Accepted Manuscript


Examining the stability of membrane proteins within SMALPs

Aiman A. Gulamhussein, Danyall Meah, Damian D. Soja, Stephen Fenner, Zakaria Saidani, Aneel Akram, Simran Lallie, Ashlyn Mathews, Culum Painter, Monique K. Liddar, Zain Mohammed, Lai Ki Chiu, Sabiha S. Sumar, Hannah Healy, Nabeel Hussain, Jaimin H. Patel, Stephen C.L. Hall, Timothy R. Dafforn, Alice J. Rothnie

PII: S0014-3057(18)31183-2
DOI: https://doi.org/10.1016/j.eurpolymj.2018.12.008
Reference: EPJ 8740

To appear in: *European Polymer Journal*

Received Date: 27 June 2018
Revised Date: 13 November 2018
Accepted Date: 5 December 2018

Please cite this article as: Gulamhussein, A.A., Meah, D., Soja, D.D., Fenner, S., Saidani, Z., Akram, A., Lallie, S., Mathews, A., Painter, C., Liddar, M.K., Mohammed, Z., Ki Chiu, L., Sumar, S.S., Healy, H., Hussain, N., Patel, J.H., Hall, S.C.L., Dafforn, T.R., Rothnie, A.J., Examining the stability of membrane proteins within SMALPs, *European Polymer Journal* (2018), doi: https://doi.org/10.1016/j.eurpolymj.2018.12.008

# Examining the stability of membrane proteins within SMALPs

Aiman A. Gulamhussein[*], Danyall Meah[*], Damian D. Soja[*], Stephen Fenner[*], Zakaria Saidani[*], Aneel Akram[*], Simran Lallie[*], Ashlyn Mathews[*], Culum Painter[*], Monique K. Liddar[*], Zain Mohammed[*], Lai Ki Chiu[*], Sabiha S. Sumar[*], Hannah Healy[*], Nabeel Hussain[*], Jaimin H. Patel[*], Stephen C. L. Hall[†], Timothy R. Dafforn[†] & Alice J. Rothnie[*].

[*]School of Life & Health Sciences, Aston University, Aston Triangle, Birmingham, B4 7ET, UK

[†]School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

Corresponding author: Dr Alice Rothnie, School of Life & Health Sciences, Aston University, Aston Triangle, Birmingham, B4 7ET, UK. a.rothnie@aston.ac.uk
Tel. +44 (0)121 204 4013

**Abstract:**

Amphipathic co-polymers such as styrene-maleic acid (SMA) have gained popularity over the last few years due to their ability and ease of use in solubilising and purifying membrane proteins in comparison to conventional methods of extraction such as detergents. SMA2000 is widely used for membrane protein studies and is considered as the optimal polymer for this technique. In this study a side-by-side comparison of SMA2000 with the polymer SZ30010 was carried out as both these polymers have similar styrene:maleic acid ratios and average molecular weights. Ability to solubilise, purify and stabilise membrane proteins was tested using three structurally different membrane proteins. Our results show that both polymers can be used to extract membrane proteins at a comparable efficiency to conventional detergent dodecylmaltoside (DDM). SZ30010 was found to give a similar protein yield and, SMALP disc size as SMA2000, and both polymers offered an increased purity and increased thermostability compared to DDM. Further investigation was conducted to investigate SMALP sensitivity to divalent cations. It was found that the sensitivity is polymer specific and not dependent on the protein encapsulated. Neither is it affected by the concentration of SMALPs. Larger divalent cations such as $Co^{2+}$ and $Zn^{2+}$ resulted in an increased sensitivity.

**Highlights:**

- SMA polymers SZ300110 and SMA2000 are comparable for protein solubilisation, yield, purity and thermostability.
- Sensitivity of SMALPs to $Mg^{2+}$ is similar for different membrane proteins.
- The sensitivity to $Mg^{2+}$ is independent of the concentration of SMALPs
- SMALPs are even more sensitive to larger divalent cations

**Keywords:**

membrane protein, SMALP, styrene-maleic acid co-polymer, detergent, divalent cation, thermostability

**Introduction:**

Biological membranes are complex and dynamic mixtures of diverse lipids and proteins. The proteins within the membrane provide the main link between the inner workings of the cell and the external environment, as such they play a vital role in intracellular communication and the control of molecular movement across the membrane. This makes them key therapeutic targets for a wide range of human diseases, and more than 50% of current therapeutics are targeted towards membrane proteins. Their location within the membrane, tightly packed with so many different proteins and lipids has made them historically extremely challenging to study. To gain a meaningful insight into the structure and function of a single protein, that protein needs to be separated from the complexity of the membrane. Traditionally, this has been achieved using detergents, however these often destabilise the protein structure and strip away the native lipid environment which is important for function [1, 2].

In 2009 this issue was partially resolved by the use of styrene-maleic acid (SMA) co-polymers for extracting small discs of membrane, termed SMA lipid particles (SMALPs) from native membranes [3]. This method has greatly simplified how membrane proteins can be purified and studied, whilst maintaining the lipid bilayer environment of the protein [3-5].

To date SMA solubilisation and subsequent affinity purification has been demonstrated to be effective for a wide range of proteins including transporters, ion channels, enzymes and G-protein coupled receptors (GPCRs), from many different expression systems [6-12]. SMALPs are small and soluble particles which are amenable to many downstream techniques including spectroscopy and ligand binding assays [6, 8, 9, 11, 13], as well as structural studies both by crystallography and electron microscopy [6, 12, 14-16]. SMALP-encapsulated proteins have been shown to have an enhanced stability compared to traditional detergent approaches [6-9, 17].

