Biochemical Journal (2016) **473** 4349–4360
DOI: 10.1042/BCJ20160723

PORTLAND PRESS

Research Article

Membrane protein extraction and purification using styrene–maleic acid (SMA) copolymer: effect of variations in polymer structure

Kerrie A. Morrison[1], Aneel Akram[1], Ashlyn Mathews[1], Zoeya A. Khan[1], Jaimin H. Patel[1], Chumin Zhou[1], David J. Hardy[1], Charles Moore-Kelly[2], Roshani Patel[1], Victor Odiba[1], Tim J. Knowles[2], Masood-ul-Hassan Javed[1,3], Nikola P. Chmel[4], Timothy R. Dafforn[2] and Alice J. Rothnie[1]

[1]School of Life & Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, U.K.; [2]School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K.; [3]College of Medicine, King Saud bin Abdulaziz University for Health Sciences, Jeddah 21423, Kingdom of Saudi Arabia; and [4]Department of Chemistry, University of Warwick, Gibbet Hill, Coventry CV4 7AL, U.K.

Correspondence: Alice J. Rothnie (a.rothnie@aston.ac.uk)

The use of styrene–maleic acid (SMA) copolymers to extract and purify transmembrane proteins, while retaining their native bilayer environment, overcomes many of the disadvantages associated with conventional detergent-based procedures. This approach has huge potential for the future of membrane protein structural and functional studies. In this investigation, we have systematically tested a range of commercially available SMA polymers, varying in both the ratio of styrene and maleic acid and in total size, for the ability to extract, purify and stabilise transmembrane proteins. Three different membrane proteins (BmrA, LeuT and ZipA), which vary in size and shape, were used. Our results show that several polymers, can be used to extract membrane proteins, comparably to conventional detergents. A styrene:maleic acid ratio of either 2:1 or 3:1, combined with a relatively small average molecular mass (7.5–10 kDa), is optimal for membrane extraction, and this appears to be independent of the protein size, shape or expression system. A subset of polymers were taken forward for purification, functional and stability tests. Following a one-step affinity purification, SMA 2000 was found to be the best choice for yield, purity and function. However, the other polymers offer subtle differences in size and sensitivity to divalent cations that may be useful for a variety of downstream applications.

Introduction

The study of the structure and function of transmembrane proteins lags significantly behind that of soluble proteins. In large part this is because, in contrast with soluble proteins, there is a need to extract or solubilise these proteins from their lipid bilayer environment. Conventionally, this has been achieved using detergents/surfactants that can disrupt the bilayer structure and form micellar structures around the hydrophobic transmembrane regions of the protein. While this approach has yielded many results, the use of detergents is not without significant challenges. In the detergent solubilised state membrane proteins tend to have limited stability and often exhibit much reduced activity when compared with native forms. This is the result of the delicate balance that needs to be struck to achieve efficient extraction without denaturing the protein. This denaturation results from the detergent's inability to reproduce the complex physical environment of the membrane in which the protein has evolved to function, and detergents often strip all lipids from the protein.

Recently, we and others have shown that a new approach to membrane protein extraction using a styrene–maleic acid (SMA) copolymer can overcome many of these problems [1–3]. The SMA polymer spontaneously inserts into membranes and forms small discs of bilayer surrounded by the

Received: 29 July 2016
Revised: 27 September 2016
Accepted: 30 September 2016

Accepted Manuscript online: 30 September 2016
Version of Record published: 25 November 2016

Biochemical Journal (2016) **473** 4349–4360
DOI: 10.1042/BCJ20160723

Table 1 Properties of the polymers
Values for the percentage of maleic acid content and the mass–average molecular masses for each polymer, as specified by the suppliers.

Polymer name	Percentage of maleic acid content	Average molecular mass (kDa)
Cray Valley		
SMA 1000	50	5.5
SMA 2000	33	7.5
SMA 3000	25	9.5
Polyscope		
XZ09006	40	7.5
XZ09008	25	10
SZ40005	42	5
SZ25010	25	10
SZ42010	42	10
SZ33030	33	30
SZ28065	28	65
SZ28110	28	110

polymer, termed SMA lipid particles (SMALPs). Membrane proteins, extracted in these discs, can be purified by affinity chromatography while retaining their annular lipid bilayer environment [4–9]. There are several examples in the literature that show proteins in SMALPs that are functional and more thermostable than detergent-solubilised proteins [4–6,9]. The SMALP disc structure is well suited for many biophysical and spectroscopic techniques [4,7,10,11], as well as structural studies using electron microscopy [4,12]. There are, of course, some limitations. First, the size of the SMALPs is typically ∼10 nm diameter [1,5,13], which can be too small for some large proteins or complexes [14]. Secondly, the SMALP structure is sensitive to divalent cations, such as Mg^{2+}, which above a certain concentration causes the SMA to precipitate out of solution [3,4].

