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

Aiman A Gulamhussein[a], Romez Uddin[a], Brian J Tighe[b], David R Poyner[a] & Alice J Rothnie[a]

[a]Life & Health Sciences or [b]Chemical Engineering & Applied Chemistry, Aston University, Aston Triangle, Birmingham, B4 7ET, UK

Corresponding author: Dr Alice Rothnie, School of Life & Health Sciences, Aston University, Aston Triangle, Birmingham, B4 7ET, UK. a.rothnie@aston.ac.uk

Abbreviations:

ABC	ATP Binding Cassette
$A_{2a}R$	adenosine A2a receptor
CGRP	Calcitonin gene related peptide
CLR	calcitonin receptor-like receptor
DIBMA	di-isobutylene maleic acid
DLS	dynamic light scattering
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
FTIR	Fourier transform infra red
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

HIGHLIGHTS

- DIBMA is able to solubilise a range of membrane proteins from different expression systems.
- For some proteins DIBMA gives a lower yield of protein and is less pure than with SMA2000.
- DIBMA encapsulated GPCRs retain ligand- and G protein-binding.
- DIBMALPs are less sensitive to divalent cations than SMALPs.
- DIBMALPs appear to be less stable over time than SMALPs.

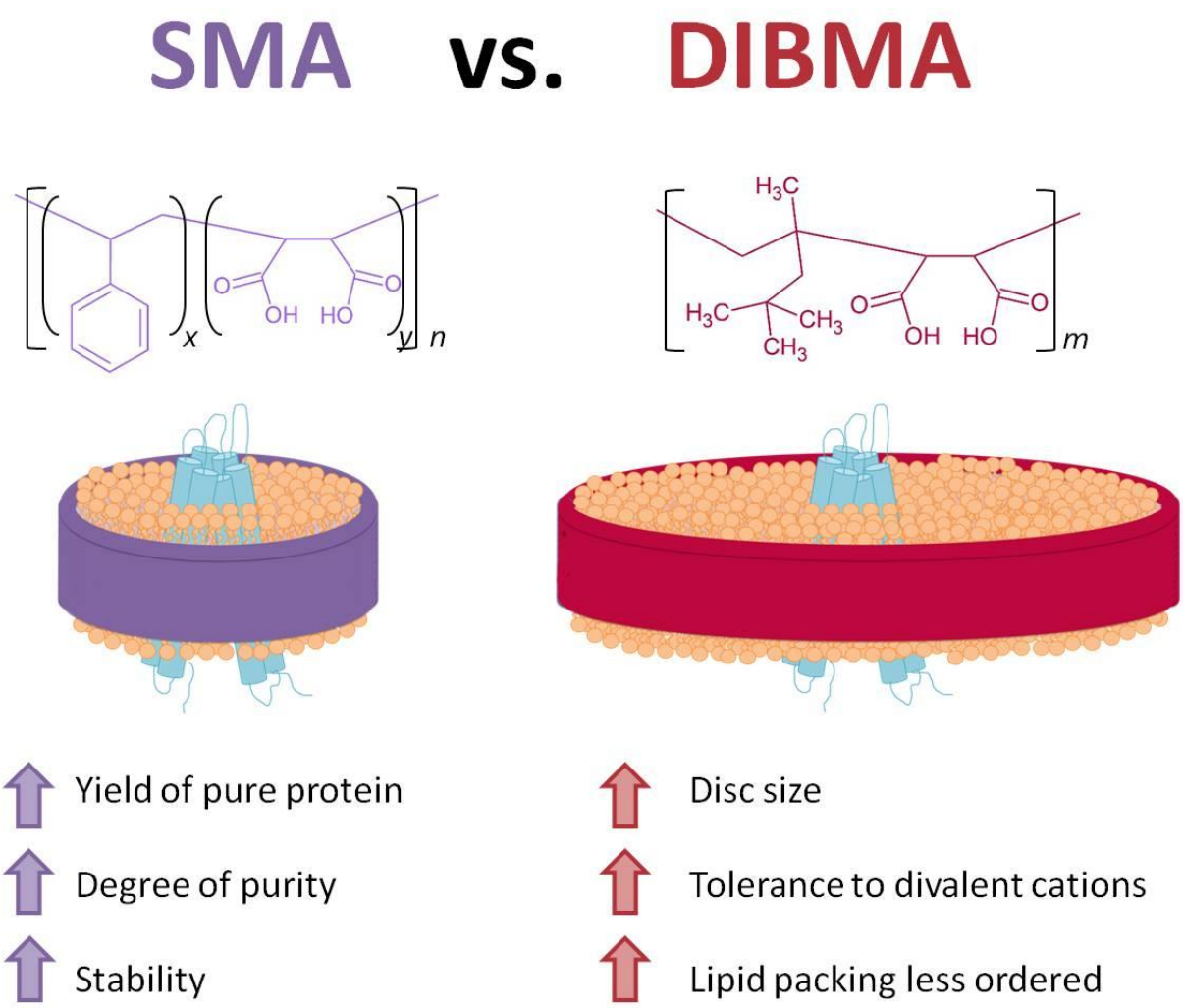
ABSTRACT

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 solubilize 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 solubilize and purify functional membrane proteins has been compared to SMA. It was found that DIBMA is able to solubilize 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.

KEYWORDS

SMALP, DIBMALP, membrane protein, GPCR, ABC transporter.

GRAPHICAL ABSTRACT



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 – 11nm 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 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 260nm 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 A2a receptor ($A_{2A}R$) and the calcitonin gene