We have previously investigated a range of different commercially available SMA polymers with varying ratios of styrene:maleic acid and varying size, and showed that SMA2000 (Cray Valley) was the optimal polymer for SMALP formation in terms of solubilisation efficiency, protein yield, purity and function [17]. SMA2000 has a reported styrene:maleic acid ratio of 2:1 and an average molecular weight ($M_w$) of 7500 Da (Supplementary Table 1). However in the past year it has become clear that researchers are beginning to use another polymer, Xiran SZ30010 from Polyscope, which is closely related to SMA2000. SZ30010 has a styrene:maleic acid ratio of 2.3:1 and a $M_w$ of 6500 Da, and thus is the most similar to SMA2000, however without a side by side comparison with SMA2000 its real performance in protein extraction remains unresolved.

In this study we systematically compare the performance of SZ30010, SMA2000 and the conventional detergent dodecylmatoside (DDM) in solubilising three different membrane proteins. We also examine the behaviour of each protein preparation during purification to determine whether SZ30010 offers any performance advantage over DDM and SMA2000.

We have previously reported that one of the disadvantages of SMALPs is the sensitivity to divalent cations, such as magnesium or calcium, which can be problematic for measuring

the function of some proteins [6, 17]. We follow up on this observation and investigate the sensitivity of each polymer to divalent cations in more detail.

**Materials & Methods:**

***Polymer preparation***

SMA2000 was a kind gift from Cray Valley (Exton, PA, USA), and SZ30010 and SZ25010 were kind gifts from Polyscope (Geleen, Netherlands). Each of the polymers was provided as a styrene maleic anhydride co-polymer, and hydrolysed to styrene maleic acid by reflux in 1 M NaOH as described previously [4, 18]. Polymers were freeze-dried and stored long term as a powder at room temperature.

***Protein expression and membrane preparation***

BmrA with a C-terminal $His_6$-tag in the vector pET-23b- BmrA was a kind gift from Prof. Jean-Michel, Jault, IBCP, Lyon. ZipA with a C-terminal $His_6$-tag in the vector pET101-ZipA was a kind gift from Dr David Roper, University of Warwick. The vector pET16b-LeuT containing LeuT with an N-terminal $His_8$-tag was from Prof. Harald Sitte, Medical University of Vienna.
BmrA, ZipA and LeuT were expressed in *E. coli* as described previously [17]. Briefly, small (5 ml) overnight cultures of *E. coli* transformed with plasmids containing the target proteins were used to inoculate 1 l cultures of LB (Luria Broth) supplemented with 100 µg/ml ampicillin. Cultures were grown at 37°C, 200 rpm until the $OD_{600}$ reached 0.6. Protein expression was induced by addition of 0.5 mM IPTG, the temperature was reduced to 25°C and the cultures incubated overnight. Cells were harvested by centrifugation (6000 g, 10 min), resuspended in buffer 1 (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 on ice (5 x 20 s bursts). A low speed spin (650 g, 20 min) was used to remove unbroken cells and debris, then membranes were harvested by ultracentrifugation (100000 g, 20 min, 4°C). Membranes were resuspended in buffer 2 (20 mM Tris pH 8, 150 mM NaCl) at 60 mg/ml (wet pellet weight) and stored in aliquots at -80°C.

***Solubilisation and purification***

Solubilisation and His-tag affinity purification were carried out as described previously [6, 17, 18]. Membranes (30 mg/ml wet pellet weight) were mixed with 2.5 %(w/v) SMA polymer or 2 %(w/v) DDM (VWR) for 1 h at room temperature. Samples were subjected to ultracentrifugation (100000 g, 20 min, 4°C) and soluble protein in the supernatant was harvested. Insoluble material within the pellet was resuspended in the same volume of buffer 2 supplemented with 2% (w/v) SDS. Solubilisation efficiency was calculated by running samples of both the supernatant and pellet on a Western blot and probing with anti-his antibody (1:1000, R&D Systems), followed by either anti-mouse alkaline phosphatase (Sigma) or anti-mouse HRP (Cell Signalling) and visualised using BCIP/NBT (Sigma) or Supersignal West chemiluminescence (Fisher) respectively. The percentage of protein solubilised was analysed by densitometry (ImageJ).

Solubilised protein was mixed with HisPur Ni-NTA resin (Fisher) at a ratio of 100 µl bed volume (bv) per ml of solubilised protein, overnight with shaking at 4°C. It was then transferred to a gravity flow column (Machery-Nagel) and the flow-through collected. The resin was washed with 50 bv buffer 2 supplemented with 20 mM imidazole, 20 bv buffer 2 supplemented with 40 mM imidazole, and 1 bv buffer 2 containing 60 mM imidazole.

Proteins were eluted in 6 fractions of ½ bv each using buffer 2 supplemented with 200 mM imidazole. For purification using DDM, all buffers also contained 0.1 %(w/v) DDM. Fractions were analysed by SDS-PAGE and stained with Instant Blue (Expedeon). Elution fractions were pooled and stored at 4°C.

***Protein quantification***

The concentration of purified protein obtained was determined by SDS-PAGE and densitometry using BSA as a standard as described previously [17, 19]. This method was used as it is not affected by imidazole, lipids or polymer present in the sample. BSA standards (0.125 – 1.25 µg) were run on SDS-PAGE alongside samples (10 µl and 20 µl) of purified protein and stained with Instant Blue. The gel was analysed by densitometry (Image J), a BSA standard curve generated and used to determine the concentration of purified protein. From the concentration the yield of protein (µg) per mg of membrane was calculated.

