Each measurement was repeated at least 6 times.

2.9. Protein stability

Stability of solubilised and purified BmrA in either SMA 2000 or DIBMA was measured over time for a period of approximately 3 weeks.

The purified protein sample was stored at 4 °C, every few days 100 µl of the sample was centrifuged at 16,000g for 10 min in a bench top Eppendorf centrifuge at room temperature. 20 µl of the supernatant was mixed with Laemmli Sample Buffer and separated using 10% SDS-PAGE. The intensity of each band was analysed via densitometry.

2.10. Radiolabelling & solubilisation of CGRP receptors

Cos7 cells were co-transfected in 10 cm dishes with haemagglutinin-tagged human calcitonin receptor-like receptor (CLR) and myc-tagged receptor activity-modifying protein 1 (RAMP1) [26]. Membranes were prepared as previously described [26]. For labelling, they were incubated with 0.5 nM of human ¹²⁵I-CGRP (Perkin Elmer) for 30 min at room temperature (with or without 1 µM unlabelled CGRP to define non-specific binding) in 2 mM MgCl₂, 20 mM Hepes pH 7.4. Membranes were pelleted and resuspended at 1 mg/ml in SMA or DIBMA copolymer (2.5% w/v final concentration in 50 mM NaCl, 12.5% glycerol, 500 mM Tris, pH 8) for 1 h at 25 °C. The undissolved membranes were pelleted and radioligand binding was performed on the CGRP receptor in the supernatant with spin columns as for the A_{2A}R. In some experiments, the soluble extract was treated with 100 µM GppNHp and left for 15 min at room temperature and the binding compared to untreated soluble extract.

2.11. Data analysis

Statistical analysis was undertaken using GraphPad Prism. A *t*-test was used for comparison of two data sets, and an ANOVA with a Bonferroni post-hoc test was used for multiple comparisons. *p* < 0.05 was considered significant.

Divalent cation sensitivity data were fitted with a normalised dose-response curve with variable slope. Radioligand binding assay data were plotted against the log unlabelled ZM214385 concentration fitted with an inhibition dose-response curve to determine IC₅₀ using Graphpad Prism with a three parameter fit.

3. Results

3.1. DIBMA polymer

The DIBMA polymer does not have an aromatic styrene group in its structure instead it has an aliphatic side chain (Fig. 1). It has an average molecular weight of 15 kDa [23], which is larger than that of the commercially available SMA polymers successfully used to date (7.5–10 kDa) [10]. It also has a 1:1 ratio of diisobutylene to maleic acid, compared with ratios of 2:1 or 3:1 styrene:maleic acid for SMA (Table 1).

The initial step was to purify DIBMA from the commercially obtained Sokolan CP9, which was supplied by BASF as a 25% (w/v) sodium salt aqueous solution with various impurities. As suggested by Oluwole *et al* [23] the method used to separate the polymer from its impurities was dialysis with a molecular weight cut off (MWCO) of 3.5 kDa. The method used for preparing DIBMA was very important, and even small variations in the protocol led to a large degree of variation in the effectiveness of the polymer for membrane protein solubilisation. We tested dialysis against water or buffer, with different ratios between the inner and outer volumes and different times. It was established that dialysis against buffer rather than water, and the volumetric ratio of DIBMA to buffer were most important factors and the conditions described in the Methods section produced the most reproducible results, so this was used for all future studies. An FTIR spectroscopy trace of this prepared polymer is shown in Supplementary Fig. 1A. which shows the characteristic C = O stretch of the carboxylic acid present in the maleic acid at 1738.61 cm⁻¹ as well as the peaks at 2900 cm⁻¹ which are indicative of C – H stretching vibrations (CH₃ and CH₂) alkane groups present in the diisobutylene, these peaks are further

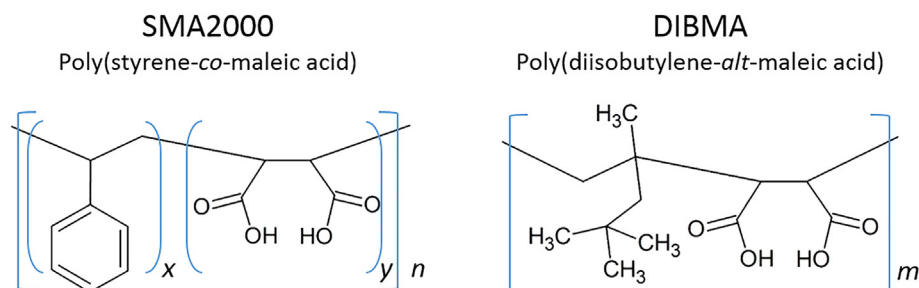


Fig. 1. Structures of the polymers. SMA2000 is a co-polymer of styrene and maleic acid at a ratio of 2:1. DIBMA is an alternating polymer of diisobutylene and maleic acid.

Table 1

Properties of the polymers according to the manufacturers. The average ratio of styrene to maleic acid within the polymers. Mw, the average molecular weight of the polymers. The number average molecular weight (Mn), which is the total weight of the polymer molecules divided by the number of molecules. The polydispersity index (PDI), which is equal to Mw/Mn and is a measure of the distribution of the molecular weights.

