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Solubilisation & purification of membrane proteins using benzylamine-modified SMA polymers

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ABSTRACT

Extraction of proteins from the membrane using styrene maleic acid *co*-polymers (SMA), forming SMA lipid particles (SMALPs), has allowed for the first time the purification of membrane proteins with their lipid bilayer environment. To date, SMA2000 has been the most effective polymer used for this purpose, with a 2:1 ratio of styrene:maleic acid, and styrene and maleic acid moieties spread statistically throughout the chain. However, SMA2000 is a highly polydisperse polymer that contains an array of different polymer lengths and sequences. RAFT polymerisation offers much better control over the polymer length; however, homogeneous distribution of styrene and maleic acid throughout the polymer is difficult to achieve. Instead, here RAFT polymerisation was used to produce a 1:1 styrene:maleic anhydride polymer, which was then modified with benzylamine. This mimics the 2:1 hydrophobic:hydrophilic nature of SMA2000, while controlling the length and obtaining a homogeneous distribution of the hydrophobic moieties (styrene and *N*-benzylmaleimide). SMA-benzylamine (SMA-BA) polymers of three different lengths (2, 4, and 7 kDa) were all able to solubilise purified lipids, cellular membranes, and a range of specific proteins. However, the larger 7 kDa polymer solubilised membranes more slowly and less efficiently than the shorter polymers. This also affected the yield of purified protein obtained by affinity purification with this polymer. The smallest 2 kDa polymer solubilised membranes the fastest but

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Abbreviations: ABC transporter, ATP Binding Cassette superfamily of transporters; Ag-GSH, Silver-glutathione; AIBN, azobisisobutyronitrile; Atm1, ABC transporter from *Novosphingobium aromaticivorans* that confers resistance to heavy metals; ATR, Attenuated total reflectance; AQP4, Human aquaporin 4; BA, Benzylamine; BPT, *S*-butyl-*S*′-(1-phenyl ethyl) trithiocarbonate; BSA, Bovine serum albumin; CD81, tetraspanin protein CD81; *Đ*, *Dispersity*; DIBMA, poly(diisobutylene-*alt*-maleic acid); DLS, Dynamic light scattering; DMF, *N,N*-dimethylformamide; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; *E.coli*, *Escherichia coli*; FTIR, Fourier transform infrared spectroscopy; GSSG, oxidised glutathione; LB, Luria broth; MAnh, maleic anhydride; MEK, Methyl ethyl ketone; M_n, Number-average molecular weight; MRP4, Multidrug resistance protein 4/ABCC4; Ni-NTA, Nickel nitriloacetic acid; *P.pastoris*, *Pichia Pastoris*; PDI, Polydispersity index; RAFT, reversible addition fragmentation chain transfer; SDS-PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis; SEC, Size exclusion chromatography; Sf9, *Spodoptera frugiperda* cell line; SMA, Styrene maleic acid co-polymer; SMA1000-BA, SMA1000 modified with benzylamine; SMA-BA 2 kDa, 1:1 SMA polymer approx. 2 kDa in size modified with benzylamine; SMA-BA 4 kDa, 1:1 SMA polymer approx. 4 kDa in size modified with benzylamine; SMA-BA 7 kDa, 1:1 SMA polymer approx. 7 kDa in size modified with benzylamine; SMALP, SMA lipid particle; SMAnh, Poly(styrene-*alt*-maleic anhydride); THF, Tetrahydrofuran; ZipA, membrane tether protein from *E. coli*.

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appeared to offer less stability to the extracted proteins. The SMA-BA polymers were more sensitive to Mg^{2+} ions than SMA2000. SMA-BA 4 kDa was otherwise comparable to SMA2000 and even gave a higher degree of purity.

1. Introduction

Membrane proteins play vital roles in various cellular functions, including mediating cellular communication, cellular recognition, controlling the entry and exit processes of cells, and various enzymatic functions. Therefore, they are targets for many current and potential future drugs. However, they are more challenging to study than soluble proteins due to their lipid bilayer-embedded environment. To study the structure and function of a membrane protein often requires its extraction from the membrane and subsequent purification. Achieving this efficiently, while retaining a correctly folded structure and activity, can be difficult. Traditionally, this was achieved using detergents that disrupt the lipid bilayer and form a micelle around the hydrophobic regions of the protein; however, detergents tend to strip away the lipid environment of the protein, which is known to be important for function, and often leads to protein instability [1,2]. Reconstitution of purified proteins into liposomes, membrane scaffold protein (MSP) nanodiscs, or other membrane mimetics re-forms the membrane environment of the protein but still requires initial detergent solubilisation [3–6]. In the last 15 years, the use of styrene maleic acid (SMA) copolymers for membrane protein extraction has offered an alternative approach. SMA can extract membrane proteins within small discs of lipid bilayer, with the polymer wrapped around the edge, termed SMA lipid particles (SMALPs) [7–10]. This ability to extract a protein directly from a membrane with its bilayer environment means that it does not lose these important protein-lipid interactions [11–13]. As a result, proteins encapsulated within SMALPs tend to be more thermostable than detergent solubilised proteins [9,14]. The small size of the SMALP disc makes it amenable to many downstream analysis techniques to study the structure and function of proteins [15]. In addition, once formed, SMALPs are stable and do not require all downstream buffers to be supplemented with polymer, as is the case for detergents, making them technically easier to work with.

Although SMALPs have many advantages for the study of membrane protein structure and function, they are not without their limitations. The polymer is sensitive to divalent cations or to low pH, causing it and any encompassed proteins and lipids to precipitate from solution [16–18]. This is problematic for proteins that require divalent cations for function, such as ABC transporters, which require Mg^{2+} for ATPase activity, or for proteins requiring a low pH. Although one of the major features of SMALPs is their increased stability, some proteins appear to be held too tightly by SMALPs, preventing them from full function/conformational changes [19,20]. Therefore, much recent work has been carried out to develop new polymers with the aim of retaining the advantages of SMA while overcoming some of the limitations [21–34]. One notable polymer is poly(diisobutylene-*alt*-maleic acid) (DIBMA). DIBMA has been shown to have a higher tolerance to divalent cations, to make larger diameter discs and to allow full function/conformational changes of proteins [19,20,22]. However, for some proteins, particularly ABC transporters, DIBMA is much less efficient at extracting proteins and has lower stability [35]. Other polymers have been developed which are reported to have better divalent metal ion resistance, including charge-free, non-ionic and zwitterionic polymers [24,25,27,36,37]. However, many of these still need to be tested with a wide range of different membrane proteins [38–41].

Another challenge with commercially available polymers, such as SMA2000, is their high molecular weight dispersity (*Đ*, previously referred to as the polydispersity index (PDI)). SMA2000 contains a wide array of polymers of different molecular weights. Furthermore, the comonomer sequence is controlled by chain growth probability, and is therefore different for every individual chain. SMA2000 is reported to have an average 2:1 ratio of styrene:maleic acid, but this is not in an ordered sequence along each polymer chain. Instead, the styrene and maleic acid moieties are spread throughout the chain statistically, while obeying the rule that two maleic acids are never adjacent to each other [42]. Commercial polymers such as SMA2000 are made by conventional free radical polymerisation, which leads to the abovementioned characteristics, such as high dispersity. This polydispersity may lead to a lack of homogeneity in the nanoparticles formed, which can be problematic for some downstream applications. It has also made it challenging to understand the optimal features of a polymer for membrane protein extraction and stabilisation. Reversible addition fragmentation chain transfer (RAFT)-mediated polymerisation enables much greater control of the size of the polymer chains formed, thereby decreasing the dispersity [43]. However, the RAFT-mediated polymerisation of a 2:1 SMA polymer typically forms an initial region of alternating styrene and maleic anhydride groups, followed by a styrene tail [42]. This does not fully mimic the non-alternating sequence of SMA2000, which has been widely found to be the most effective polymer to date. Efforts have been made to reproduce this distribution using RAFT polymerisation, but this is much more challenging than synthesising a 1:1 polymer [42]. Unfortunately, the 1:1 polymer is not effective for membrane protein solubilisation [16]. Therefore, the aim of this study was to create a series of 1:1 styrene maleic anhydride copolymers with distinct molecular weights by RAFT-mediated polymerisation. These polymers were modified by the reaction of benzylamine with the maleic anhydride groups. This would form a polymer with a 2:1 ratio of hydrophobic to hydrophilic moieties along its length, thus mimicking the structure of SMA2000 but with a more homogeneous chemical structure (Fig. 1, Table 1). It needs to be stressed that one important difference between the modified alternating SMA and SMA2000 exists. In SMA2000, the carboxylic acid functionalities always appear in pairs, whereas after conversion of the maleic anhydride moieties into so-called amic-acids with benzylamine, the carboxylic acids occur in isolated form, flanked by aromatic groups from styrene and benzylamine. The modified polymers were tested for solubilisation and purification of a range of different membrane proteins and compared with SMA2000.