To estimate the purity of each protein, samples (1 – 2 µg) were run on SDS-PAGE and stained with Instant Blue. The whole lane was analysed by densitometry and the signal from the target protein calculated as a percentage of the total [17].

***Preparation of lipid-only SMALPs***

DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) lipids (Sigma) 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. This was mixed at a 1:1 ratio with 2.5 %(w/v) polymer, which almost instantly clarified. Excess polymer was removed by size exclusion chromatography using a Superdex 200 30/10 column (GE Healthcare).

***Dynamic light scattering***

100 µl of either lipid-only SMALPs 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.

***Thermostability assay***

The aggregation of purified protein upon heating was analysed using a gel based assay. Purified protein (100 µl aliquots at 30 µg/ml) was heated for 10 minutes at temperatures from 4°C - 90°C followed by centrifugation at 10,000 g for 10 min to remove aggregated protein. Samples of the supernatant were then run on SDS-PAGE and stained with Instant Blue. Gels were analysed by densitometry (ImageJ) and the amount of protein remaining in solution for each temperature normalised to that obtained at 4°C.

***Divalent cation assay***

The sensitivity to divalent cations was assayed as described previously [17]. Purified protein (100 µl aliquots at 30 µg/ml) was mixed with 0- 10 mM $MgCl_2$ (or other divalent cation) for 10 min at room temperature, then centrifuged at 100,000 g for 20 min, 4°C. The supernatants containing soluble protein were harvested and the pellets containing insoluble protein resuspended in 100 µl buffer 2. Samples of both supernatant and pellet were run on SDS-PAGE and stained with Instant Blue. Gels were analysed by densitometry (ImageJ) and the proportion remaining soluble at each concentration of $MgCl_2$ calculated.

Alternatively lipid-only SMALPs (100 µl aliquots) were mixed with 0- 10 mM $MgCl_2$ (or other divalent cation) for 10 min at room temperature in a 96-well plate. Light scattering was then measured at 390 nm.

***Data analysis***

Statistical analysis was carried out using GraphPad Prism. A one-way ANOVA was used for multiple comparisons with a Tukey's post-hoc test. A value of $p<0.05$ was considered to be significant.

**Results:**

***Comparison of SZ30010 with SMA2000***

The initial aim of this study was to compare the performance of the hydrolysed form of SZ30010 with SMA2000, which has previously been found to be the most effective SMA polymer for membrane protein purification [17]. We also included the detergent DDM to provide a comparison with more conventional methods. To do this, three test membrane proteins were used which have a range of different structures and sizes. ZipA is a membrane tether important in *E. coli* cell division and comprises a single transmembrane helix with a large cytoplasmic domain [20]. It has a molecular weight of 39kDa but is known to run on SDS-PAGE around 52 kDa [4, 21]. BmrA (65 kDa) is a multidrug efflux pump of the ABC (ATP binding cassette) transporter superfamily. It forms a homodimer, with each monomer contributing 6 transmembrane helices and a cytosolic domain [22]. LeuT (57 kDa) is an amino acid:sodium symporter of the NSS (neurotransmitter:sodium symporter) family. It comprises 12 transmembrane helices and is located almost entirely within the membrane [23].

Protein solubilisation experiments are shown in Figure 1A which shows that SZ30010 was able to solubilise 57-67% of each target protein. This is comparable to that achieved with SMA2000 and there are no significant differences in solubilisation efficiency obtained for either SMA polymer or DDM.

All three proteins were successfully purified with both SMA polymers and DDM (Supplementary Figure 1). The yield obtained for LeuT is slightly lower than ZipA or BmrA, but there are no significant differences in yield between the two SMA polymers or DDM (Figure 1B). We previously showed that one advantage of SMA2000 is the degree of purity achieved [17]. As shown in Figure 1C the degree of purity achieved with SMA2000 and SZ30010 was significantly higher than with DDM for all three proteins.

Finally we characterised the size of the particles formed using SMA2000 in comparison to SZ30010 by DLS. As can be seen in Figure 1D, both polymers form very similarly sized particles, with an average diameter of just under 10 nm.

Thus, in terms of solubilisation efficiency, protein yield and purity, SZ30010 is comparable to SMA2000.

***Thermostability – aggregation assay***

The next step was to examine the thermostability of proteins purified using SZ30010. We and others have previously shown that SMA2000 provides an increased thermostability in comparison to conventional detergents [6-8, 17]. We also included the SMA polymer SZ25010 in this analysis. SZ25010 has a 3:1 ratio of styrene:maleic acid and a $M_w$ of 10kDa. It was previously found to be capable of purifying membrane proteins but was suggested to be less stable than SMA2000 [17]. To assess thermostability we utilised an aggregation assay using SDS-PAGE to visualise samples. Example gels for the protein BmrA are shown in Figure 2A. For DDM it can be seen that at 4°C a strong protein band is visible. As the temperature is increased above 40°C the intensity of the band decreases with increasing temperature. In

contrast for SMA2000 the intensity of the protein band remains approximately constant until 70°C. A very similar profile is observed for SZ30010 as for SMA2000, suggesting both polymers are able to confer a significant increase in resistance to aggregation compared to DDM. However whilst SZ25010 might be slightly better than DDM it is not able to confer the same stabilizing effect as the two polymers with the 2:1 ratio of styrene:maleic acid. Average results for BmrA are shown in Figure 2B and the shift in stability afforded by both SMA2000 and SZ30010 are clear. A very similar profile is observed for LeuT (Figure 2C). However for ZipA the results are somewhat different. Even with DDM ZipA appears to avoid aggregation even at high temperatures. There are no real differences observed between any of the different polymers/detergents.