Name	Supplier	Ratio S/DIB:MA	Mn (g/mol)	Mw (g/mol)	PDI
SMA2000	Cray Valley	2:1	3000	7500	2.5
DIBMA	BASF	1:1	8400 ^a	15,300 ^a 12,000 ^b	1.8 ^a

^a As calculated experimentally in Oluwole et al. (2017) *Angew. Chem. Int. Ed.* 56, 1919–1924 [23].

^b As provide by BASF data sheet.

supported by the presence of strong peaks in the fingerprint region of the spectrum between the range of 1200–1300 cm^{-1} also indicating the presence of C – H alkane groups in the polymer.

NMR characterisation of the polymer was also undertaken. The ^1H NMR (Supplementary Fig. 1B) shows a characteristic peak at $\delta = 3.0$ ppm due to the CH groups and at $\delta = 3.7$ ppm due to the OH groups present in maleic acid. The CH_3 and CH_2 protons within the 2,4,4-trimethylpent-1-ene monomer seem to be present between $\delta = 0.9$ –1.1 and $\delta = 1.2$ –1.8 ppm respectively. The sharp peak observed at $\delta = 2.0$ ppm is indicative of the presence of impurities or a solvent, possibly acetone. The ^{13}C NMR spectra (Supplementary Fig. 1C) contain several peaks at the lower chemical shift of the spectra indicative of the presence of aliphatic groups. The peaks that are identified in order of shielding (lower chemical shift) are $\delta = 29$ –33 ppm that relate to the primary and secondary alkyl groups. A weaker signal observed at $\delta = 41$ ppm is indicative of quaternary carbons that are present in the structure where the central C atom is bonded to four other R groups. Carboxylic acid peaks for the maleic acid are observed at a chemical shift of $\delta = 180$ –190 ppm. Sharp peaks observed around $\delta = 55$ –63 ppm in the C-NMR structure of DIBMA indicate the presence of residual solvents.

Another challenge associated with working with DIBMA was that it caused substantial streaking on SDS-PAGE, obscuring visualisation of the sample (Supplementary Fig. 2A), as reported previously [23]. This also affected migration and detection of solubilised proteins via western blots as shown in Supplementary Fig. 2B. Therefore for solubilisation efficiency measurements it was decided to measure the intensity of the insoluble band (which did not contain polymer) and compare it to that of a negative control. Following purification of proteins, where the bulk of the excess polymer was removed this streaking effect was no longer an issue.

3.2. Protein solubilisation & purification

Both SMA2000 and DIBMA were tested for their ability to solubilise the membrane proteins ZipA and BmrA (Fig. 2). ZipA is a membrane

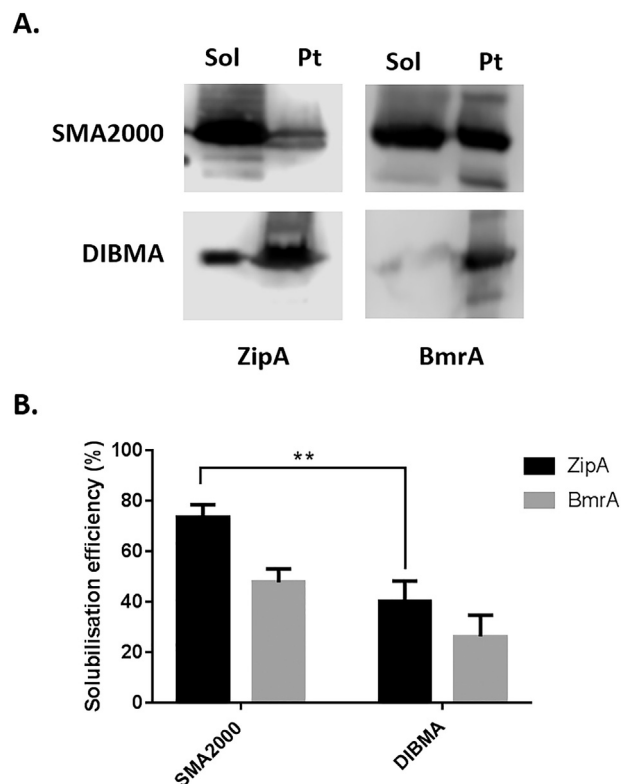


Fig. 2. Solubilisation of membrane proteins with DIBMA is less efficient than with SMA2000. Membranes (30 mg/ml wet pellet weight) from *E. coli* over-expressing either ZipA or BmrA were solubilised with 2.5%(w/v) polymer for 1 h at room temperature, then subjected to ultracentrifugation. The supernatant containing solubilised protein (Sol) was harvested and the insoluble material (Pt) resuspended in an equivalent volume of buffer supplemented with 2% (w/v) SDS, and samples of each analysed by western blotting. A; Representative western blots. Primary antibody is an anti-his antibody, followed by a secondary anti-mouse HRP, and detected by chemiluminescence. B; Western blots as shown in A were analysed by densitometry to determine solubilisation efficiency. Due to issues with free excess polymer obscuring bands in the soluble fractions, the amount of protein remaining insoluble was always compared to that of a negative (buffer only) control. Data are mean \pm sem, $n \geq 6$. Data were analysed by an ANOVA with a Bonferroni posthoc test, $**p < 0.01$.

tether with a single transmembrane helix, whereas BmrA is an ABC transporter, which is a homodimer with a total of 12 transmembrane helices. Both polymers were able to solubilise both membrane proteins, although SMA2000 had a significantly higher solubilisation efficiency for ZipA than DIBMA, with only ~25% of the protein remaining insoluble in the pellet for SMA2000, compared to ~50% for DIBMA.