Many different SMA copolymers are commercially available, which can vary in the ratio of styrene:maleic acid and in their total length and size. To date, the polymers used to extract membrane proteins are SMA(2:1) [1,4–7,9,12] and SMA(3:1) [8,10,11,15,16], which have styrene:maleic acid ratios of 2:1 and 3:1, and are relatively small with an average molecular mass of 7.5 and 10 kDa, respectively. In the present study, we systematically assessed a selection of SMA copolymers (Table 1), with varying ratios of styrene:maleic acid, and a range of molecular masses. We aimed to determine whether these polymers were effective for extracting and purifying membrane proteins, and if so whether they could overcome the limitations in size and Mg^{2+} sensitivity of the SMA copolymers currently in use.

Our study assessed the performance of these polymers in the extraction process for three different bacterial membrane proteins of varying size and structure, namely BmrA, LeuT and ZipA. BmrA is a multidrug efflux pump of the ABC (ATP-binding cassette) superfamily from *Bacillus subtilis* [17]. It forms a homodimer, where each monomer provides six transmembrane α-helices and a cytosolic nucleotide-binding domain. Thus, it has a large transmembrane region and a large cytosolic region. LeuT, an amino acid:sodium symporter of the NSS (neurotransmitter:sodium symporter) family from *Aquifex aeolicus*, comprising 12 transmembrane helices, is located almost entirely within the membrane [18]. Finally, ZipA, a membrane tether involved in cell division in *Escherichia coli*, has just a single transmembrane helix with a large cytosolic domain [19].

Experimental procedures

SMA preparation

SMA 2000, SMA 1000 and SMA 3000 were obtained from Cray Valley (Exton, PA, USA). XZ09008, XZ09006, SZ25010, SZ40005, SZ42010, SZ33030, SZ28065 and SZ28110 were from Polyscope (Geleen, Netherlands). The commercially available polymers are provided as styrene–maleic anhydride copolymer and need to be converted into styrene and maleic acid by hydrolysis using NaOH [20,21]. A solution of each styrene–maleic anhydride

Biochemical Journal (2016) **473** 4349–4360
DOI: 10.1042/BCJ20160723

copolymer, 10% (w/v) in 1 M NaOH, was refluxed for 2 h and allowed to cool to room temperature. The polymers were precipitated by the addition of excess concentrated HCl and washed extensively with distilled water. The washed polymer was dissolved in 0.6 M NaOH to give a pH of 8 and freeze-dried. Styrene–maleic acid copolymer powder was stored at room temperature.

Protein production and membrane preparation

C41 (DE3) *E. coli* cells were transformed with the vector pET23b-BmrA containing the gene for BmrA expressed as a fusion to a C-terminal His_6 tag (kind gift from Prof. Jean-Michel Jault, IBCP, Lyon, France). BL21 (DE3) *E. coli* cells were transformed with the vector pET101-ZipA with a V5 epitope and a C-terminal His_6 tag (kind gift from Dr David Roper, University of Warwick) or with pET16b-LeuT with an N-terminal His_8 tag (kind gift from Prof. Harald Sitte, Medical University of Vienna).

Small overnight cultures (5 ml) were used to inoculate 1 l flasks of Luria broth supplemented with 100 μg/ml ampicillin and grown at 37°C, 200 rpm until OD_{600} reached 0.6. Protein synthesis was induced by the addition of 0.5 mM IPTG and the temperature was reduced to 25°C. Cells were harvested 18–20 h later by centrifugation (6000×***g***, 10 min).

The *E. coli* cell pellets were resuspended in buffer 1 (50 mM Tris pH 7.4, 250 mM sucrose and 0.25 mM $CaCl_2$) supplemented with protease inhibitors (1 μM pepstatin, 1.3 μM benzamidine and 1.8 μM leupeptin). Cells were disrupted on ice using sonication. Unbroken cells and debris were removed by a low-speed spin (650 ***g***, 20 min, 4°C), and then membranes were harvested by ultracentrifugation (100 000×***g***, 20 min, 4°C). Membranes were resuspended in buffer 2 (20 mM Tris pH 8 and 150 mM NaCl) at a final concentration of 60 mg/ml wet membrane weight, aliquoted and stored at −80°C.

Solubilisation trials

Initial trials compared SMA 2000 with the conventional detergents octyl-β-D-glucoside (OG) and dodecyl-β-D-maltoside (DDM). Membranes containing each target protein (30 mg/ml wet weight) in buffer 2 were mixed with 2.5% (w/v) SMA 2000, 2% (w/v) OG (Sigma) or 2% (w/v) DDM (VWR) for 1 h at room temperature, with gentle shaking. Samples were then centrifuged (100 000×***g***, 20 min) and the supernatant containing solubilised protein was retained. The pellet containing insoluble material was resuspended in the same total volume of buffer 2 supplemented with 2% (w/v) SDS. Samples of soluble and insoluble material were analysed by Western blot, probed with an anti-his 1° primary antibody (1:1000, R&D systems). Blots were visualised using a 2° antibody of either anti-mouse alkaline phosphate and BCIP/NBT (Sigma) or anti-mouse HRP and the SuperSignal West Chemiluminescent kit (ThermoFisher). The percentage of total protein solubilised was determined from the Western blots using densitometry (ImageJ).