2. Methods

2.1. Synthesis of SMAnh polymers

Poly(styrene-*alt*-maleic anhydride) (SMAnh) samples with different molecular weights were prepared via RAFT-mediated polymerisation in methyl ethyl ketone (MEK). A typical polymerisation was conducted as follows: in a 100 mL 3-neck flask, a solution of styrene (3.12 g, 30.0 mmol), maleic anhydride (MAnh, 2.94 g, 30.0 mmol), *S*-butyl-*S*′-(1-phenyl ethyl) trithiocarbonate (BPT) RAFT agent (0.68 g, 2.5 mmol) and azobisisobutyronitrile (AIBN) (0.08 g, 0.5 mmol) in 15 mL MEK was degassed for 45 min. The flask was placed in a preheated oil bath at 65 °C and stirred for 1.5 h. The polymer was isolated via precipitation (twice) from diethyl ether and dried overnight in a vacuum oven at 40 °C producing 4.8 g (∼ 70 % yield).

The polymers were characterized by size exclusion chromatography (SEC) in *N*,*N*-dimethylformamide (DMF). The theoretical molecular weight ($M_{n,th}$), experimental molecular weight ($M_{n,exp}$), and dispersity (*Đ*) of the three samples are summarized in Table 1.

2.2. Synthesis of SMA-benzylamine polymers

The obtained polymers (in addition to a commercial SMA1000 (Cray Valley)) were modified with benzylamine as follows. A solution of benzylamine in DMF (4.24 g, 39.6 mmol in 10 mL) was added slowly (over 30 min) to a DMF solution of SMAnh (4 g in 15 mL) in a 100 mL flask. Upon amine addition, a rapid formation of a white precipitate was observed, characteristic of ring opening of MAnh, as primary amines rapidly reacted with MAnh to form the amic-acid. The reaction was left to continue stirring at room temperature for 2 h before isolating the polymer by filtration (or centrifugation) and washing with diethyl ether. The white product obtained was dried under vacuum at 40 °C for 16 h. The dry product was subsequently suspended in water before adding 1 M NaOH solution until all white solids were dissolved. The polymer was obtained either via precipitation from tetrahydrofuran (THF) or acetone, or by freeze-drying.

2.3. *Attenuated total reflectance (ATR) Fourier transform infrared (FTIR) spectroscopy*

ATR-FTIR spectroscopy was conducted on a Thermo Scientific Nicolet iS10 Smart iTR with diamond/ZnSe internal reflection crystal ATR accessory. Polymer samples were analysed in dry state i.e., powder form at room temperature. The method includes 128 scans from 600 cm^{-1}–4000 cm^{-1}, including 64 background scans prior to the analysis of each sample.

2.4. *pH sensitivity testing & DLS*

SMA2000 from Cray Valley was used for comparison throughout the study, and was hydrolysed and prepared as previously described [16,44].

To test pH sensitivity polymer stock solutions (5 % *w/v* in water) were mixed with buffers of different pH in a 96 well plate to obtain a final polymer concentration of 3.3 % (*w/v*). Sodium acetate buffer was used for pH 4 and 5, sodium phosphate buffer for pH 6 and 7, Tris buffer for pH 8 and 9, and CHES buffer for pH 10, all supplemented with 150 mM NaCl. Samples were read at 640 nm using a plate reader (Anthos Zenyth 340 RT), where large light scattering signals were obtained when the polymer precipitated from solution, whereas signals were low when the polymer remained in solution.

To examine the size of discs formed with the various polymers, lipid only SMALPs were formed and analysed by dynamic light scattering (DLS). DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) lipids were dissolved in 2:1 chloroform: methanol, dried under nitrogen, and resuspended in water to form a 10 mg/mL suspension of DMPC. The resuspended DMPC was passed 11× through a 200 nm filter to create liposomes of uniform size (Mini-Extruder, Avanti Polar lipids). For DLS measurements, polymers at pH 7 were mixed with 200 nm DMPC vesicles to obtain a final concentration of 2.5 % (*w/v*) polymer and 1.25 mg/mL lipid. The samples were incubated at room temperature for 1 h in a 96 well plate (Greiner flat black polystyrene wells with a micro-clear bottom). DLS signal was measured using a multi-well plate DLS instrument (Wyatt DynaPro). Scattering intensity data was used to estimate the formation and size of the polymer-lipid particles (Dynamics Software).

Table 1
Characterization of the polymers.

Sample code	$M_{n,th}$	$M_{n,exp}$*	Đ*
WH069	2500	2100	1.32
WH070	5000	4500	1.36
WH073	8100	7000	1.34

[*] SEC was used to determine the experimental molecular weight and dispersity values utilising DMF as mobile phase and calibrated against poly(methyl methacrylate) standards.

2.5. *Lipid solubilisation screening*

DMPC lipids were dissolved in 2:1 chloroform: methanol and dried under nitrogen. The lipid film was resuspended in buffer 1 (20 mM Tris, pH 8, 150 mM NaCl) to form a 2 % (*w/v*) suspension of DMPC liposomes. Liposomes (50 μL) were mixed with 50 μL of each polymer at various concentrations (0–5 % (w/v)) in a 96-well plate and incubated at room temperature for 1 h. Light scattering was measured at 390 nm using a Multiskan Go plate reader.

2.6. *Lipid only SMALP formation*

DMPC liposomes (2 % (w/v)) were mixed with an equal volume of 1 % (*w/v*) polymer (or 5 % for SMA-BA 7 kDa). To remove excess free polymer the sample was run on a Superdex 200 10/300 column (GE Healthcare), equilibrated with buffer 1, at a flow rate of 0.5 mL/min, and absorbance detected at 260 nm. The fractions corresponding to the SMALP peak were collected and stored at 4 °C.

2.7. *Light scattering kinetics*

*Sf*9 insect cell membranes (50 μL) at 60 mg/mL (wet pellet weight) were mixed rapidly with 50 μL 5 % (w/v) polymers or buffer 1 in a cuvette with a 3 mm pathlength, and the light scattering was monitored over time at 390 nm using a spectrophotometer.