The solubilised proteins were then purified using affinity chromatography. Both proteins could be purified with both polymers (Fig. 3). However it should be noted that purification of ZipA with DIBMA

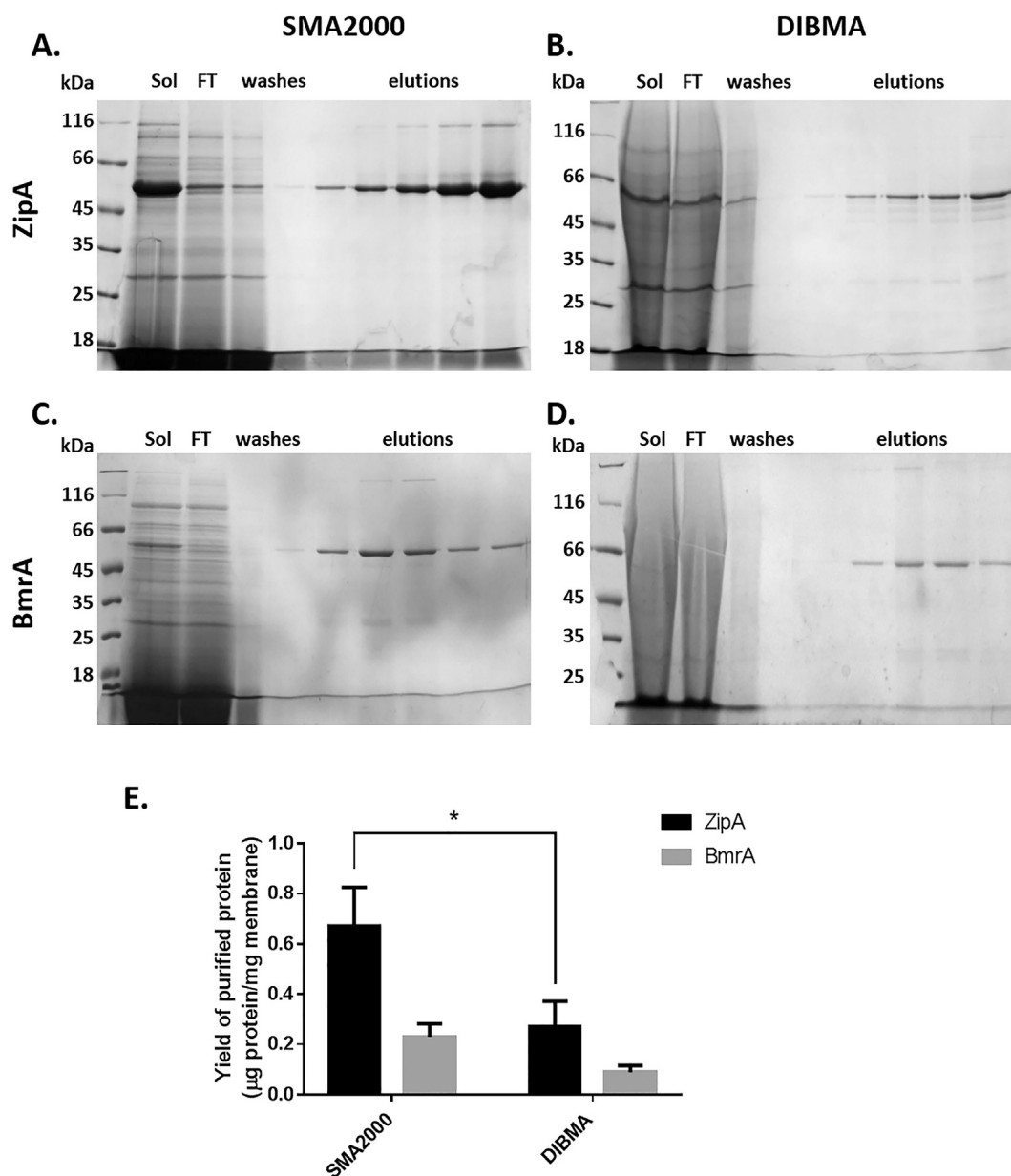


Fig. 3. Lower yields of purified protein are obtained with DIBMA compared to SMA2000. Solubilised protein was purified by Ni^{2+} -NTA affinity chromatography. Fractions of the purification were analysed by SDS-PAGE. A-D; Representative SDS-PAGE showing purification of ZipA with SMA2000 (A), ZipA with DIBMA (B), BmrA with SMA2000 (C) or BmrA with DIBMA (D). E; Elution fractions from purifications as in A-D were pooled together and quantified using an SDS-PAGE densitometric method with BSA as a standard, in order to determine the protein yield. Data are mean \pm sem, $n \geq 6$. Data were analysed by ANOVA with a Bonferroni post-hoc test, $*p < 0.05$.

(Fig. 3B) gave much less intense bands of ZipA, and more contaminating proteins were present than was seen with SMA2000 (Fig. 3A). Similarly for BmrA, DIBMA appeared to give a lower yield of protein than SMA2000 (Fig. 3C & D). When average yields were compared (Fig. 3E) it was found that DIBMA gave a significantly lower yield of purified ZipA than SMA 2000.