***Divalent cation sensitivity***

SMA is known to have an affinity for divalent cations. In the bound state the polymer becomes insoluble. When the SMA is integrated into a SMALP the interaction between the SMA and the divalent cation means that the SMA disassociates from the SMALP causing the lipid and the encapsulated protein to precipitate. In this study we investigate the sensitivity of SMALPs made using different SMA variations to divalent cations. A representative gel for this assay using BmrA purified with SMA2000 is shown in Figure 3A. It can be seen that in the absence of $Mg^{2+}$ the polymer is unaffected and the purified protein remains almost entirely soluble. At an $Mg^{2+}$ concentration of approximately 4 mM the SMA starts to be affected and a proportion of the protein is now in the insoluble pellet, and in the presence of 10 mM $Mg^{2+}$ almost all of the protein becomes insoluble. Average results for BmrA in SMA2000 are shown in Figure 3B alongside results for ZipA and LeuT. It can be seen that the effect of $Mg^{2+}$ is comparable for all three proteins and there are no differences in sensitivity caused by the individual proteins. In contrast Figure 3C shows the results obtained for BmrA in SMALPs formed from different polymers. Whilst SZ30010 is not significantly different to SMA2000, for SZ25010 the curve has clearly shifted showing an increased sensitivity to $Mg^{2+}$. Similar results for the three different polymers were also observed using lipid-only SMALPs, LeuT and ZipA (Supplementary Figure 2A, B & C). However for each polymer there were no significant differences between the different proteins (Supplementary Figure 2D & E).

Next we investigated the effect of different divalent cations. As can be seen in Figure 3D for ZipA in SMA2000 SMALPs the curves for $Ca^{2+}$ and $Ni^{2+}$ are both shifted to the left indicating an increased sensitivity, such that approximately 50% of the protein is insoluble in the presence of 4 mM $Ca^{2+}$, and all of the protein is insoluble in the presence of 4 mM $Ni^{2+}$. $Co^{2+}$ and $Zn^{2+}$ cannot be tolerated at any of the concentrations tested (≥ 1 mM). A similar trend was also observed using lipid-only or BmrA SMALPs (Supplementary Figure 3A & BB), or when SZ30010 or SZ25010 polymers were used (Supplementary Figure 3C & D).

Finally we investigated how the concentration of SMALPs affected the sensitivity to $Mg^{2+}$. Figure 3E shows the sensitivity curves for lipid-only SMALPs and it can be seen that the sensitivity to $Mg^{2+}$ is not altered despite changing the SMALP concentration 10-fold or 100-fold.

**Discussion:**

The initial aim of this study was to carry out a side-by-side comparison of the two polymers SMA2000 and SZ30010, and the conventional detergent DDM for purification of membrane proteins. No significant differences were found between them in terms of solubilisation efficiency or protein yield. This agrees with the previous study which showed no difference between SMA2000 and DDM [17], and shows that SZ30010 also behaves comparably. When examining the degree of purity obtained we previously found that SMA2000 gave a higher degree of purity than DDM [17], this was observed again in this study. In addition we found that SZ30010 also affords the improved purity observed with SMA2000. Despite having small differences in average size and ratio of styrene:maleic acid, the particles produced using SMA2000 and SZ30010 were very similar in size at just under 10 nm diameter, which agrees well with previous reports using these two polymers [3, 17, 24, 25].

To assess thermostability we utilised a gel-based aggregation assay. For BmrA and LeuT, which both comprise multiple transmembrane spanning helices, the polymers SMA2000 and SZ30010 both offer a significant increase in resistance to aggregation over the conventional detergent DDM. SMA2000 has previously been shown in many studies to offer an increased thermostability to proteins [6-9], and this study shows that SZ30010 is comparable in this feature. In contrast the polymer SZ25010, which has a styrene:maleic acid ratio of 3:1 and a larger average molecular weight than SMA2000 and SZ30010, shows little improvement in thermostability compared to DDM.

The protein ZipA displayed no real differences in thermostability for any of the solubilisation agents, and was remarkably resistant to aggregation even in detergent. This may reflect its structure with only a single transmembrane helix and a large soluble domain [20], which perhaps enables it to behave more like a soluble protein. If so the presence of detergent may even help prevent aggregation at high temperatures [26].

Previous studies on the thermodynamics of phospholipid solubilisation by SMA2000 [27] and SZ30010 [24] indicate that these polymers have similar thermodynamic efficiencies. SMA2000 exhibits a larger negative free energy change associated with the polymer, indicating it is more efficient at solubilising phospholipids. However, the free energy change associated with the lipids is less positive for SMALPs formed by SZ30010. This suggests that the solubilised phospholipids experience a more similar thermodynamic environment to a vesicular bilayer in SZ30010 SMALPs compared to SMA2000 SMALPs. When compared to the thermodynamics of SMALP self-assembly by the SZ25010 3:1 polymer [28], SMA2000 has a larger negative free energy change during SMALP self-assembly than SZ25010, while SZ30010 is slightly less negative. However, SZ30010 leads to less disruption to the thermodynamic environment of the phospholipids than either SMA2000 or SZ25010. Our results align well with these studies where proteins solubilised by both SMA2000 and SZ30010 display a higher thermostability when compared to both DDM and SZ25010 solubilised proteins. This suggests that while SZ30010 is less thermodynamically efficient than SMA2000 and SZ25010, the more native-like phospholipid environment encapsulating the solubilised membrane protein compensates for the smaller overall driving force. In the case of SMA2000, the large negative free energy change associated with the polymer

appears to trump the less favourable phospholipid environment leading to a similarly high thermostability. From the results presented here, there is not a direct link between the overall thermodynamic efficiency of a polymer in the solubilisation of phospholipids and the solubilisation of membrane proteins. In the case of solubilisation from native membranes, both polymers are able to obtain similar yields of purified membrane proteins. This is likely due to the substantially more complex lipid environment in a native membrane when compared to the simplified model membranes necessary for thermodynamic characterisation.