2.8. *Cell culture and membrane preparation*

ZipA was expressed in BL21 (DE3) *E. coli* as described previously [14,16]. The construct for *Novosphingobium aromaticivorans* Atm1 (NaAtm1) was a gift from Douglas Rees (Addgene plasmid # 78308; http://n2t.net/addgene:78308; RRID:Addgene_78,308) [45]. The

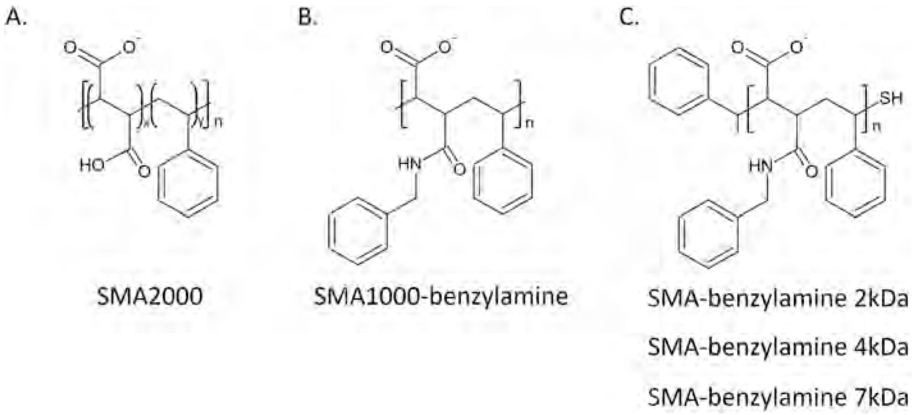

Fig. 1. Structures of benzylamine-modified SMA polymers.

construct was transformed into BL21 (DE3) *E. coli*. Small (5 mL) cultures were grown overnight in Luria broth (LB) supplemented with 100 μg/mL ampicillin (LB-amp). These were used to inoculate large 1 L flasks of LB-amp, which were grown at 37 °C, 200 rpm until an OD_{600} of 0.6 was reached. Expression was induced by addition of 0.5 M isopropyl β-D-1-thiogalactopyranoside(IPTG), and the cultures were left to grow overnight. Cells were resuspended in buffer 2 (50 mM Tris pH 7.4, 250 mM sucrose and 0.25 mM $CaCl_2$, 1 μM pepstatin, 1.3 μM benzamidine and 1.8 μM leupeptin) and disrupted by sonication (5 × 20 s bursts, on ice). A low-speed spin (650 ×*g*, 20 min) removed unbroken cells and debris, then membranes were harvested by ultracentrifugation (100,000 ×*g*, 20 min, 4 °C). Membranes were resuspended in buffer 1 at 60 mg/mL (wet pellet weight) and stored in aliquots at −80 °C.

Human MRP4/ABCC4 (multidrug resistance protein 4/ ATP Binding Cassette transporter C subfamily member 4) was expressed in Sf9 insect cells, disrupted using nitrogen cavitation, and membranes were harvested as described previously [46].

Human AQP4 and CD81 were expressed in *Pichia pastoris* using the pPICZ expression vector. CD81 expression was carried out in baffled shake flasks at 30 °C and induced by the addition of methanol, as previously described [47]. For AQP4, yeast transformed with pPICZB-AQP4 were grown on YPD plates containing 0.1 mg/mL zeocin (Thermo Fisher) at 30 °C for three days and single colonies were picked for 50 mL BMGY starter cultures (20 g/L peptone, 10 g/L yeast extract, 100 mM potassium phosphate buffer pH 6.0, yeast nitrogen base (YNB), 0.4 μg/mL biotin, 0.5 % *v*/v glycerol, 0.1 mg/mL zeocin) at 30 °C in baffled flasks shaken at 220 rpm. After 24 h, 2 mL of starter culture was inoculated into a 2 L Applikon bioreactor vessel containing 1 L BMGY with a proportional-integral-derivative control set to maintain a pH of 6.0, temperature of 30 °C, and dissolved oxygen at 30 % of maximum. Upon starvation (determined by a dissolved oxygen spike), a glycerol-fed batch was initiated using 50 % *v*/v glycerol for four hours at 14 mL/h. Once the glycerol was consumed (determined by dissolved oxygen spike), protein expression was induced using a 50 % v/v methanol feed added at 4.8 mL/h for 36–48 h at 30 °C. Yeast cells were harvested in breaking buffer (5 % glycerol, 2 mM EDTA, 100 mM NaCl, 50 mM sodium phosphate buffer pH 7.4) supplemented with cOmplete protease inhibitor cocktail (Merck) and homogenised using an EmulsiFlex C3 (Avestin). The cell lysate was centrifuged at 4000 ×*g* for 20 min to remove unbroken cells and cell debris, followed by ultracentrifugation of the supernatant at 150,000 ×*g* for 90 min to obtain membranes. The membranes were resuspended in membrane resuspension buffer (20 mM Tris-HCl pH 8, 20 mM NaCl, 10 % glycerol) at a concentration of 180 mg/mL and stored at −80 °C or used immediately.

2.9. Solubilisation of membrane proteins

Membranes (60 mg/mL wet pellet weight) were mixed with an equal volume of 5 % (*w*/*v*) polymer for 1 h at room temperature with shaking. The samples were then subjected to ultracentrifugation (100,000 ×*g* for 30 min, 4 °C). The supernatant containing the solubilised material was harvested, and the pellet containing the insoluble material was resuspended in an equal volume of buffer 1 supplemented with 2 % SDS. Samples of solubilised and insoluble material were analysed by western blotting. For Atm1 and ZipA, a primary anti-His antibody was used (R&D Systems) at a dilution of 1:5000, followed by either anti-mouse HRP (Cell Signalling) or anti-mouse alkaline phosphatase (Sigma), and developed with chemiluminescence (Pierce) and a C-Digit scanner (Licor) or BCIP/NBT (Sigma). For MRP4, an anti-MRP4 antibody M4I-10 (Enzo) was used at 1:100 dilution with an anti-rat HRP secondary antibody (Sigma), developed using chemiluminescence, and imaged using a C-Digit scanner. For AQP4, an anti-AQP4 antibody was used (Abcam, ab128906, dilution 1:1000). For CD81, anti-CD81 2 s131 antibody [48] was used at 1:100, followed by anti-mouse HRP secondary antibody. Solubilisation efficiency was determined by densitometric analysis of western blot images (Image J).

2.10. Ni-NTA affinity purification

Solubilised Atm1 or ZipA membranes were mixed with HisPur Ni-NTA resin (100 μL resin/mL solubilised protein) overnight with shaking at 4 °C. The mixture was then poured into a gravity flow column and the flow-through was collected. The resin was washed with 50 bed volumes (bv) of buffer 1 supplemented with 20 mM imidazole, then 20bv of buffer 1 supplemented with 40 mM imidazole. Proteins were eluted from the column using six washes of 0.5 bed volume with buffer 1, supplemented with 200 mM imidazole.

Elution fractions containing purified protein were pooled, and samples were run on SDS-PAGE alongside bovine serum albumin (BSA) standards (0.25–1.25 μg). The gels were stained with Instant Blue and analysed by densitometry (ImageJ) to determine the yield of purified protein.

2.11. Magnesium sensitivity assays

Samples of purified protein were mixed with various concentrations of $MgCl_2$ (0–10 mM) and then ultracentrifuged (100,000 ×g, 20 min, 4 °C). The supernatant containing soluble protein/SMALPs was harvested, and the pellet was resuspended in an equal volume of buffer 1. Samples of the supernatant and pellet for each concentration of $MgCl_2$ were run on SDS-PAGE, stained with Instant Blue, and analysed by densitometry.

SEC-purified lipid-only SMALPs were mixed with various concentrations of $MgCl_2$ (0–10 mM) in a 96-well plate, and light scattering was measured at 390 nm using a Multiskan Go plate reader.

2.12. Size exclusion chromatography

500 μL of pooled elution fractions following Ni-NTA purification were loaded on a Superdex 200 10/300 column, equilibrated in buffer 1. The column was run at 0.5 mL/min, and 0.5 mL fractions were collected. Fractions corresponding to peaks 1 and 2 were harvested.