3.3. Characterisation of DIBMALPs

To characterize the size of the polymer lipid particles formed from SMA2000 or DIBMA, DLS was carried out. As can be seen in Fig. 4A, SMALPs are approximately 10 nm in diameter, whereas DIBMALPs are approximately 25 nm in diameter.

It was previously reported that DIBMALPs were less sensitive than SMALPs to divalent cations such as magnesium [23]. So next we tested

if this was still the case when proteins were encapsulated within the lipid particles. As shown in Fig. 4B when the concentration of magnesium exceeds 4 mM, protein within SMALPs loses solubility, with all the protein insoluble at 8-10 mM MgCl_2 . However for the DIBMALPs this was not the case and at 10 mM MgCl_2 there was still > 80% of the protein remaining soluble.

3.4. Solubilisation of GPCRs

Having established that DIBMA could be used to solubilise and purify two different membrane proteins from *E. coli*, next it was investigated whether DIBMA could effectively extract functional GPCRs. First, solubilisation of the adenosine A_{2a} receptor ($A_{2a}R$) from *Pichia pastoris* yeast membranes was tested. Table 2 shows the pK_i values derived from radioligand binding assays on both SMA2000 and DIBMA

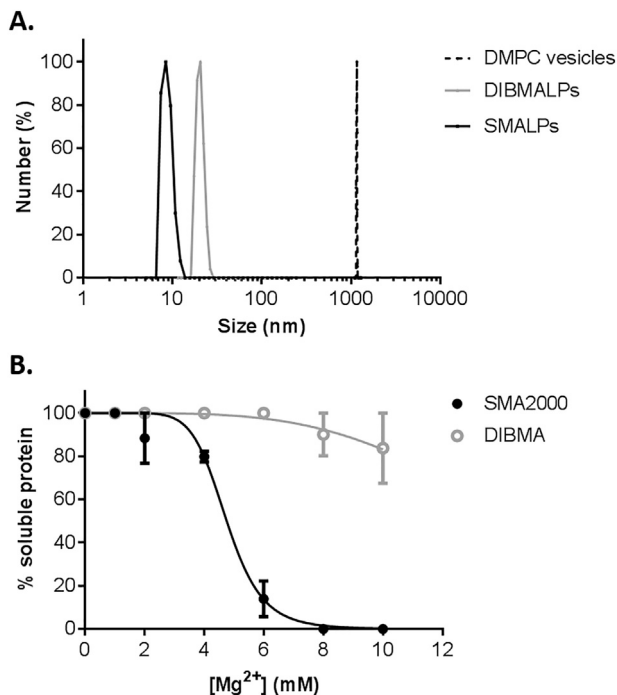


Fig. 4. DIBMALPs are larger than SMALPs and display increased tolerance to divalent cations. A; Vesicles formed from DMPC lipids were mixed with buffer (DMPC vesicles, black dashed line), 2.5% (w/v) SMA (SMALPs, solid black) or 2.5% (w/v) DIBMA (DIBMALPs, grey) and analysed by DLS using a Brookhaven NanoBrook 90plus Zeta instrument (640 nm) and 1.0 cm path length disposable cuvettes. B; BmrA purified using either SMA2000 (black closed circles) or DIBMA (grey open circles) were mixed with 0-10 mM MgCl₂, centrifuged to remove aggregates and samples of both supernatant and pellet run on SDS-PAGE to monitor sensitivity to divalent cations. Data are mean \pm sem, $n = 3$.

Table 2

A_{2A}R solubilised with either SMA2000 or DIBMA binds ligand effectively. Membranes from *Pichia pastoris* overexpressing A_{2A}R were solubilised with 2.5% (w/v) SMA2000 or DIBMA for either 1 h or 18 h at room temperature, then ultracentrifuged to harvest the soluble fraction. The ligand ³H-ZM241385 was used at 100 nM, with varying concentrations of unlabelled ZM241385. Specific binding was plotted against log [ZM241385], and data were fitted with an inhibition dose-response curve to determine IC₅₀. pKi was calculated from the IC₅₀ values using the Cheng-Prusoff equation. Data are mean \pm sem, $n = 3$. A negative control of the supernatant from pelleted membranes was also tested and showed negligible binding.

Polymer	pKi	
	1 h solubilisation	18 h solubilisation
SMA2000	8.15 \pm 0.27	7.81 \pm 0.65
DIBMA	8.31 \pm 0.48	8.50 \pm 0.20

solubilised membranes using the radioactive ligand ³H-ZM241385, which competitively binds to the active site of A_{2A}R. After solubilisation for 1 h both A_{2A}R-SMALP and A_{2A}R-DIBMALP showed specific binding to ZM241385, with pKi values of 8.15 \pm 0.27 and 8.31 \pm 0.47 respectively. No improvement was found from increasing the solubilisation time to 18 h.