A similar procedure was followed to screen the different SMA polymer variations. Each polymer was used at a final concentration of 2.5% (w/v), and the amount of protein solubilised was normalised to that obtained with SMA 2000.

Ni-NTA affinity purification

Solubilised protein was mixed with HisPur Ni^{2+}-NTA resin (ThermoFisher) at a ratio of 100 μl resin per ml of solubilised protein at 4°C overnight with gentle rotation. The sample was transferred to a gravity flow column (Macherey-Nagel), and the flow-through containing unbound material was collected. The resin was washed five times with 10 bed volumes (BVs) of buffer 2 supplemented with 20 mM imidazole, twice with 10 BVs of buffer 2 containing 40 mM imidazole and once with 2 BVs of buffer 2 supplemented with 60 mM imidazole. Proteins were eluted with buffer 2 supplemented with 200 mM imidazole, and six fractions of one BV were collected. For purifications using DDM, all wash and elution buffers were also supplemented with 0.1% (w/v) DDM. Fractions were analysed using 7.5% SDS–PAGE and InstantBlue stain (Expedeon). Elution fractions containing the target protein were pooled and stored at 4°C.

Purified protein quantification

The concentration of purified protein samples was determined from SDS–PAGE using BSA as a standard as described previously [22]. Unlike many colorimetric methods, this method does not suffer from interference from lipids, imidazole or SMA. Briefly, samples (10 and 20 μl) of purified proteins were separated using 7.5% SDS–PAGE alongside BSA standards (0.25, 0.5, 0.75, 1 and 1.25 μg) and stained with InstantBlue (Expedeon). The intensity of each band was analysed by densitometry (ImageJ), and a standard curve was constructed for

Biochemical Journal (2016) **473** 4349–4360
DOI: 10.1042/BCJ20160723

BSA. Using the standard curve, the concentration of purified LeuT/ZipA/BmrA was calculated. From the concentration, the yield of purified protein per litre of culture could be calculated.

To estimate the purity of each target protein, 1–2 μg of purified protein sample was loaded on 7.5% SDS–PAGE and stained with InstantBlue. The whole lane was analysed by densitometry, and the intensity of the protein of interest as a percentage of the total staining intensity was determined.

BmrA substrate-binding assay

Substrate binding to BmrA was measured using a tryptophan fluorescence quenching assay as described previously [17]. Using centrifugal filter concentrators (Amicon Ultra, 30K cut-off), purified BmrA was concentrated and exchanged into buffer 2 to remove imidazole. Tryptophan fluorescence of BmrA (50 μg/ml) was monitored using a PerkinElmer LS55 fluorimeter, with an excitation wavelength of 280 nm (slit width 10 nm) and emission measured at 310–400 nm (slit width 20 nm). Fluorescence quenching by the successive addition (1–50 μM) of Hoechst 33342 or doxorubicin was measured at 335 nm (λ_{max}). Fluorescence intensities were corrected for the effects of dilution and the inner filter effect using *N*-acetyl tryptophanamide. Results were analysed by non-linear regression using GraphPad Prism (Graphpad Software, Inc.) to fit a one-site binding hyperbola.

Thermostability

The aggregation of purified BmrA upon heating was measured using right-angle light scattering. BmrA (50 μg/ml) was heated from 25 to 95°C in increments of 10°C for 10 min each. After incubation at each temperature, the light scattering was measured using a PerkinElmer LS55 fluorimeter at a wavelength of 390 nm (slit width 10 nm).

Thermostability was also monitored by heating samples of purified BmrA (50 μg/ml) at specific temperatures for 10 min, followed by centrifugation to remove aggregates (10 000×***g***, 10 min). Soluble protein remaining in the supernatant was analysed by SDS–PAGE.

Mg^{2+} sensitivity assay

SMA solubilised and purified ZipA (50 μg/ml) was mixed with various concentrations of $MgCl_2$ (0–10 mM) at room temperature for 10 min. Samples were centrifuged (100 000×***g***, 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 SDS–PAGE, stained with InstantBlue and the percentage of protein remaining in solution was calculated by densitometry.

Dynamic light scattering

Purified LeuT was concentrated using Amicon Ultra filter concentrators to 100 μg/ml in buffer 2. Dynamic light scattering (DLS) data were recorded using a Malvern Zetasizer Nano S (633 nm) and 1.0 cm pathlength disposable plastic cuvettes (Brand BMBH, Germany). Measurements were taken at 20°C with 300 s equilibration time. Automated instrument parameters were used. Each measurement was repeated at least 11 times.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism and used an ANOVA for multiple comparisons, with a Tukey *post hoc* test; a value of $P < 0.05$ was considered significant.