Investigation of the divalent cation sensitivity using a range of different proteins and different polymers showed that the sensitivity is entirely polymer dependent and not protein specific. SZ30010 displayed similar sensitivity to magnesium as observed with SMA2000, however as reported previously SZ25010 was even more sensitive to magnesium [17]. Interestingly the sensitivity is also independent of SMALP concentration, at least over the 100-fold range tested. It is still not known exactly how many polymer molecules surround each SMALP disc, or if this is the same for all the different polymers. We previously showed that SMALPs formed from SZ25010 are smaller than those from SMA2000 and it is more sensitive to magnesium [17]. Perhaps the precipitation occurs when a certain proportion of the maleic acid groups surrounding a SMALP have bound the divalent cation.

When magnesium was exchanged for other divalent cations, even greater sensitivity was observed. Calcium and nickel both shifted the curve to the left, whilst zinc and cobalt caused complete precipitation at all concentrations tested. The sensitivity to $Ni^{2+}$ was particularly interesting given that we routinely use Ni-NTA affinity chromatography to purify SMALP-encapsulated proteins. Binding of SMALP-encapsulated proteins to Ni-NTA resin has previously been reported to be problematic at times [5, 14, 18, 29], but this has generally been considered to result from interactions between the resin and excess free SMA [18, 29], and despite these issues there have been many reported success stories using Ni-NTA [6-11, 14, 16, 17, 29]. This suggests that the effective concentration of $Ni^{2+}$ in the bead slurry must be below 2 mM or perhaps the spatial arrangement of $Ni^{2+}$ attached to the beads doesn't allow multiple nickel ions to bind to the same SMALP in the manner that leads to precipitation. Interestingly the SZ25010 polymer is more sensitive to divalent cations than SMA2000 or SZ30010, and we previously observed a loss of protein during the purification process with this polymer [17]. With SZ25010 a lot more of each protein did not bind to the resin and was instead present in the flow-through, compared to SMA2000 or SZ30010 (Supplementary Figure 1). Perhaps the increased sensitivity to divalent cations might explain this loss of protein during purification.

In conclusion, we have shown here in a side-by-side comparison that SMA2000 and SZ30010 are comparable in terms of membrane protein solubilisation, purification yield, degree of purity and thermostability, and both offer an advantage over the conventional detergent DDM. We have also shown that the sensitivity to divalent cations is polymer specific and independent of the protein encapsulated by the polymer or the SMALP concentration. Finally we have shown that SMALPs display even greater sensitivity to larger divalent cations.

**Acknowledgements:**

We thank Professor Jean-Michel Jault (IBCP, Lyon) for providing the BmrA expression construct, Prof. Harald Sitte & Dr Thomas Stockner (Medical University of Vienna) for providing the LeuT expression plasmid, and Dr David Roper (Warwick University) for the ZipA expression plasmid. Thanks to Dr Steve Russell for help in the lab. The underlying data for this publication can be found at https://doi.org/10.17036/researchdata.aston.ac.uk.00000360.

**Funding:**

AJR was the recipient of a Biochemical Society Eric Reid Methodology Fund. SCLH was supported by an Engineering and Physical Sciences Research Council (EPSRC: EP/M506461/1) and Diamond Light Source studentship. TRD received funding from the Biotechnology and Biological Sciences Research Council (BBSRC: BB/M018261/1, BB/J017310/1).

**Figure Legends:**

**Figure 1. Solubilisation efficiency, yield and purity with SZ30010 compared to SMA2000 and DDM. A**; Membranes (30 mg/ml wet pellet weight) from *E.coli* cells overexpressing either BmrA, LeuT or ZipA were mixed with either 2 %(w/v) DDM or 2.5 %(w/v) SMA polymer in 20 mM Tris pH, 150 mM NaCl for 1 hour at room temp. Samples were centrifuged at 100,000 g for 20 mins. The soluble and insoluble fractions were harvested and run on a Western blot using an anti-his primary antibody. The solubilisation efficiency was calculated using densitometry. Data are mean±sem, n≥3. **B**; Solubilised proteins were subjected to Ni-NTA affinity chromatography and the yield of pure protein calculated. Data are mean±sem, n≥3. **C**; The degree of purity was analysed from SDS-PAGE using densitometry. Data are mean±sem, n≥3. Data were analysed using an ANOVA with a Tukey post-hoc test, *$p<0.05$, ***$p<0.001$ purity is significantly higher than that obtained using DDM. **D**; Size of DMPC vesicles and lipid only-SMALP particles formed using either SMA2000 or SZ30010 as determined by DLS.

**Figure 2. Thermostability measured by a gel based aggregation assay.** Purified proteins (30 µg/ml) in either DDM micelles or SMALPs were heated for 10 min at temperatures ranging from 4-90°C. Samples were centrifuged (15000 g, 10 min) to remove aggregated protein, the samples of the supernatant were run on SDS-PAGE and stained with InstantBlue. **A**; Example SDS-PAGE gels for BmrA. The % soluble protein at each temperature was analysed by densitometry for **B**; BmrA, **C**; LeuT and **D**; ZipA. Data are mean±sem, n≥3.