Secondly the ability to extract CGRP receptors from Cos7 cells was tested. Cos7 cell membranes expressing CGRP receptors were labelled with ¹²⁵I-CGRP before solubilisation as this ligand binds to SMA, precluding labelling after solubilisation. Following SMALP extraction for 1 h at 25 °C, 64 \pm 8% ($n = 5$) of the ¹²⁵I-CGRP specifically bound to the membranes was recovered in the soluble fraction, with 14 \pm 1% of the specifically bound ligand left in the insoluble pellet. Very similar

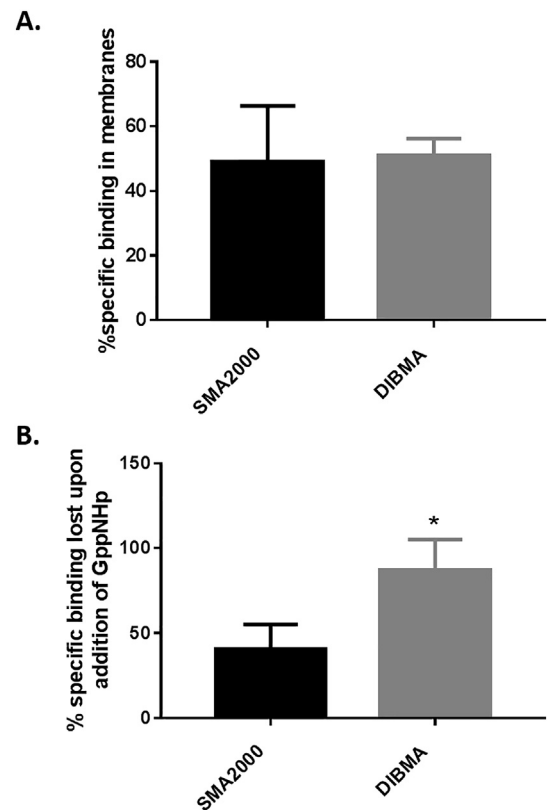


Fig. 5. Radiolabelled CGRP receptor can be extracted from cells with either SMA2000 or DIBMA. A; Recovery of ¹²⁵I-CGRP binding after pre-labelling with radioligand and extraction with 2.5% SMA or DIBMA. Binding is expressed as a % of that found in the membranes prior to extraction. B; Effect of 100 μ M GppNHp on ¹²⁵I-CGRP pre-bound to membranes and then extracted with 2.5% SMA or DIBMA. Binding is expressed as % reduction of that found in polymer extract prior to addition of GppNHp. Data are mean \pm SD, $n = 3$. Data were analysed by a *t*-test, * $p < 0.05$.

results were observed with DIBMA (Fig. 5A). There was no advantage to a 24 h solubilisation at 4 °C (supernatant recovery 49 \pm 22%, $n = 3$). As CGRP is an agonist, the majority of the receptor to which it binds would be predicted to be coupled to a G protein. Addition of 100 μ M GppNHp (a non-hydrolysable GTP analogue) would be expected to cause the G protein to dissociate from the receptor and therefore cause a decrease in affinity for agonist binding. As shown in Fig. 5B GppNHp reduced specific binding by 60 \pm 8% ($n = 3$) with the SMA extract and virtually 100% with DIBMA, showing the extracted CGRP receptors were bound to G proteins.

3.5. Stability over time

Finally because SMALPs have been reported many times to improve the stability of membrane proteins, the long term stability of DIBMA samples was investigated. Fig. 6A shows a radioligand binding assay on SMA and DIBMA solubilised A_{2A}R membranes which had been stored at 4 °C for 6 days. The SMA sample behaved almost identically to that of the fresh sample (Table 2), with a pKi of 8.34 \pm 0.40. For the DIBMA sample, there is almost no change in the affinity of binding with a pKi of 8.58 \pm 0.16, however there does appear to be a decrease in the amount of ligand bound.

Rather than functional stability Fig. 6B examines the propensity of the nanoparticle encapsulated protein to remain soluble over time. Purified BmrA stored in the fridge was sampled and centrifuged to remove any aggregates at various time points over 3 weeks. It can be seen that BmrA purified within SMALPs remains relatively stable with approximately 80% still soluble after 24 days, however BmrA within