Results

Solubilisation of proteins using SMA 2000 and conventional detergents

The initial aim of the study was to determine whether SMA 2000, the most widely used SMA polymer to date, was able to effectively solubilise three different membrane proteins: LeuT, ZipA and BmrA. It can be seen in Figure 1 that for each protein, ∼55% of the total was solubilised using 2.5% (w/v) SMA 2000. This was comparable to the solubilisation efficiency observed for each protein using the conventional detergent DDM. For ZipA, a similar solubilisation efficiency was also observed for the shorter chain conventional detergent OG; however, for LeuT and BmrA, the solubilisation with OG was significantly less efficient ($P < 0.05$, ANOVA).

Biochemical Journal (2016) **473** 4349–4360
DOI: 10.1042/BCJ20160723

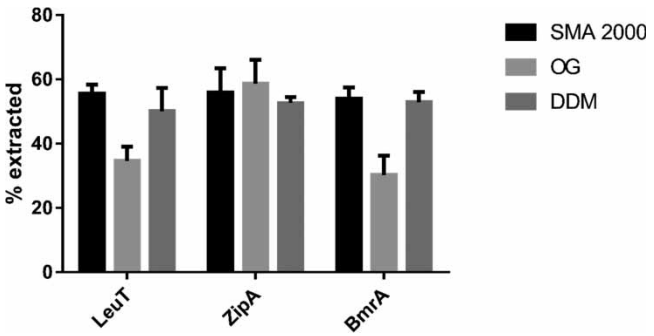

Figure 1. Comparison of SMA 2000 with conventional detergents for extraction/solubilisation efficiency.
Membranes (30 mg/ml wet weight) from *E. coli* cells overexpressing each protein were solubilised with either 2.5% (w/v) SMA 2000, 2% (w/v) octyl-β-D-glucoside (OG) or 2% (w/v) dodecyl-β-D-maltoside (DDM) in 20 mM Tris pH 8 and 150 mM NaCl for 1 h at room temperature, while gently shaking. Samples were centrifuged at 100 000×***g*** for 20 min at 4°C. The supernatant containing solubilised protein was harvested, and the pellet containing insoluble material was resuspended in an equal volume of 2% (w/v) SDS. Samples of both solubilised and insoluble material were run on a Western blot using an anti-his 1° antibody (1:1000), and the % of total protein solubilised was determined by densitometry. Data are mean ± SEM, $n \geq 3$.

Screening SMA polymer variations for protein solubilisation

Having established that SMA 2000 could successfully solubilise all three proteins, the next step was to screen the new SMA polymers to establish whether they were capable of extracting membrane proteins. The new SMA polymers tested vary in size from 5 to 110 kDa and had ratios of styrene:maleic acid ranging from 3:1 to 1:1 (Table 1). As shown in Figure 2, three of the polymers, SMA 3000, XZ09008 and SZ25010, stand out as being effective at solubilising membrane proteins, giving results that are comparable to SMA 2000. SMA 3000 has been previously used to successfully solubilise several membrane proteins [8,10,11,15,16], so it is not surprising that it is effective. XZ09008 and SZ25010 have very similar properties to SMA 3000, with a 3:1 styrene:maleic acid ratio and a reported molecular mass of 10 kDa. However, most of the other polymers were not effective at all. The larger polymers, SZ33030, SZ28065 and SZ28110, with average molecular masses of 30–110 kDa, were very poor at extracting proteins, despite having styrene:maleic acid ratios comparable to SMA 2000 and SMA

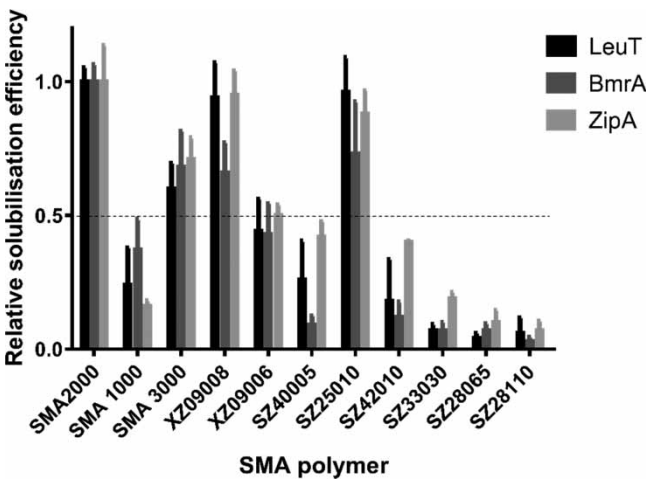

Figure 2. Screen of SMA polymer variants for extraction of membrane proteins compared with SMA 2000.
Membranes (30 mg/ml wet weight) from *E. coli* cells overexpressing each protein were incubated with 2.5% (w/v) of each polymer for 1 h at room temperature, while gently shaking. Samples were centrifuged at 100 000×***g*** for 20 min at 4°C. The supernatant containing solubilised protein was harvested. Samples were run on a Western blot using an anti-his 1° antibody (1:1000), and the degree of solubilisation relative to SMA 2000 was determined by densitometry. Data are mean ± SEM, $n \geq 3$.