2.13. Mass photometry

Samples of purified Atm1 corresponding to peaks 1 and 2 from size-exclusion chromatography were analysed using an MPTwo mass photometer (Refeyn). Samples were pre-diluted to approximately 100 nM in PBS, then diluted tenfold into a PBS droplet on the photometer stage to give a final concentration of approximately 10 nM. Data were collected for 60 s, with the image size set to “regular”.

2.14. Ligand binding assays

Following Ni-NTA purification, imidazole was removed from Atm1 using a PD-10 column. Tryptophan fluorescence of purified Atm1 was measured using a Perkin Elmer LS55 fluorimeter with an excitation wavelength of 280 nm (slit width 10 nm) and emission measured from to 290–500 nm (slit width 20 nm), 3–5 scans were averaged for each condition. Quenching of the peak fluorescence (338 nm) upon sequential addition of 0.02–2.1 mM Ag-GSH (silver glutathione) or 0.05–15.9 mM GSSG (oxidised glutathione) was monitored. Ag-GSH was formed by mixing $AgNO_3$ (0.1 M) and GSH (0.2 M) in 1 M Tris pH 8.2 overnight in the dark and then heated at 100 °C for 5 min. A stock solution of GSSG was prepared at 0.1 M in 1 M Tris pH 8.2.

2.15. Data analysis

Statistical analyses were performed using the GraphPad Prism software. An ANOVA with Dunnett’s *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, and tryptophan quenching ligand binding data were fitted with a binding isotherm by non-linear regression.

3. Results

3.1. Generation & characterization of benzylamine-modified SMA polymers

A series of 1:1 styrene:maleic anhydride polymers with different molecular weights (M_n) and relatively narrow molecular weight distributions were synthesised by RAFT-mediated polymerisation (FTIR spectra in Supplementary Fig. 1). These polymers, along with commercially available SMA1000, were modified by reaction with benzylamine (Table 2, Fig. 1). Apart from the modification of the maleic anhydride groups, benzylamine modification resulted in a reduction of the RAFT end group to a thiol group. SMA2000 was used as a control.

The pH sensitivity of each polymer was investigated by mixing polymers with buffers ranging from pH 2 to 10 and monitoring precipitation by light scattering (Supplementary Fig. 2). Similar to SMA2000, benzylamine-modified polymers precipitated from solution at low pH. Some of them, particularly the higher-molecular-weight polymers, also precipitated slightly at pH 10. The best working pH range appeared to be pH 7 to 9, so further experiments were carried out at pH 8, which has become the standard for SMA2000.

The polymers were then tested for their ability to solubilise purified lipids from DMPC vesicles (Fig. 2). All polymers were effective at a concentration of 2.5 % (*w/v*) polymer, with the solutions quickly turning optically clear. SMA2000, SMA1000-BA, SMA-BA 2 kDa and SMA-BA 4 kDa were all effective at solubilising lipids down to a concentration of 0.5 % (w/v). However, SMA-BA 7 kDa was less effective at concentrations below 1 % (w/v). Therefore, a standard polymer concentration of 2.5 % (w/v) was used for further experiments.

Analysis of the formed SMALPs by size exclusion chromatography showed that SMALPs formed using SMA-BA 4 kDa were very similar to those formed using SMA2000, with a peak elution at 11.5 mL (Supplementary Fig. 3 A). The excess free polymer results in peaks at 17–18 mL, and a lower dispersity of the RAFT-derived polymer compared to that of SMA2000 can be seen by the narrower peak for the free polymer. The size of the particles formed from each polymer was analysed by DLS, and there were no significant differences between the different polymers and the standard SMA2000 (Supplementary Fig. 3B).

3.2. Kinetics of membrane solubilisation

To try to investigate more subtle differences between the polymers, the kinetics of solubilisation were investigated. To do this, Sf9 insect cell membranes were used rather than purified lipids, because they represent a complex biological membrane, including both lipids and proteins. The solubilisation of Sf9 membranes was monitored by measuring light scattering in a spectrophotometer (Fig. 3).

It can be seen that SMA2000 causes a rapid decrease in light scattering, with a > 50 % decrease in signal within 4 s, and appearing to plateau within 1 min (Fig. 3A). The 2 kDa and 4 kDa benzylamine-modified polymers also cause a rapid decrease in signal, but notably, the 2 kDa polymer has a faster initial solubilisation than the 4 kDa polymer. The 7 kDa benzylamine polymer shows much slower solubilisation kinetics, and includes an initial increase in light scattering. Despite the differences in the initial kinetics, after 1 h of incubation, the signal is very similar for all polymers (Fig. 3B).

Table 2
Properties of the polymers. SMA2000 and the unmodified SMA1000 polymer were manufactured by Cray Valley. The other polymers were synthesised via RAFT-mediated polymerisation. The polymers were fully modified by the addition of benzylamine to the maleic anhydride groups.

Polymer name	Properties of unmodified polymers			Modification
	Styrene:maleic anhydride	M_n (kDa)	Đ	
SMA2000	2:1	3	2.5	hydrolysed to maleic acid
SMA1000-benzylamine	1:1	2	2.75	benzylamine
SMA-benzylamine 2 kDa	1:1	2.1	1.32	benzylamine
SMA-benzylamine 4 kDa	1:1	4.5	1.36	benzylamine
SMA-benzylamine 7 kDa	1:1	7	1.34	benzylamine

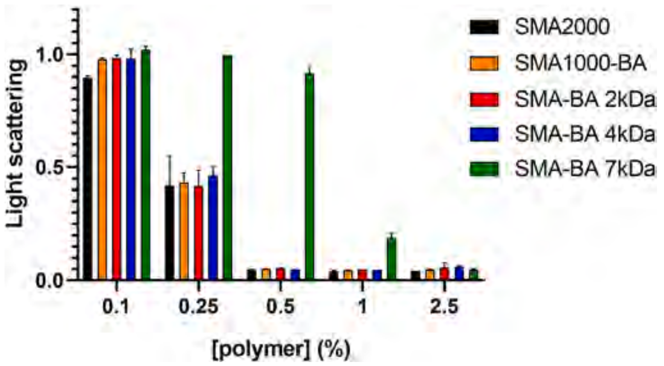

Fig. 2. Solubilisation of DMPC vesicles using benzylamine-modified polymers. DMPC vesicles (50 μL of 2 % (w/v) suspension) were mixed with 50 μL 5 % (w/v) polymers or buffer 1, in a 96 well plate. Light scattering was measured at 390 nm and normalised to the signal obtained upon addition of buffer. Data are presented as mean ± SD ($n = 2$).

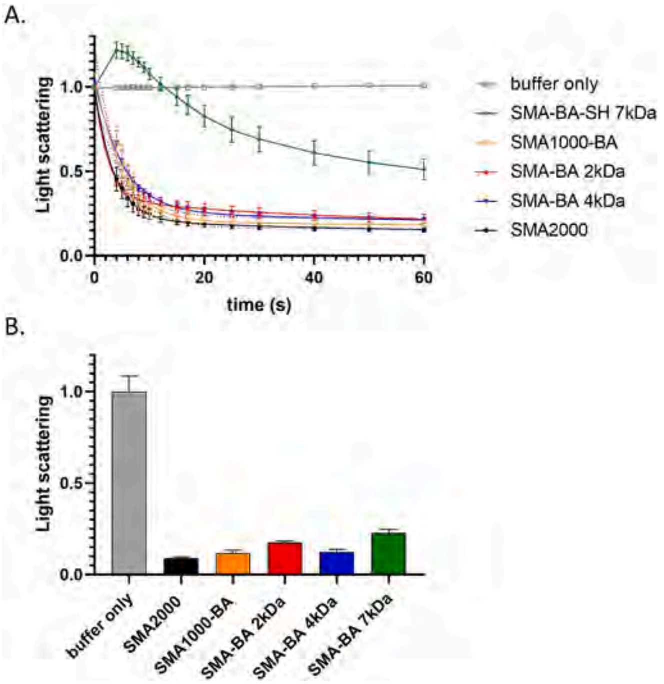

Fig. 3. Kinetics of solubilisation of Sf9 insect cell membranes. A; Sf9 insect cell membranes (50 μL) at 60 mg/mL (wet pellet weight) were mixed rapidly with 50 μL 5 % (*w/v*) polymers or buffer 1, in a cuvette with 3 mm pathlength, and the light scattering monitored over time at 390 nm. Data are mean ± sem ($n \geq 3$). B; Samples prepared as in A, but measured after 1 h incubation at room temperature.