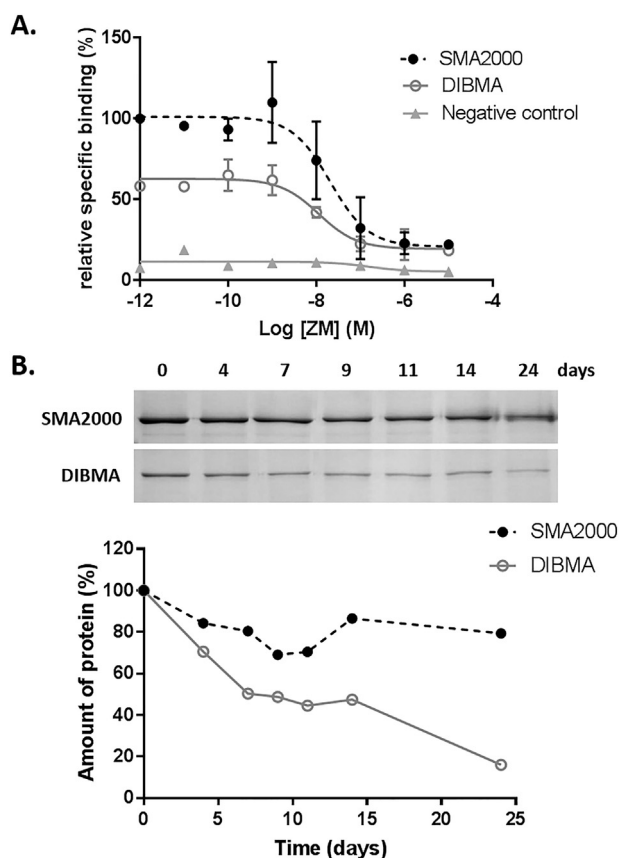


Fig. 6. Stability of proteins within SMALPs or DIBMALPs. A; $A_{2A}R$ solubilised from *Pichia pastoris* using either SMA2000 (black closed circles) or DIBMA (grey open circles), or a negative control (triangles) were stored at 4 °C for 6 days, then analysed by radioligand binding using 3H -ZM241385, $n = 2$. B; BmrA purified using either SMA2000 (black closed circles) or DIBMA (grey open circles) was stored at 4 °C for 0–24 days, subjected to centrifugation (16,000g), the supernatant loaded on SDS-PAGE and analysed by densitometry.

DIBMALPs shows a steady decrease in solubility over time.

4. Discussion

In this study the membrane protein solubilisation and purification efficiency of commercially available SMA was compared to that of its aliphatic variant DIBMA. A range of different membrane proteins with varying shapes and sizes, from different expression systems were tested to identify any protein/expression system preferences of the polymers.

DIBMA did not offer any improvements over SMA in terms of membrane protein solubilisation efficiency. In fact for the protein ZipA expressed in *E. coli*, DIBMA was significantly less efficient than SMA. In contrast DIBMA was able to solubilise comparable amounts of functional GPCRs, $A_{2A}R$ and CGRP receptor, from *P. pastoris* and Cos7 cells respectively.

Following affinity purification of solubilised proteins from *E. coli*, lower yields of purified protein were obtained with DIBMA than with SMA (2.5-fold lower). This was a larger decrease than seen in solubilisation efficiency (which was 1.8-fold lower), suggesting that some solubilised protein was lost during the purification procedure. In addition the purified protein contained more contaminants, particularly for ZipA. In agreement with previous reports [23,27] we found that the DIBMALPs are larger in diameter than SMALPs. This larger size may be responsible for the greater number of impurities as there is more space within the disc to contain other proteins, especially with ZipA which only has one transmembrane helix.

It was previously shown that lipid-only DIBMALPs have a greater

tolerance to divalent cations than SMALPs [23]. Here we have shown that this is also observed when the DIBMALPs contain a membrane protein. That DIBMALPs are more tolerant to divalent cations seems counterintuitive because it has been hypothesized to be the interaction between divalent cations and maleic acid parts of SMA that are responsible for this sensitivity [10], yet DIBMA still contains maleic acid groups, and at a higher percentage than SMA. Perhaps the difference is caused by the larger diameter of DIBMALPs, meaning any induced conformational change does not cause the same strain on the polymer surrounding the disc. Alternatively, as proposed previously [28], the greater proportion of maleic acid groups present in DIBMA means it is more hydrophilic overall and can therefore tolerate a greater degree of neutralization by binding to divalent cations. In fact it was shown previously that addition of Mg^{2+} or Ca^{2+} can increase the efficiency of solubilisation when using DIBMA [28].

Effective solubilisation of GPCRs using SMA and characteristic binding of ligands has been reported previously [13,29] and here we show that DIBMA can also achieve this, with pKi values showing good comparison to those seen within natural membranes [13]. We also show for the first time the extraction of a class B GPCR, namely the CGRP receptor, which is a complex comprised of CLR (a GPCR) and RAMP1 (a single transmembrane helix accessory protein, essential for CGRP binding to CLR) [30]. Both SMA and DIBMA extracted CGRP receptor from Cos7 cells with bound CGRP ligand, and importantly in both cases the extracted receptor was also bound to a G protein. It is worth noting that binding of GppNHp requires magnesium. The concentration of magnesium used was kept below the concentration which causes SMA to precipitate, however the chelation of magnesium by SMA may explain the slightly larger effect of GppNHp observed with DIBMALP encapsulated CGRP.

Finally we examined the stability of proteins within DIBMALPs over time. SMALP encapsulated proteins have been shown in many other studies to have an increased stability [7,9–15]. We found that $A_{2A}R$ within DIBMALPs displayed no change in pKi after storage at 4 °C for 6 days, however there was a decrease in the amount of ligand bound, which therefore is likely to reflect a decrease in the B_{max} (i.e. the number of functional receptors). We also showed that long term storage of BmrA within DIBMALPs showed a time dependent increase in aggregation, which was not apparent with SMALPs. It was reported previously that DIBMA perturbs the lipid packing within the disc less than SMA [23]. The styrene groups of SMA are known to insert into the core of the bilayer and increase the rigidity of lipids in the outer layer [8]. With DIBMA this effect may be less, and in addition the size of the disc is larger meaning there is more lipid in total. This may be the cause of the lower stability of proteins in DIBMALPs compared to SMALPs. However, the upside to this is that the DIBMALP may represent a better environment for protein conformational changes and dynamics to occur.

In conclusion we have shown that DIBMA can be used as an alternative to SMA for solubilisation and purification of a wide range of membrane proteins. Whilst for some proteins yield, purity and stability are lower with DIBMA than with SMA, DIBMA offers a tolerance to divalent cations and larger disc size with less ordered lipids. Thus the choice of which polymer will be best to use will depend upon the features of a protein to be investigated.