Biochemical Journal (2016) 473 4349–4360
DOI: 10.1042/BCJ20160723

3000. The small polymers with a high percentage of maleic acid, SMA 1000, SZ40005, XZ09006 and SZ42010, were also not particularly effective at solubilising proteins, despite having similar molecular masses to SMA 2000 and SMA 3000. It is interesting to note that the results are strikingly similar for all three target proteins, despite their differences in size and shape. It appears that the effectiveness of each polymer is not protein-specific. The target proteins tested here were all expressed in *E. coli*; however, the same pattern of results is also observed for MRP4/ABCC4 expressed in *Sf9* insect cells and MRP1/ABCC1 in H69AR cancer cells (Supplementary Figure S1), showing the effectiveness of each polymer is not expression system-specific. Thus, it seems that a styrene:maleic acid ratio of 2:1 or 3:1, and a molecular mass of 7.5–10 kDa, is optimal for membrane protein solubilisation.

Purification of membrane proteins

The three polymers capable of solubilising the membrane proteins effectively (SMA 3000, XZ09008 and SZ25010) were taken to protein purification trials, alongside SMA 2000 and the conventional detergent DDM. Also included was the polymer XZ09006, which solubilised almost half as much protein as SMA 2000. Each protein was purified by a single-step Ni^{2+}-NTA affinity chromatography procedure as shown in Figure 3A. The concentration and volume of purified protein obtained were used to calculate the yield per litre culture for each protein with each solubilisation agent, and the average results are shown in Figure 3B. The average yield using SMA 2000 was 1.2 ± 0.1, 1.0 ± 0.1 and 0.8 ± 0.1 mg/l, for ZipA, BmrA and LeuT, respectively. With the conventional detergent DDM, the yields were comparable to those of SMA 2000. However, for each of the other polymers, the yield of pure protein was decreased compared with SMA 2000. XZ09006 gave the lowest yield for each protein. This is not surprising given that XZ09006 solubilised half as much protein as SMA 2000 or DDM. However, SMA 3000, XZ09008 and SZ25010 were capable of solubilising almost the same amount of protein as SMA 2000. The decrease in yield observed must be due to a loss at some point during the purification procedure.

The purity of protein samples obtained with this single-step affinity procedure was also analysed. It has recently been reported that SMA 2000 typically yields a more pure sample than conventional detergents [4]. This observation was reiterated with all three of the proteins studied here, and is illustrated in Figure 3C, where a sample of BmrA purified using DDM clearly contains many more contaminating proteins than the sample purified using SMA 2000. The average measurements for the degree of purity, measured using densitometric analysis of gels loaded with 1–2 μg total protein, are shown in Figure 3D. The purity of each protein obtained using DDM was significantly lower than when using SMA 2000. Thus, despite achieving similar total yields with DDM and SMA 2000, the purity of the SMA 2000 encapsulated proteins was superior. The polymers XZ09008 and XZ09006 also gave samples of significantly lower purity than SMA 2000; however, SMA 3000 and SZ25010 showed no significant differences to SMA 2000, despite the lower yields.

Ligand binding

We investigated whether the different polymers tested affected the membrane protein structure and function by measuring ligand binding to BmrA using a tryptophan fluorescence quenching assay [17]. Two substrates were tested: Hoechst 33342 and doxorubicin. The binding affinity (K_d) and maximal quenching (%) parameters are summarised in Table 2. It can be seen that BmrA in DDM micelles or SMA 2000 SMALPs has comparable ligand-binding properties, displaying a greater affinity and a larger degree of quenching for Hoechst 33342 than doxorubicin, as has been previously observed [17]. XZ09006 purified BmrA also shows no differences in SMA 2000 or DDM. However, for SMA 3000 (Figure 4D), XZ09008 and SZ25010, a significantly lower affinity is observed for Hoechst 33342. BmrA encapsulated within these polymers still appears to bind Hoechst 33342: the same maximal degree of quenching is obtained, but with a lower affinity. Interestingly, only the Hoechst 33342 binding is affected, while all samples show comparable affinities and a maximal degree of quenching with doxorubicin.

Influence of polymer type on SMALP size

One important aspect of the SMALP method is the size of the particle that contains the protein. Larger particles could allow proteins with more extensive transmembrane domains to be solubilised (currently, the limit seems to be 36 transmembrane helices [12]). Conversely, smaller particles might be of more use in techniques like NMR where rapid tumbling times are important.