related peptide (CGRP) receptor, expressed in *Pichia Pastoris* and Cos-7 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 1M NaOH overnight. The solution was then refluxed for 2 hours 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 10min 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 0.6M 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 (85ml) was placed inside snake skin dialysis tubing with a molecular weight cut off of 3.5KDa, and dialysed against 1l buffer 2 (20mM Tris pH8, 150mM NaCl) for 12-15h at 4°C, stirring continuously. Following dialysis the polymer had been diluted approximately 3-fold, and the pH was decreased to pH8-9. 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 – 650cm^{-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 ^{1}H and ^{13}C-NMR on a Bruker Avance-300 spectrometer at room temperature overnight and recorded using a 5mm normal dual detection probe. 60µl of DIBMA sample was dissolved in 0.6 – 0.8ml of deuterated water D_2O. The ^{1}H NMR spectra were recorded at 300.13 MHz using a high-resolution dual (^{1}H ^{13}C) gradients probe. Spectra were recorded using the zg30 pulse program with 16 or 128 scans and referenced to the DMSO peak at 2.50ppm**.** ^{13}C NMR spectra were obtained at 75 MHz for carbon. The pendant pulse program was used with waltz16 decoupling during acquisition

with 6k scans and phased for CH_3/CH positive and quaternary carbons and CH_2 negative with spectra referenced to DMSO peak at 40.0 ppm.

2.3 BmrA & ZipA Expression & Membrane Preparation

Overnight (5ml) cultures containing LB supplemented with 100µg/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 1L flasks of LB-amp incubated at 37^{o}C at a speed of 200rpm. When OD_{600} reached 0.6, 0.5mM IPTG was added to induce protein synthesis and the culture incubated overnight at 25°C, 200rpm.