3.3. Solubilisation of membrane proteins using benzylamine-modified SMA polymers

Having established that the polymers can solubilise both lipids and biological membranes, the next step was to investigate the solubilisation of specific membrane proteins. A selection of different proteins and expression systems were tested. Atm1 is an ABC transporter that was recombinantly expressed in *E. coli*. The functional protein is a dimer, and each monomer contains six transmembrane helices and a cytosolic domain. ZipA was also expressed in *E. coli,* but has a different structure, with only a single transmembrane helix and a large cytosolic domain. MRP4 is another ABC transporter, but is a monomer of 12 transmembrane helices and was expressed in Sf9 insect cells. CD81 is a tetraspanin and AQP4 is a water channel (the functional protein is a tetramer), both of which were expressed in *Pichia pastoris*.

As shown in Fig. 4, the degree of solubilisation achieved using SMA2000 varied among the different proteins and expression systems, with MRP4 achieving the highest mean solubilisation efficiency (84 %), followed by Atm1 (73 %), CD81 (64 %), ZipA (61 %), and AQP4 (49 %). A significantly lower degree of solubilisation for Atm1, ZipA, MRP4 and CD81 was observed with SMA-BA 7 kDa, but otherwise there were no significant differences in solubilisation efficiency between the various polymers and SMA2000 for these proteins. In contrast, for AQP4 expressed in *P. pastoris* SMA1000-BA, SMA-BA 2 kDa and SMA-BA 7 kDa all gave significantly lower solubilisation efficiency than SMA2000. Interestingly, the smaller 2 kDa polymer also seemed to stabilise AQP4 less well, with a degradation product visible on the western blots (Supplementary Fig. 4). Overall, however, each of the polymers worked for each protein family and expression system to some degree.

3.4. Purification of membrane proteins using benzylamine-modified SMA polymers

Having established that all the polymers were able to solubilise membranes and membrane proteins, their use in protein purification was investigated. As shown in Fig. 5, all the different polymers were capable of purifying the ABC transporter Atm1 using Ni^{2+} affinity chromatography. Strong bands are observed in the eluted fractions at approximately 60 kDa for each of the polymers tested. Similar results were obtained for ZipA (Supplementary Fig. 5).

There is some variation in the apparent degree of purity and the intensity of the bands; therefore, the yield and purity were quantified across a number of different purifications (Fig. 6). An average degree of purity of 87 ± 10 % was achieved using SMA2000. There is little difference in the degree of purity obtained with the modified polymers, although SMA-BA-4 kDa does give a significantly higher degree of purity than SMA2000. The average yield of Atm1 obtained using SMA2000 was 0.68 ± 0.24 μg protein/mg membrane, and comparable yields were obtained with SMA1000-BA, SMA-BA-2 kDa and SMA-BA-4 kDa. However, SMA-BA 7 kDa gave a significantly decreased yield of the purified protein.

3.5. Characterization of purified membrane proteins

Since ABC transporters require Mg^{2+} ions for their ATPase activity, and sensitivity to divalent cations is one of the current limitations of SMA2000, the sensitivity of the SMALPs formed with benzylamine-modified polymers was investigated. As shown in Fig. 7, protein-lipid nanoparticles formed using SMA2000 show a dose-dependent response to increasing $MgCl_2$ concentrations, with approximately 50 % of the protein precipitating and being lost from solution at 5 mM $MgCl_2$. Unfortunately, nanoparticles formed with the benzylamine-modified polymers were even more sensitive to divalent cations, as seen by the shift in the curves to the left, meaning that the SMALPs/proteins precipitate from solution at lower $MgCl_2$ concentrations.

Despite the increased sensitivity to magnesium, the SMA-BA 4 kDa polymer showed promising results, with comparable solubilisation efficiency, comparable pure protein yield, and increased purity compared to SMA2000. Therefore, this polymer was used for a more detailed analysis of the purified protein-lipid particles in comparison to SMA2000. Following Ni^{2+} affinity purification, as shown in Fig. 5, the eluted proteins for both polymers were analysed using size exclusion chromatography (SEC) (Fig. 8A). Both SMA-BA 4 kDa and SMA2000 gave very similar SEC profiles, with one main peak eluting at approx. 10 mL (designated peak 2), and a shoulder on this peak eluting before it (approx. 9 mL, designated peak 1). Samples from these two peaks were collected and analysed using mass photometry. Peak 1 contained particles of approximately 200 kDa in size, but also some much larger

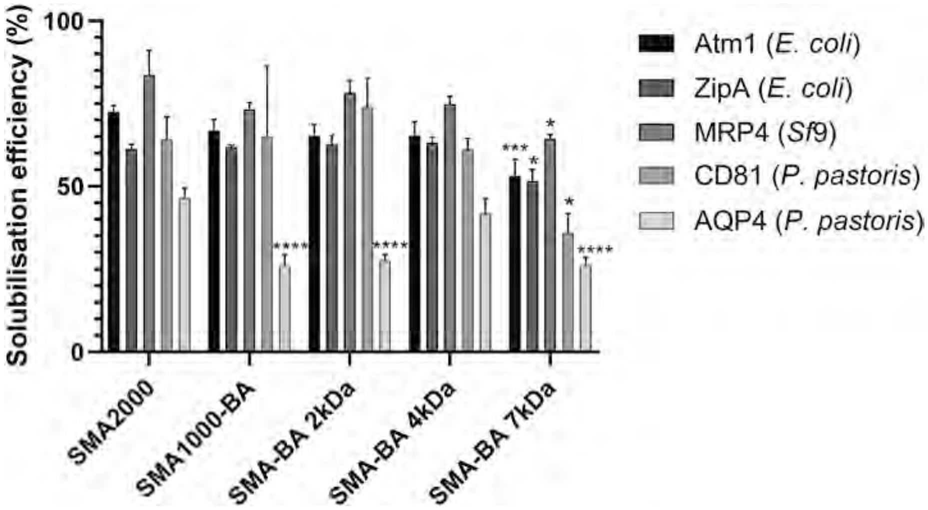

Fig. 4. Benzylamine modified SMA polymers are effective at solubilising membrane proteins. The ABC transporter Atm1 or the membrane tether ZipA were expressed in E.coli. The ABC transporter MRP4/ABCC4 was expressed in Sf9 insect cells. CD81 and Aquaporin 4 (AQP4) were expressed in P. pastoris. Membranes (30 mg/mL wet weight) were solubilised with 2.5 % (*w/v*) polymer for 1 h at room temperature. Samples were ultracentrifuged (100,000 ×g, 30 min, 4 °C). Supernatant (containing solubilised protein) and resuspended pellet (containing insoluble material) were analysed by western blot using an anti-his primary antibody, anti-MRP4 primary antibody (M_4I), anti-CD81 primary antibody or anti-AQP4 primary antibody, and analysed by densitometry (Image J). Data are mean ± sem, $n \geq 8$ (Atm1), $n = 3$ (ZipA, MRP4, CD81, AQP4). Data were analysed by ANOVA with a Dunnett's post-hoc test, $*p < 0.05$, $**p < 0.001$, significantly different to SMA2000.