Biochemical Journal (2016) **473** 4349–4360
DOI: 10.1042/BCJ20160723

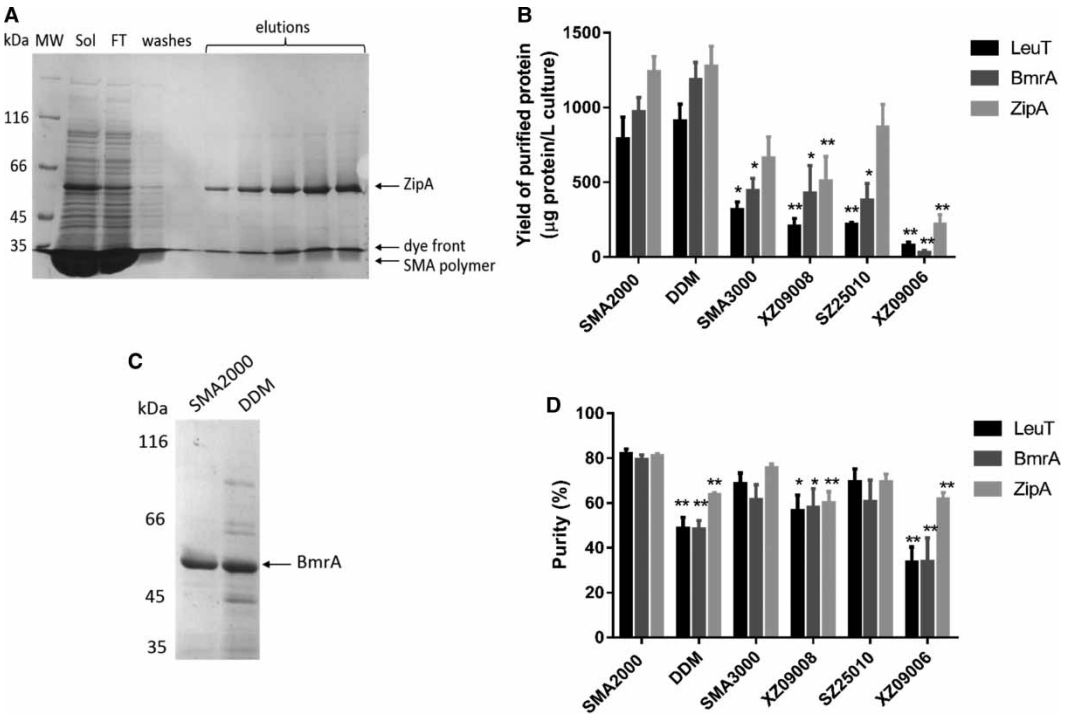

Figure 3. Yield and purity obtained using SMA polymer variants.

Solubilised proteins were mixed with Ni-NTA resin (100 μl resin per ml solubilised protein) overnight at 4°C, transferred to a gravity flow column and washed extensively with buffer supplemented with 20–60 mM imidazole. Proteins were eluted using buffer supplemented with 200 mM imidazole. (**A**) Example purification gel for ZipA solubilised using SZ25010, showing solubilised protein (Sol), flow-through (FT), 20 mM imidazole washes and 200 mM imidazole elution fractions on a 7.5% SDS–PAGE stained with InstantBlue. (**B**) The concentration of purified protein was determined and total yield per litre culture was calculated. Data are mean ± SEM, $n \geq 3$. (**C**) SDS–PAGE stained with InstantBlue highlighting the difference in purity obtained for BmrA solubilised and purified using SMA 2000 compared with DDM. (**D**) The degree of purity for each protein preparation was determined by densitometry. Data are mean ± SEM, $n \geq 3$. Data were analysed using an ANOVA with a Tukey *post hoc* test, $^{*}P < 0.05$, $^{**}P < 0.01$ yield/purity is significantly lower than that obtained using SMA 2000.

Table 2 Binding assay parameters for BmrA

Quenching of the intrinsic tryptophan fluorescence of BmrA (50 μg/ml), purified with each polymer or DDM, upon binding of substrates Hoechst 33342 or doxorubicin was measured. Data are mean ± SEM and $n \geq 3$. Data were analysed using an ANOVA.

Polymer/detergent	Hoechst 33342		Doxorubicin	
	K_d (μM)	Maximal quenching (%)	K_d (μM)	Maximal quenching (%)
DDM	5.9 ± 1.4	55 ± 4	16 ± 5	22 ± 4
SMA 2000	4.0 ± 0.8	52 ± 5	16 ± 2	39 ± 5
SMA 3000	16 ± 2*	49 ± 6	16 ± 5	39 ± 10
XZ09008	18 ± 3**	51 ± 9	12 ± 5	31 ± 8
SZ25010	15 ± 5*	47 ± 5	21 ± 9	30 ± 10
XZ09006	4.5 ± 1.2	44 ± 5	15 ± 6	29 ± 7

$^{*}P < 0.05$,
$^{**}P < 0.01$ significantly different from SMA 2000.