**Figure 3. Sensitivity to divalent cations.** Purified proteins within SMALPs were mixed with various concentrations of $MgCl_2$ (0-10 mM), then centrifuged at 100000 g for 20 min. Supernatant (S) and pellet (P) were harvested and run on SDS-PAGE. The % soluble protein was analysed by densitometry. Alternatively DMPC lipid-only SMALPs were mixed with various concentrations of $MgCl_2$ (0-10 mM) and light scattering measured at 390 nm. **A**; Example SDS-PAGE for BmrA in SMA2000 SMALPs. **B**; Comparison of different proteins within SMA2000 SMALPs. **C**; Comparison of different polymers/detergents for BmrA. **D**; Effect of various different divalent cations on ZipA in SMA2000 SMALPs. **E**; The effect of SMALP concentration using DMPC lipid-only SMALPs made with SMA2000. Data are mean±sem, n≥3.

**References:**


1. Dawaliby, R., et al., *Allosteric regulation of G protein-coupled receptor activity by phospholipids.* Nat Chem Biol, 2016. **12**(1): p. 35-9.
2. Zoghbi, M.E., R.S. Cooper, and G.A. Altenberg, *The Lipid Bilayer Modulates the Structure and Function of an ATP-binding Cassette Exporter.* J Biol Chem, 2016. **291**(9): p. 4453-61.
3. Knowles, T.J., et al., *Membrane proteins solubilized intact in lipid containing nanoparticles bounded by styrene maleic acid copolymer.* J Am Chem Soc, 2009. **131**(22): p. 7484-5.
4. Lee, S.C., et al., *A method for detergent-free isolation of membrane proteins in their local lipid environment.* Nat Protoc, 2016. **11**(7): p. 1149-62.
5. Pollock, N.L., et al., *Structure and function of membrane proteins encapsulated in a polymer-bound lipid bilayer.* Biochim Biophys Acta, 2018. **1860**(4): p. 809-817.
6. Gulati, S., et al., *Detergent-free purification of ABC (ATP-binding-cassette) transporters.* Biochem J, 2014. **461**(2): p. 269-78.
7. Dorr, J.M., et al., *Detergent-free isolation, characterization, and functional reconstitution of a tetrameric K+ channel: the power of native nanodiscs.* Proc Natl Acad Sci U S A, 2014. **111**(52): p. 18607-12.
8. Jamshad, M., et al., *G-protein coupled receptor solubilization and purification for biophysical analysis and functional studies, in the total absence of detergent.* Biosci Rep, 2015. **35**(2).
9. Swainsbury, D.J., et al., *Bacterial reaction centers purified with styrene maleic acid copolymer retain native membrane functional properties and display enhanced stability.* Angew Chem Int Ed Engl, 2014. **53**(44): p. 11803-7.
10. Prabudiansyah, I., et al., *Characterization of the annular lipid shell of the Sec translocon.* Biochim Biophys Acta, 2015. **1848**(10 Pt A): p. 2050-6.
11. Logez, C., et al., *Detergent-free Isolation of Functional G Protein-Coupled Receptors into Nanometric Lipid Particles.* Biochemistry, 2016. **55**(1): p. 38-48.
12. Postis, V., et al., *The use of SMALPs as a novel membrane protein scaffold for structure study by negative stain electron microscopy.* Biochim Biophys Acta, 2015. **1848**(2): p. 496-501.
13. Sahu, I.D., et al., *DEER EPR measurements for membrane protein structures via bifunctional spin labels and lipodisq nanoparticles.* Biochemistry, 2013. **52**(38): p. 6627-32.
14. Broecker, J., B.T. Eger, and O.P. Ernst, *Crystallogenesis of Membrane Proteins Mediated by Polymer-Bounded Lipid Nanodiscs.* Structure, 2017. **25**(2): p. 384-392.
15. Parmar, M., et al., *Using a SMALP platform to determine a sub-nm single particle cryo-EM membrane protein structure.* Biochim Biophys Acta, 2018. **1860**(2): p. 378-383.
16. Sun, C., et al., *Structure of the alternative complex III in a supercomplex with cytochrome oxidase.* Nature, 2018. **557**(7703): p. 123-126.
17. Morrison, K.A., et al., *Membrane protein extraction and purification using styrene-maleic acid (SMA) copolymer: effect of variations in polymer structure.* Biochem J, 2016. **473**(23): p. 4349-4360.
18. Rothnie, A.J., *Detergent-Free Membrane Protein Purification.* Methods Mol Biol, 2016. **1432**: p. 261-7.
19. Rothnie, A., et al., *The topography of transmembrane segment six is altered during the catalytic cycle of P-glycoprotein.* J Biol Chem, 2004. **279**(33): p. 34913-21.
20. Hale, C.A. and P.A. de Boer, *Direct binding of FtsZ to ZipA, an essential component of the septal ring structure that mediates cell division in E. coli.* Cell, 1997. **88**(2): p. 175-85.
21. Hale, C.A., A.C. Rhee, and P.A. de Boer, *ZipA-induced bundling of FtsZ polymers mediated by an interaction between C-terminal domains.* J Bacteriol, 2000. **182**(18): p. 5153-66.
22. Steinfels, E., et al., *Characterization of YvcC (BmrA), a multidrug ABC transporter constitutively expressed in Bacillus subtilis.* Biochemistry, 2004. **43**(23): p. 7491-502.