The bacterial culture was centrifuged for 10min at 6000g, 4^{o}C, and the *E. coli* cell pellets resuspended in buffer 1 (50mM Tris pH7.4, 250mM sucrose, 0.25mM $CaCl_2$, 1 µM pepstatin, 1.3 µM benzamidine 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 20mins at 750g, 4^{o}C. The cell membranes were further harvested by ultra-centrifugation at 100,000g for 20 min at 4^{o}C. The membranes were resuspended in buffer 2 (20mM Tris pH 8, 150mM NaCl) and aliquoted at a final concentration of 60 mg/ml wet membrane weight and stored at –80^{o}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 10,000 x g for 30mins. The membranes were collected by ultracentrifugation (100,000g in a Beckman type Ti 70 rotor for 45min). The membrane pellets were collected and resuspended in buffer 2 (20mM Tris and 150mM NaCl at pH 8) and aliquoted at a final concentration of 60mg/ml wet membrane weight and rapidly stored at -80^{o}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 30mg/ml membrane (wet pellet weight) and 2.5% (w/v) polymer. This was incubated for 1 hour 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 Ni^{2+}-NTA resin (ThermoFisher) at 100µl 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 20mM imidazole, twice with 10 BVs of buffer 2 containing 40mM imidazole and once with 1 BV of buffer 2 supplemented with 60mM imidazole. Proteins were eluted with buffer 2 supplemented with 200mM 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 (100µl) was mixed with various concentrations of $MgCl_2$ ranging from 0–10mM. 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 (0.001µM – 1mM) 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 ^{3}H-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 5µl of ^{3}H-ZM214385. 500µl 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 30,000 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. 100µl of lipid-only SMALPs, lipid-only DIBMALPs or 2 % (w/v) DMPC lipid suspension was added to 1900µl of buffer 2. Dynamic light scattering (DLS) data were recorded using a Brookhaven NanoBrook 90plus Zeta instrument (640nm) and 1.0cm 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^{o}C, every few days 100µL of the sample was centrifuged at 16,000g for 10 min in a bench top Eppendorf centrifuge at room temperature. 20µL 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

Cos 7 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 ^{125}I-CGRP (Perkin Elmer) for 30 min at room temperature (with or without 1 µM unlabelled CGRP to define non-specific binding) in 2mM $MgCl_2$, 20 mM Hepes pH 7.4. Membranes were pelleted and resuspended at 1mg/ml in SMA or DIBMA co-polymer (2.5 % w/v final concentration in 50mM NaCl, 12.5% glycerol, 500mM Tris, pH 8) for 1 h at 25^{o} C. The undissolved membranes were pelleted and radioligand binding was performed on the CGRP receptor in the supernatant with spin columns as for the $A2_AR$. 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 IC_{50} 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 (Figure 1). It has an average molecular weight of 15kDa[23], which is larger than that of the commercially available SMA polymers successfully used to date (7.5 – 10kDa) [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 3.5KDa. 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 Figure 1A. which shows the characteristic C = O stretch of the carboxylic acid present in the maleic acid at 1738.61cm^{-1} as well as the peaks at 2900cm^{-1} which are indicative of C – H stretching vibrations (CH_3 and CH_2) alkane groups present in the diisobutylene, these peaks are further supported by the presence of strong peaks in the fingerprint region of the spectrum between the range of 1200 – 1300cm^{-1} also indicating the presence of C – H alkane groups in the polymer.

NMR characterization of the polymer was also undertaken. The ^{1}H-NMR (Supplementary Figure 1B) shows a characteristic peak at δ=3.0ppm due to the CH groups and at δ=3.7ppm 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 δ=0.9 – 1.1 and δ=1.2 – 1.8ppm respectively. The sharp peak observed at δ=2.0ppm is indicative of the presence of impurities or a solvent, possibly acetone. The ^{13}C-NMR spectra (Supplementary Figure 1C) contains 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 δ=29 - 33ppm that relate to the primary and secondary alkyl groups. A weaker signal observed at δ = 41ppm 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 δ= 180 – 190ppm. Sharp peaks observed around δ = 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 Figure 2A), as

reported previously [23]. This also affected migration and detection of solubilised proteins via western blots as shown in Supplementary Figure 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 (Figure 2). ZipA is a membrane 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 (Figure 3). However it should be noted that purification of ZipA with DIBMA (Figure 3B) gave much less intense bands of ZipA, and more contaminating proteins were present than was seen with SMA2000 (Figure 3A). Similarly for BmrA, DIBMA appeared to give a lower yield of protein than SMA2000 (Figure 3C & D). When average yields were compared (Figure 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 Figure 4A, SMALPs are approximately 10nm in diameter, whereas DIBMALPs are approximately 25nm in diameter.