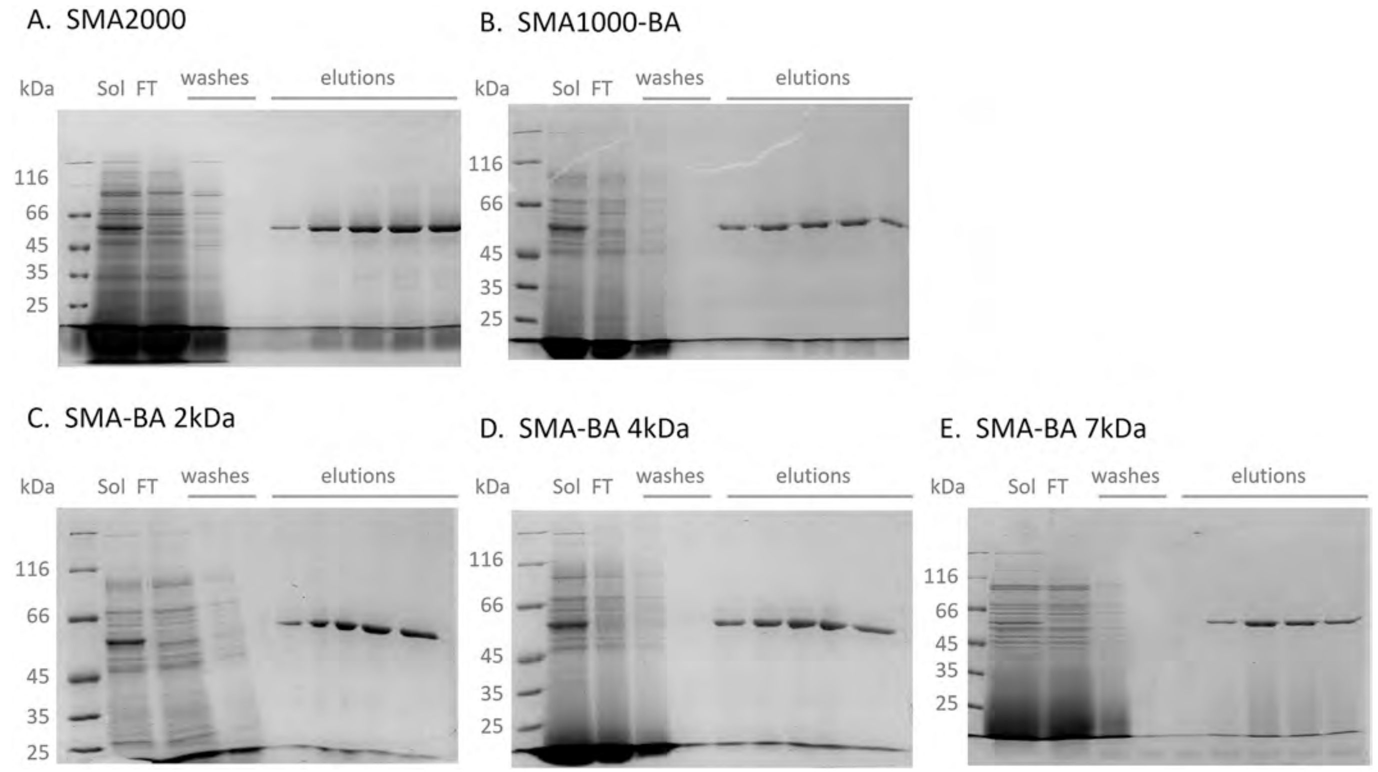

Fig. 5. Atm1 can be purified using benzylamine modified polymers. Polymer solubilised membranes (Sol) from E.coli expressing Atm1 were mixed with HisPur resin (100 μL/mL soluble protein) overnight at 4 °C, then transferred to a gravity flow column and the flowthrough (FT) collected. The resin was washed with 50bv buffer containing 20 mM imidazole and 20bv containing 40 mM imidazole. Atm1 was eluted in 0.5bv aliquots using buffer supplemented with 200 mM imidazole. Samples were run on SDS-PAGE and stained with InstantBlue. Representative images of ≥8 repeats.

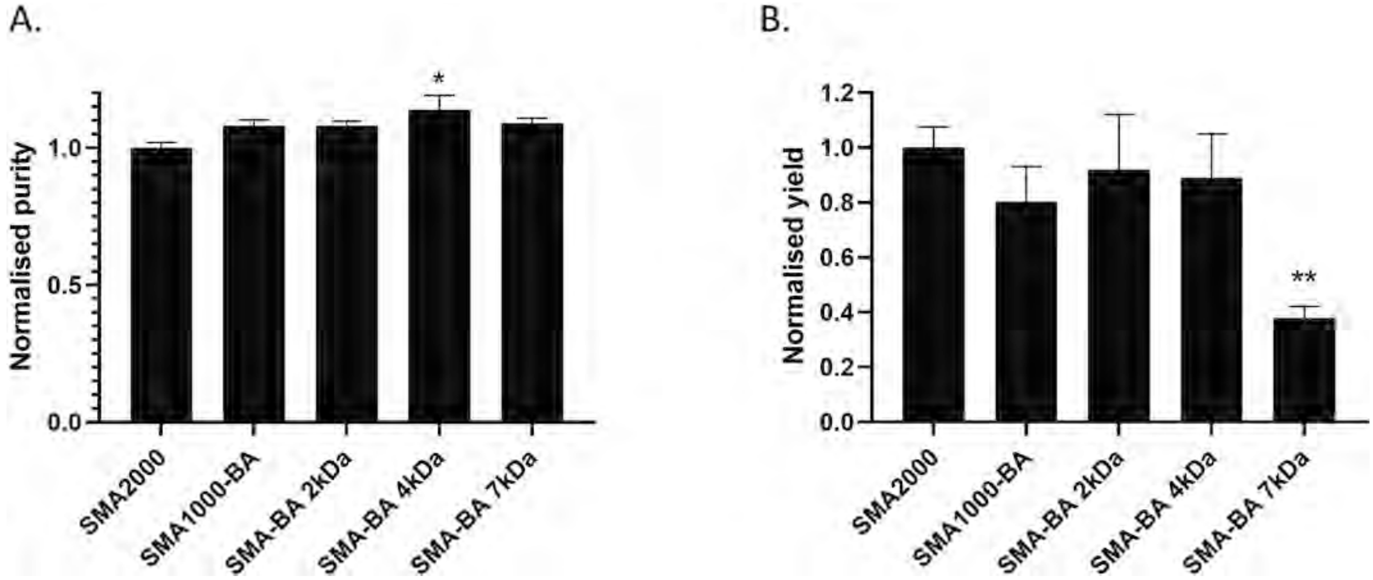

Fig. 6. Polymer size affects the yield of purified Atm1. A; The degree of purity achieved following affinity purification was measured by densitometric analysis (ImageJ) of a single lane of purified protein run on SDS-PAGE. Data are mean ± sem, $n \geq 7$. B; The yield of purified protein was quantified by densitometric analysis of samples run on SDS-PAGE alongside BSA standards (0.25–1.5 μg). Data are mean ± sem, $n \geq 4$. All values obtained were corrected for membrane batch-to-batch variation in purity and yield by normalising to the values obtained with SMA2000 using the same membrane preparation. Data were analysed by a one-way ANOVA with a Dunnett's post-hoc test. $^{*}p < 0.05$, $^{**}p < 0.01$, significantly different to SMA2000.

particles (Supplementary Fig. 6). However, for both polymers, peak 2 appeared to be relatively homogenous, with very similar distributions and an average size of 207 kDa (Fig. 7B). This corresponds well to a SMALP particle containing a protein of 133 kDa and associated lipids and polymers.