23. Yamashita, A., et al., *Crystal structure of a bacterial homologue of Na+/Cl--dependent neurotransmitter transporters.* Nature, 2005. **437**(7056): p. 215-23.
24. Grethen, A., et al., *Thermodynamics of nanodisc formation mediated by styrene/maleic acid (2:1) copolymer.* Sci Rep, 2017. **7**(1): p. 11517.
25. Jamshad, M., et al., *Structural analysis of a nanoparticle containing a lipid bilayer used for detergent-free extraction of membrane proteins.* Nano Research, 2015. **8**(3): p. 774-789.
26. Haji Abdolvahab, M., et al., *The effects of dodecyl maltoside and sodium dodecyl sulfate surfactants on the stability and aggregation of recombinant interferon Beta-1b.* J Interferon Cytokine Res, 2014. **34**(11): p. 894-901.
27. Hall, S.C.L., et al., *Influence of Poly(styrene- co-maleic acid) Copolymer Structure on the Properties and Self-Assembly of SMALP Nanodiscs.* Biomacromolecules, 2018. **19**(3): p. 761-772.
28. Cuevas Arenas, R., et al., *Influence of lipid bilayer properties on nanodisc formation mediated by styrene/maleic acid copolymers.* Nanoscale, 2016. **8**(32): p. 15016-26.
29. Smirnova, I.A., et al., *Isolation of yeast complex IV in native lipid nanodiscs.* Biochim Biophys Acta, 2016. **1858**(12): p. 2984-2992.

Figure 1.

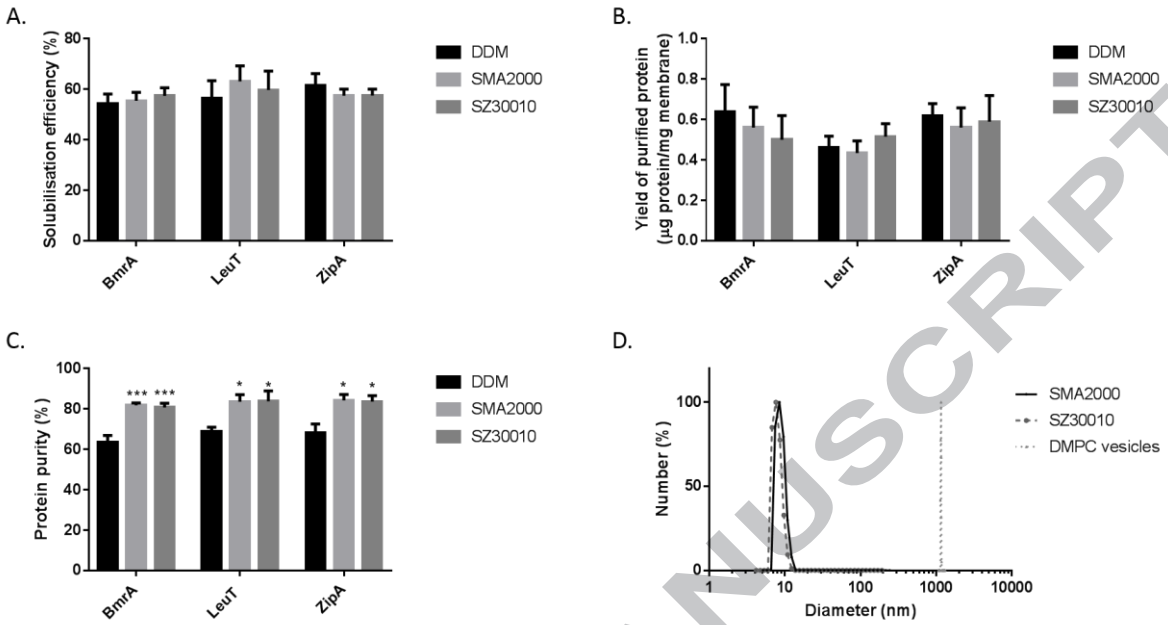


Figure 2.