It was previously reported than 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 Figure 4B when the concentration of magnesium exceeds 4mM, protein within SMALPs loses solubility, with all the protein insoluble at 8-10mM $MgCl_2$. However for the DIBMALPs this was not the case and at 10mM $MgCl_2$ there was still more than 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 A2a receptor ($A_{2A}R$) from *Pichia pastoris* yeast membranes was tested. Table 2 shows the pKi values derived from radioligand binding assays on both SMA2000 and DIBMA solubilised membranes using the radioactive ligand ^{3}H-ZM241385, which competitively binds to the active site of $A_{2A}R$. After

solubilisation for 1 hour 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 hours.

Secondly the ability to extract CGRP receptors from Cos-7 cells was tested. Cos 7 cell membranes expressing CGRP receptors were labelled with ^{125}I-CGRP before solubilisation as this ligand binds to SMA, precluding labelling after solubilisation. Following SMALP extraction for 1 h at 25^{o} C, 64 ± 8% (n=5) of the ^{125}I-CGRP specifically bound to the membranes was recovered in the soluble fraction, with 14 ± 1% of the specifically bound ligand left in the insoluble pellet. Very similar results were observed with DIBMA (Figure 5A). There was no advantage to a 24 h solubilisation at 4^{o} C (supernatant recovery 49 ± 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 µM 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 Figure 5B GppNHp reduced specific binding by 60 ± 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. Figure 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 figure 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 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 solubilize comparable amounts of functional GPCRs, $A_{2A}R$ and CGRP receptor, from *P. pastoris* and Cos7 cells respectively.

Following affinity purification of solubilized 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 solubilized 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 affect 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.

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TABLES

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 *	15300 * 12000 **	1.8 *

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. * as calculated experimentally in Oluwole et al (2017) Angew. Chem. Int. Ed. 56, 1919-1924 [23]. ** as provide by BASF data sheet.

Polymer	pKi	
	1 hour solubilisation	18 hour solubilisation
SMA2000	8.15 ± 0.27	7.81 ± 0.65
DIBMA	8.31 ± 0.48	8.50 ± 0.20

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 hour or 18 hours at room temperature, then ultracentrifuged to harvest the soluble fraction. The ligand ^{3}H-ZM241385 was used at 100nM, 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 IC_{50}. pKi was calculated from the IC_{50} values using the Cheng-Prusoff equation. Data are mean±sem, n=3. A negative control of the supernatant from pelleted membranes was also tested and showed negligible binding.

FIGURE LEGENDS

Figure 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.

Figure 2. Solubilisation of membrane proteins with DIBMA is less efficient than with SMA2000. Membranes (30 mg/ml wet pellet weight) from *E.coli* overexpressing either ZipA or BmrA were solubilised with 2.5%(w/v) polymer for 1 hour 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±sem, n≥6. Data were analysed by an ANOVA with a Bonferroni posthoc test, ** $p<0.01$.

Figure 3. Lower yields of purified protein are obtained with DIBMA compared to SMA2000. Solubilised protein was purified by Ni-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 ±sem, n≥6. Data were analysed by ANOVA with a Bonferroni post-hoc test, * $p<0.05$.

Figure 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) or 2.5% (w/v) DIBMA (DIBMALPs, grey) and analysed by DLS using a Brookhaven NanoBrook 90plus Zeta instrument (640nm) and 1.0cm path length disposable cuvettes.. B; BmrA purified using either SMA2000 (black closed circles) or DIBMA (grey open circles) were mixed with 0-10mM $MgCl_2$, centrifuged to remove aggregates and samples of both supernatant and pellet run on SDS-PAGE to monitor sensitivity to divalent cations. Data are mean ±sem, n=3.

Figure 5. Radiolabelled CGRP receptor can be extracted from cells with either SMA2000 or DIBMA. A; recovery of ^{125}I-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 mM GppNHp on ^{125}I-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±SD, n= 3. Data were analysed by a t test, *$p<0.05$.

Figure 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 ^{3}H-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.