CRedit author contribution

Aiman Gulamhussein: Conceptualization, Investigation, Analysis, Validation, Writing – original draft. **Romez Uddin:** Investigation, Analysis, Writing – review & editing. **Brian Tighe:** Supervision, Resources, Writing – review & editing. **David Poyner:** Investigation, Analysis, Resources, Supervision, Writing – review & editing. **Alice J. Rothnie:** Conceptualization, Methodology, Analysis, Supervision, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Dr. Khaled Doudin for assistance with NMR & FTIR spectroscopy. The underlying data for this study can be found at the Aston Explorer Data Repository (<https://doi.org/10.17036/researchdata.aston.ac.uk.00000462>).

Funding sources

AG was the recipient of an Aston University overseas student excellence bursary and RU the recipient of a Life & Health Sciences school studentship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbamem.2020.183281>.

References

- [1] D. Hardy, E. Desuzinges Mandon, A.J. Rothnie, A. Jawhari, The yin and yang of solubilization and stabilization for wild-type and full-length membrane protein, *Methods* 147 (2018) 118–125.
- [2] R. Dawaliby, C. Trubbia, C. Delporte, M. Masurel, P. Van Antwerpen, B.K. Kobilka, C. Govaerts, Allosteric regulation of G protein-coupled receptor activity by phospholipids, *Nat. Chem. Biol.* 12 (2016) 35–39.
- [3] M.E. Zoghbi, R.S. Cooper, G.A. Altenberg, The lipid bilayer modulates the structure and function of an ATP-binding cassette exporter, *J. Biol. Chem.* 291 (2016) 4453–4461.
- [4] I.G. Denisov, S.G. Sligar, Nanodiscs for structural and functional studies of membrane proteins, *Nat. Struct. Mol. Biol.* 23 (2016) 481–486.
- [5] M. Zoonens, J.L. Popot, Amphipols for each season, *J. Membr. Biol.* 247 (2014) 759–796.
- [6] S. Poulos, J.L. Morgan, J. Zimmer, S. Faham, Bicyclic coming of age: an empirical approach to bicelle crystallization, *Methods Enzymol.* 557 (2015) 393–416.
- [7] T.J. Knowles, R. Finka, C. Smith, Y.P. Lin, T. Dafforn, M. Overduin, Membrane proteins solubilized intact in lipid containing nanoparticles bounded by styrene maleic acid copolymer, *J. Am. Chem. Soc.* 131 (2009) 7484–7485.
- [8] M. Jamshad, V. Grimard, I. Idini, T.J. Knowles, M.R. Dowle, N. Schofield, P. Sridhar, Y.P. Lin, R. Finka, M. Wheatley, O.R.T. Thomas, R.E. Palmer, M. Overduin, C. Govaerts, J.M. Ruysschaert, K.J. Edler, T.R. Dafforn, Structural analysis of a nanoparticle containing a lipid bilayer used for detergent-free extraction of membrane proteins, *Nano Res.* 8 (2015) 774–789.
- [9] S. Gulati, M. Jamshad, T.J. Knowles, K.A. Morrison, R. Downing, N. Cant, R. Collins, J.B. Koenderink, R.C. Ford, M. Overduin, I.D. Kerr, T.R. Dafforn, A.J. Rothnie, Detergent-free purification of ABC (ATP-binding-cassette) transporters, *The Biochemical journal* 461 (2014) 269–278.
- [10] K.A. Morrison, A. Akram, A. Mathews, Z.A. Khan, J.H. Patel, C. Zhou, D.J. Hardy, C. Moore-Kelly, R. Patel, V. Odiba, T.J. Knowles, M.U. Javed, N.P. Chmel, T.R. Dafforn, A.J. Rothnie, Membrane protein extraction and purification using styrene-maleic acid (SMA) copolymer: effect of variations in polymer structure, *The Biochemical journal* 473 (2016) 4349–4360.
- [11] A.A. Gulamhussein, D. Meah, D.D. Soja, S. Fenner, Z. Saidani, A. Akram, S. Lallie, A. Mathews, C. Painter, M.K. Liddar, Z. Mohammed, L.K. Chiu, S.S. Sumar, H. Healy, N. Hussain, J.H. Patel, S.C.L. Hall, T.R. Dafforn, A.J. Rothnie, Examining the stability of membrane proteins within SMALPs, *Eur. Polym. J.* 112 (2019) 120–125.
- [12] J.M. Dorr, M.C. Koorengel, M. Schafer, A.V. Prokofyev, S. Scheidelaar, E.A. van der Crujssen, T.R. Dafforn, M. Baldus, J.A. Killian, Detergent-free isolation, characterization, and functional reconstitution of a tetrameric K⁺ channel: the power of native nanodiscs, *Proc. Natl. Acad. Sci. U. S. A.* 111 (2014) 18607–18612.
- [13] M. Jamshad, J. Charlton, Y.P. Lin, S.J. Routledge, Z. Bawa, T.J. Knowles, M. Overduin, N. Dekker, T.R. Dafforn, R.M. Bill, D.R. Poyner, M. Wheatley, G-protein coupled receptor solubilization and purification for biophysical analysis and functional studies, in the total absence of detergent, *Biosci. Rep.* 35 (2015).