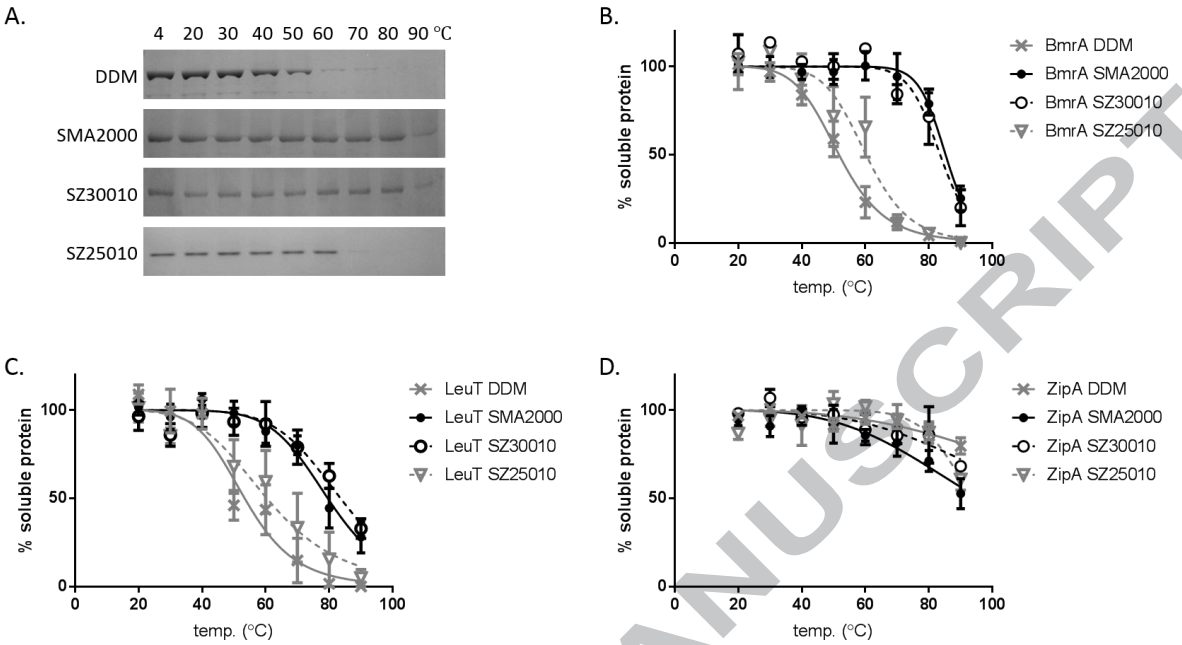
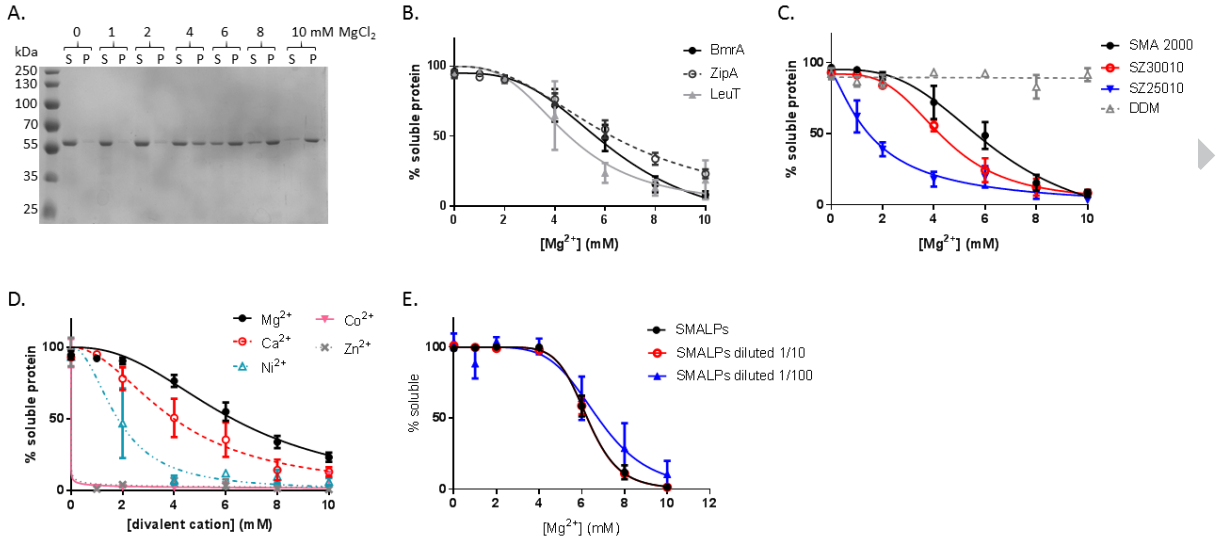
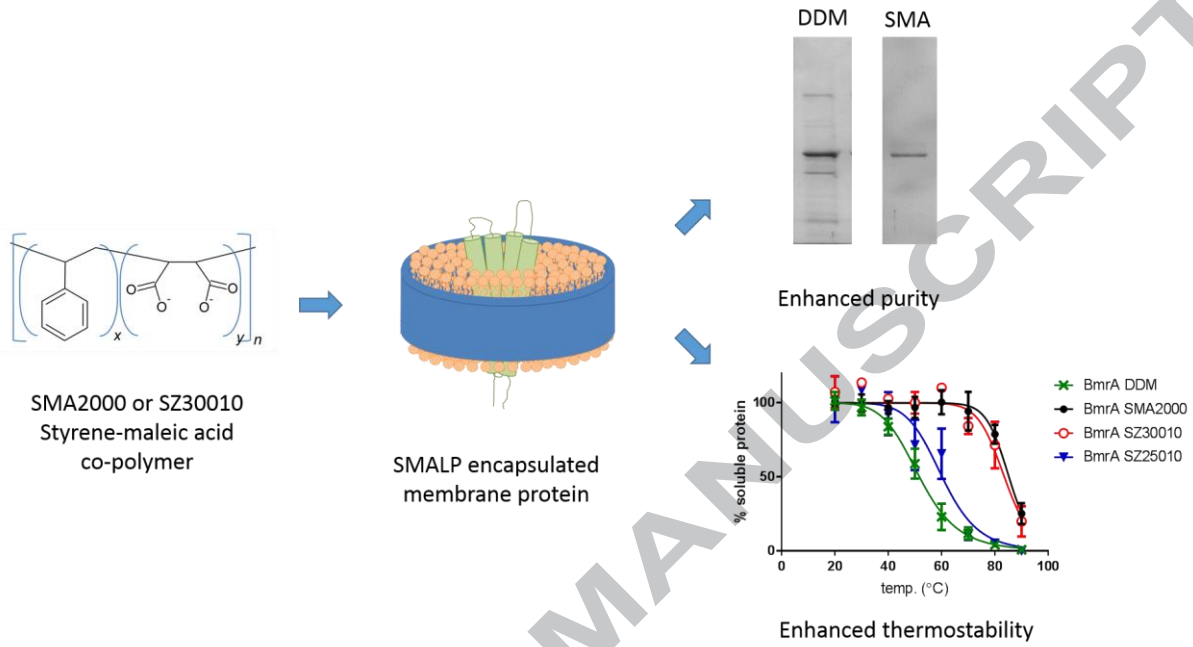


Figure 3.



**Graphical abstract**



**Highlights:**

- SMA polymers SZ300110 and SMA2000 are comparable for protein solubilisation, yield, purity and thermostability.
- Sensitivity of SMALPs to $Mg^{2+}$ is similar for different membrane proteins.
- The sensitivity to $Mg^{2+}$ is independent of the concentration of SMALPs
- SMALPs are even more sensitive to larger divalent cations