Finally, the ligand-binding function of the purified Atm1 was measured using a tryptophan fluorescence quenching assay [49]. Clear dose-dependent fluorescence quenching upon the addition of Ag-GSH

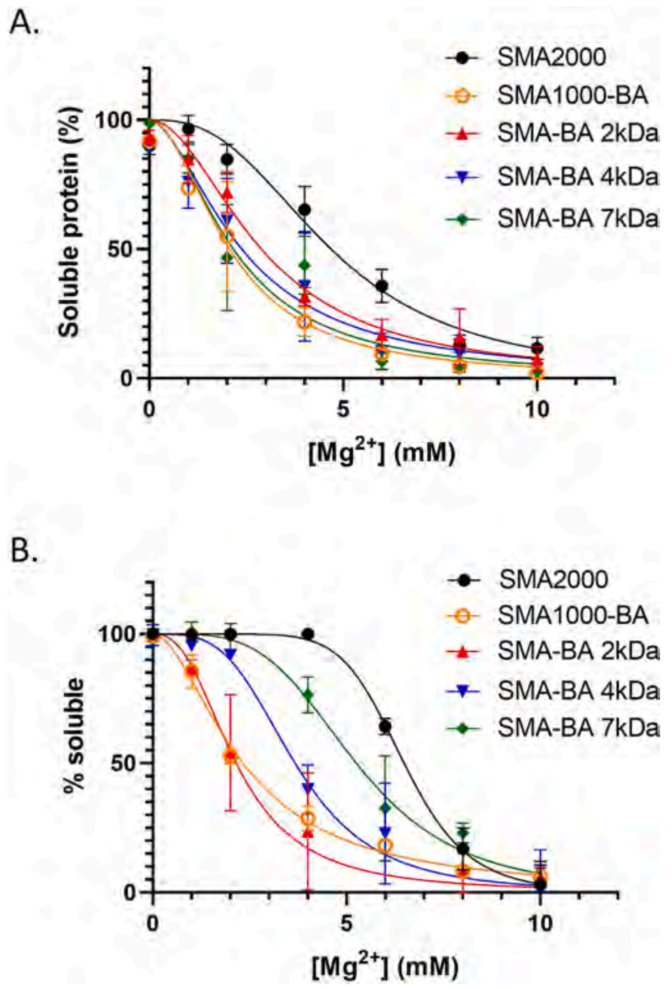

Fig. 7. Benzylamine-modified SMALPs are even more sensitive to divalent cations. A; Purified Atm1 in each polymer was mixed with $MgCl_2$ (0–10 mM) and ultracentrifuged (100,000 ×g 30 min, 4 °C). Supernatant was harvested and pellet resuspended, and both run on SDS-PAGE. Stained with InstantBlue and analysed by densitometry (ImageJ). B; Lipid only SMALPs were formed from DMPC with each polymer and purified by SEC. They were mixed with $MgCl_2$ (0–10 mM) in a 96-well plate and light scattering measured at 395 nm. Data are mean ± sem ($n \geq 2$), and were fitted with a dose-response curve.

(Fig. 8C) and GSSG (Fig. 8D) was observed, with little difference between the two polymers. The K_d for Ag-GSH is 0.23 mM for SMA2000 and 0.28 mM for SMA-BA 4 kDa, whereas for GSSG it is 7.4 mM for SMA2000 and 5.5 mM for SMA-BA 4 kDa.

4. Discussion

In this study, benzylamine-modified 1:1 SMA RAFT polymers were generated with the aim of mimicking the hydrophilic/hydrophobic balance of SMA2000, yet allowing much tighter control over the polymer length and homogeneity. All polymers generated were able to solubilise lipids and membrane proteins. When looking at the kinetics of solubilisation of Sf9 insect cells, it was notable that the smaller polymer (M_n 2 kDa) gave an initial rate of solubilisation that was faster than the 4 kDa polymer, and the 7 kDa polymer was much slower still (Fig. 3). However, AQP4 protein solubilised with the smaller 2 kDa polymer appeared to be less stable than that solubilised with the larger polymers (Supplementary Fig. 4). This agrees with previous reports on fractionated SMA2000, or SMA polymers of different lengths produced by RAFT polymerisation, which found that polymers with a lower M_n solubilise lipids more quickly and efficiently, but SMALPs formed from larger polymers are more stable [42,50]. Notably, those studies found no differences in the size of SMALPs formed using polymers of different lengths, which is in agreement with our results (Supplementary Fig. 3). Another interesting observation from the kinetics results is the initial increase in light scattering observed upon addition of SMA-BA 7 kDa. While we do not know exactly what this represents, it could be hypothesised that it represents the initial association of the polymer with liposomes, or the initial steps of solubilisation, such as the formation of pores in the lipid bilayer, as visualised previously by electron microscopy for a DIBMA variant [51] or predicted by molecular modelling [52]. If it is an initial step in the molecular process of SMALP formation, although this initial increase in scattering is only seen for the SMA-BA 7 kDa polymer, it would likely also occur with other polymers, including SMA2000, but happens too quickly to be observed with the experimental setup used. In the future, it would be interesting to study this initial step using stopped-flow spectroscopy.

The degree of solubilisation of specific membrane proteins from a variety of expression systems showed variability between proteins, even with the control SMA2000 polymer. However, the solubilisation efficiencies measured for MRP4 from insect cells [53], ZipA from *E. coli* [16,21] and CD81 from *P. pastoris* [47] agree well with previous studies, and Atm1 is comparable to other ABC transporters extracted from *E. coli* [16,21]. For each of these proteins, no significant differences were observed between SMA2000 and the benzylamine-modified polymers, except for SMA-BA 7 kDa, which resulted in a lower solubilisation efficiency for all proteins. Again, this would fit with the suggestion that larger polymers are less efficient for membrane solubilisation [42,50]. AQP4 expressed in *P. pastoris*, however, showed the lowest degree of solubilisation and much more variability among the different polymers. This difference would appear to be protein specific, and not due to the expression system used, since CD81 was also expressed in *P. pastoris*. Given that the solubilisation of membranes by SMA is often thought to be driven by an interaction between the polymer and lipids, it is interesting that the protein can impact it. Perhaps AQP4 interacts with specific lipids or affects the physical properties of surrounding lipids, and impacts solubilisation indirectly, or perhaps the protein can directly affect solubilisation and SMALP formation. Further studies are needed to fully understand the molecular mechanisms by which SMALPs are formed.

Affinity purification of proteins encapsulated in SMALPs formed by each of the different polymers was successful. The SMA-BA 7 kDa protein gave a significantly lower yield, as might be expected given its lower solubilisation efficiency. Combined with the apparent lower stability of proteins in SMALPs formed from the 2 kDa polymer, this would suggest that a polymer with an M_n of approximately 4 kDa is the best choice. Following affinity purification, analysis of the eluted proteins by size exclusion chromatography showed similar profiles for both SMA-BA 4 kDa and SMA2000, with a main peak at approximately 10 mL, which yielded a relatively monodisperse sample when analysed by mass photometry (Fig. 8). Mass photometry revealed a particle size of approximately 200 kDa, suggesting that the SMALPs contained a single transporter protein. The active protein is a dimer with molecular weight of 133 kDa, and the additional mass is from the lipids and polymer within the particle [54]. Both samples also showed a shoulder before the main peak, which contained larger particles. This shoulder might represent higher oligomers of the protein, or it could just be aggregates, but notably this shoulder appeared to be larger for SMA2000 than for SMA-BA 4 kDa.