Biochemical Journal (2016) **473** 4349–4360
DOI: 10.1042/BCJ20160723

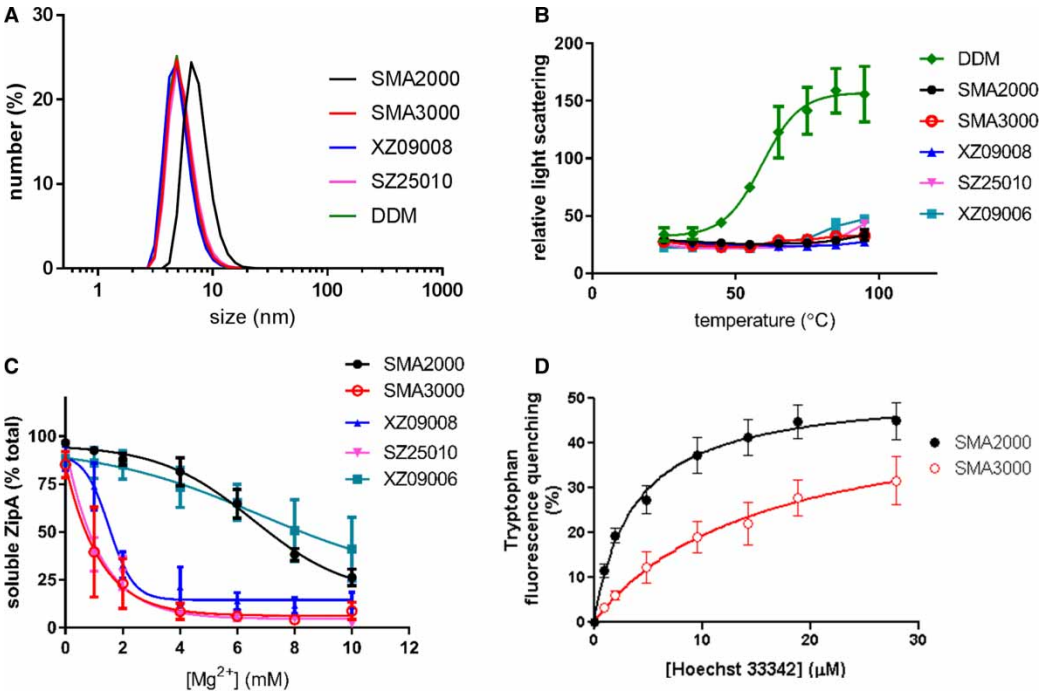

Figure 4. Size and stability of SMALPs formed from the different polymers.

(**A**) LeuT solubilised and purified with each polymer and DDM was analysed using DLS. Results are presented as number-weighted particle size distributions. (**B**) Thermal aggregation of BmrA purified using each polymer or DDM was monitored using right-angle light scattering at a wavelength of 390 nm. (**C**) Magnesium sensitivity of purified ZipA with each polymer was assessed by centrifugation at 100 000×***g*** for 20 min, and samples of both supernatant and pellet were run on SDS–PAGE and analysed by densitometry. (**D**) Binding of Hoechst 33342 to BmrA extracted and purified with either SMA 2000 (closed circles) or SMA 3000 (open circles) as monitored by tryptophan fluorescence quenching. Data are mean ± SEM, $n \geq 3$.

To measure the size of the SMALPs produced with various polymers, we used DLS and the protein LeuT (since this protein is predominantly located within the membrane bilayer). This allowed the disc size to be measured with minimal interference from cytosolic regions of the protein. The results in Figure 4A show that SMA 2000 SMALPs display an average diameter of 8–9 nm, whereas SMA 3000, XZ09008 and SZ25010 give a smaller diameter of ∼5 nm, despite these polymers having a larger molecular mass than SMA 2000.

Influence of polymer type on stability

A key observation of previous SMALP encapsulations has been an increase in the stability of the encapsulated protein. In this experiment, we examined whether different SMA types confer different stabilities. The thermostability of purified samples was assessed using a right-angle light scattering assay that was able to measure the thermally induced aggregation of the sample. As shown in Figure 4B, BmrA purified in DDM micelles showed a clear temperature-dependent increase in aggregation; however, this was not observed when BmrA was purified within SMA 2000 SMALPs. To confirm that the increase in light scattering was due to protein aggregation, samples were also analysed by centrifugation and SDS–PAGE (Supplementary Figure S2). A clear loss of DDM-solubilised BmrA from solution is seen at 65°C, whereas this is not the case for BmrA in SMA 2000 SMALPs. Similar light scattering results to those obtained with SMA 2000 were also observed for BmrA within SMA 3000 and XZ09008 polymers. For SZ25010 and XZ09006, there was a slight increase in scattering at the highest temperatures, but this was very small when compared with the DDM sample.