- [14] D.J. Swainsbury, S. Scheidelaar, R. van Grondelle, J.A. Killian, M.R. Jones, Bacterial reaction centers purified with styrene maleic acid copolymer retain native membrane functional properties and display enhanced stability, *Angew Chem Int Ed Engl* 53 (2014) 11803–11807.
- [15] S. Rehan, V.P. Jaakola, Expression, purification and functional characterization of human equilibrative nucleoside transporter subtype-1 (hENT1) protein from Sf9 insect cells, *Protein Expr. Purif.* 114 (2015) 99–107.
- [16] J. Broecker, B.T. Eger, O.P. Ernst, Crystallogenesis of membrane proteins mediated by polymer-bounded lipid nanodiscs, *Structure* 25 (2017) 384–392.
- [17] C. Sun, S. Benlekber, P. Venkatakrishnan, Y. Wang, S. Hong, J. Hosler, E. Tajkhorshid, J.L. Rubinstein, R.B. Gennis, Structure of the alternative complex III in a supercomplex with cytochrome oxidase, *Nature* 557 (2018) 123–126.
- [18] W. Qiu, Z. Fu, G.G. Xu, R.A. Grassucci, Y. Zhang, J. Frank, W.A. Hendrickson, Y. Guo, Structure and activity of lipid bilayer within a membrane-protein transporter, *Proc. Natl. Acad. Sci. U. S. A.* 115 (2018) 12985–12990.
- [19] S.C. Lee, R. Collins, Y.P. Lin, M. Jamshad, C. Broughton, S.A. Harris, B.S. Hanson, C. Tognoloni, R.A. Parslow, A.E. Terry, A. Rodger, C.J. Smith, K.J. Edler, R. Ford, D.I. Roper, T.R. Dafforn, Nano-encapsulated *Escherichia coli* divisome anchor ZipA, and in complex with FtsZ, *Sci. Rep.* 9 (2019) 18712.
- [20] D.J.K. Swainsbury, S. Scheidelaar, N. Foster, R. van Grondelle, J.A. Killian, M.R. Jones, The effectiveness of styrene-maleic acid (SMA) copolymers for solubilisation of integral membrane proteins from SMA-accessible and SMA-resistant membranes, *Biochim. Biophys. Acta Biomembr.* 1859 (2017) 2133–2143.
- [21] N.L. Pollock, S.C. Lee, J.H. Patel, A.A. Gulamhussein, A.J. Rothnie, Structure and function of membrane proteins encapsulated in a polymer-bound lipid bilayer, *Biochim. Biophys. Acta* 1860 (2018) 809–817.
- [22] S.C. Lee, T.J. Knowles, V.L. Postis, M. Jamshad, R.A. Parslow, Y.P. Lin, A. Goldman, P. Sridhar, M. Overduin, S.P. Muench, T.R. Dafforn, A method for detergent-free isolation of membrane proteins in their local lipid environment, *Nat. Protoc.* 11 (2016) 1149–1162.
- [23] A.O. Oluwole, B. Danielczak, A. Meister, J.O. Babalola, C. Vargas, S. Keller, Solubilization of membrane proteins into functional lipid-bilayer nanodiscs using a diisobutylene/maleic acid copolymer, *Angew Chem Int Ed Engl* 56 (2017) 1919–1924.
- [24] A.J. Rothnie, Detergent-free membrane protein purification, *Methods Mol. Biol.* 1432 (2016) 261–267.
- [25] A. Rothnie, J. Storm, J. Campbell, K.J. Linton, I.D. Kerr, R. Callaghan, The topography of transmembrane segment six is altered during the catalytic cycle of P-glycoprotein, *J. Biol. Chem.* 279 (2004) 34913–34921.
- [26] J. Barwell, A. Conner, D.R. Poyner, Extracellular loops 1 and 3 and their associated transmembrane regions of the calcitonin receptor-like receptor are needed for CGRP receptor function, *Biochim. Biophys. Acta* 1813 (2011) 1906–1916.
- [27] A.O. Oluwole, J. Klingler, B. Danielczak, J.O. Babalola, C. Vargas, G. Pabst, S. Keller, Formation of lipid-bilayer nanodiscs by diisobutylene/maleic acid (DIBMA) copolymer, *Langmuir: The ACS Journal of Surfaces and Colloids* 33 (2017) 14378–14388.
- [28] B. Danielczak, A. Meister, S. Keller, Influence of Mg(2+) and Ca(2+) on nanodisc formation by diisobutylene/maleic acid (DIBMA) copolymer, *Chem. Phys. Lipids* 221 (2019) 30–38.
- [29] C. Logez, M. Damian, C. Legros, C. Dupre, M. Guery, S. Mary, R. Wagner, C. M'Kadmi, O. Nosjean, B. Fould, J. Marie, J.A. Fehrentz, J. Martinez, G. Ferry, J.A. Boutin, J.L. Baneres, Detergent-free isolation of functional G protein-coupled receptors into nanometric lipid particles, *Biochemistry* 55 (2016) 38–48.
- [30] L.M. McLatchie, N.J. Fraser, M.J. Main, A. Wise, J. Brown, N. Thompson, R. Solari, M.G. Lee, S.M. Foord, RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor, *Nature* 393 (1998) 333–339.