Unfortunately, benzylamine-modified polymers had an even greater sensitivity to divalent cations than SMA2000. This is similar to what was previously reported for the partially esterified polymer SMA1440 [21]. While the mechanism of sensitivity to divalent cations is not fully understood, it adds weight to the argument that it relates to the overall hydrophobicity of the polymer [21]. The benzylamine-modified 1:1 SMA polymer mimics SMA2000 in that there are two hydrophobic aromatic groups to one hydrophilic acid group; however, it is just a single

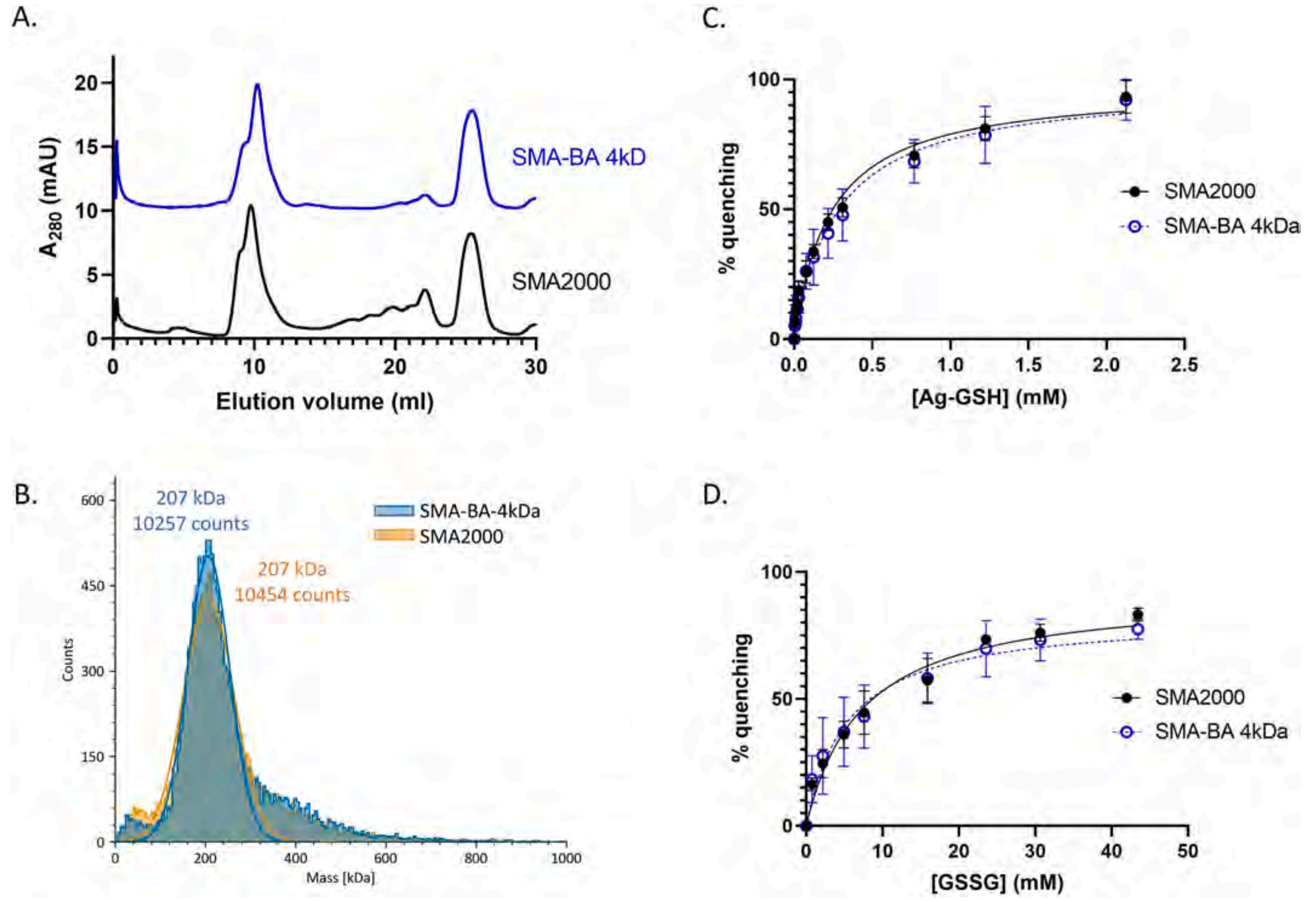

Fig. 8. Atm1 purified using SMA-BA 4 kDa is comparable to SMA2000. A; Atm1 purified by Ni-NTA chromatography using either SMA2000 (black) or SMA-BA 4 kDa (blue) was analysed by size exclusion chromatography using a superdex200 10/300 column. B; Samples corresponding to peak 2 of the size exclusion trace were analysed by mass photometry. C & D; Binding of the substrates Ag-GSH (C) or GSSG (D) to purified Atm1 was analysed by tryptophan fluorescence quenching. Data are mean ± SD, $n \geq 4$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

carboxylic acid group compared to the two carboxyl groups of maleic acid. Therefore, the polymer has a higher degree of hydrophobicity than SMA2000. Due to the sensitivity of all polymers to $Mg^{2+,}$ it was not possible to measure Atm1 function in terms of ATPase activity. However, ligand binding assays showed that purified Atm1 was able to bind known ligands with affinities comparable to those reported in the literature, and there was no difference between SMA-BA-SH 4 kDa and SMA2000 [49].

Despite not overcoming the issue of divalent cation sensitivity, it can be concluded that the approach of modifying a 1:1 RAFT polymer with benzylamine to increase the ratio of hydrophobic to hydrophilic moieties to mimic the structure of SMA2000, whilst controlling polymer length and sequence, does work. In the future, it would be interesting to investigate alternative modifications of benzylamine. In terms of protein solubilisation, purification, and characterization, SMA-BA 4 kDa was comparable to SMA2000 and gave a higher degree of purity. It will be interesting going forward to investigate whether the lower polydispersity of SMA-BA 4 kDa compared to SMA2000 is beneficial for downstream applications, such as structural studies.

CRediT authorship contribution statement

Aneel Akram: Investigation, Formal analysis. **Waled Hadasha:** Investigation, Formal analysis. **Gestél C. Kuyler:** Investigation, Formal analysis. **Michael-Phillip Smith:** Investigation, Formal analysis. **Shauna Bailey-Dallaway:** Investigation. **Aiden Preedy:** Investigation. **Caolan Browne:** Investigation. **Luke Broadbent:** Investigation. **Adam Hill:** Investigation. **Tahreem Javaid:** Investigation. **Haroon Nazar:** Investigation. **Nikita Samra:** Investigation. **Anadil Naveed:** Investigation. **Holly Tregunna:** Investigation. **Hetal Joshi:** Investigation. **Nusheen Akhtar:** Investigation. **Aneesa Javed:** Investigation. **Jessica Bowater:** Investigation. **Joel Ravenhill:** Investigation. **Patrik Hajdu:** Investigation. **Yazdan Ali:** Investigation. **Yanik Tailor:** Investigation. **Sabreen Mumtaz:** Investigation. **Mohammed Hamza:** Investigation. **Kiran Gill:** Investigation. **Jemma Gillett:** Investigation. **Faye Patton:** Investigation. **Huma Arshid:** Investigation. **Maria Zaheer:** Investigation. **Hannah Qureshi:** Investigation. **Isabel Edwards:** Investigation. **Shreya Patel:** Investigation. **Aaminah Azadi:** Investigation. **Naomi Pollock:** Writing – review & editing, Methodology, Investigation. **Philip Kitchen:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Bert Klumperman:** Writing – review & editing, Supervision, Resources, Methodology, Formal analysis, Conceptualization. **Alice J. Rothnie:** Writing – original draft, Supervision, Resources, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors have no conflicting interests to declare that are relevant to the content of this article. GCK and BK are directors of Nanosene (Pty) Ltd., a company that commercializes amphiphilic copolymers for the isolation of membrane proteins.

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

Data availability

The underlying data for this study can be found at the Aston Explorer Data Repository (https://doi.org/10.17036/researchdata.aston.ac.uk.00000647).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bpc.2024.107343.

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