Susceptibility of SMALPs to Mg^{2+}

One of the deficiencies of the SMALP is its susceptibility to Mg^{2+} [4]. Magnesium ions are thought to bind to the SMA polymer surrounding an SMALP causing the polymer and the protein it encapsulates to precipitate

Biochemical Journal (2016) **473** 4349–4360
DOI: 10.1042/BCJ20160723

out of solution. We examined the sensitivity to magnesium (Mg^{2+}) of protein encapsulated by other SMA types. As shown in Figure 4C, for ZipA purified within SMA 2000 SMALPs, a concentration of $MgCl_2$ below 4 mM is tolerated, but at concentrations above this, a dose-dependent loss of solubility is observed. ZipA within XZ09006 SMALPs displays a similar response to Mg^{2+}. However, ZipA within SMA 3000, X09008 and SZ25010 SMALPs is even more sensitive to Mg^{2+}, with concentrations of 1–2 mM causing complete precipitation.

Discussion

The recent application of SMA polymers for the solubilisation and purification of membrane proteins has the potential to revolutionise the field of membrane protein structural and functional studies, resolving many issues currently posed by the use of conventional detergents. The SMALPs provide a solubilisation method that preserves the lipid bilayer environment of membrane proteins, making it possible to maintain near-native structure, function and stability.

In the present study, we aimed to screen a variety of commercially available SMA polymer variations, to establish which features of the polymer are important for efficient protein extraction, purification and downstream analysis. We used a set of three different membrane proteins, with varying size and shape to reduce protein-specific issues.

The first step was to assess the solubilisation efficiency for each protein with conventional detergents compared with SMA 2000. The solubilisation of both LeuT and BmrA was less effective with the shorter chain conventional detergent OG. This is not surprising since it is well established that short-chain detergents are less effective for initial solubilisation, but better for crystallography. Notably, published LeuT crystal structures utilised DDM to solubilise the protein, before switching to OG for crystallisation [18]. For each of the three proteins in this study, the solubilisation efficiency with the conventional detergent DDM was just over 50%. This is comparable to that reported previously for BmrA with DDM [17]. SMA 2000 solubilised ∼55% of each protein; thus, SMA 2000 is comparable to or better than conventional detergents. This concurs with a previous study of other protein targets [4].

During the solubilisation screening, none of the alternative polymers offered an improvement on the solubilisation efficiency achieved with SMA 2000, though SMA 3000, XZ09008 and SZ25010 did give results comparable to SMA 2000. These three polymers each have a styrene:maleic acid ratio of 3:1, and an average molecular mass of 10 kDa, compared with SMA 2000, which has a 2:1 ratio of styrene:maleic acid and is a little smaller at 7.5 kDa. It should be considered, however, that all of the commercially available polymers used here have high polydispersity indexes, and the molecular masses detailed in Table 1 are the average of a mixture of different sizes. These result from the methods used to synthesise the polymers, which yield samples containing a distribution of sizes, and also variations in the sequence of styrene and maleic acid groups [3]. We do not currently know if all of the different polymers within each distribution are functional in solubilisation and forming SMALPs or if it is a small subset. However, given that more defined preparations are not easily available, it was reasonable to test the commercially available polymers. The results showed clearly that a styrene:maleic acid ratio of either 2:1 or 3:1, combined with an average molecular mass of 7.5–10 kDa, was required for efficient solubilisation of membrane proteins, and any deviation from this resulted in poor protein extraction.

Ni^{2+}-NTA affinity purification of proteins within SMA 2000 SMALPs gave yields that were comparable to those obtained using DDM; however, the purity achieved using SMA 2000 was significantly higher than that achieved using DDM, as has been reported previously for other proteins [4]. Surprisingly, the yields of protein achieved using SMA 3000, XZ09008 and SZ25010 were lower than using SMA 2000, despite showing comparable solubilisation efficiencies, suggesting that, with these polymers, protein was lost during the purification procedure. One possibility is less efficient binding to the Ni^{2+}-NTA resin, as it is known that the binding interaction between SMALP-encapsulated proteins and the Ni^{2+}-NTA resin can be of low affinity [3]. An alternative could be that SMALPs formed from these polymers are less stable than SMA 2000 SMALPs, leading to loss of protein from solution during washing steps. It remains to be determined at which stage(s) the protein is lost; however, under the standard conditions tested here, it would seem that SMA 2000 gives the greatest yield and purity of purified membrane protein.

The size of SMALPs formed using SMA 2000 reported in the literature shows considerable variation [3]. This may be the result of differences in the method used to make the measurement. DLS [1,7,23], small-angle neutron scattering [13] and electron microscopy [1,5,7] have all been used. For some proteins, these measurements are complicated by the presence of large cytosolic domains, which will contribute to the overall

Biochemical Journal (2016) 473 4349–4360
DOI: 10.1042/BCJ20160723

dimensions. To overcome this, we used LeuT-SMALPs, where the protein is predominantly located within the bilayer region. Our results using DLS gave an average diameter for LeuT-SMA 2000 of 8–9 nm, which is smaller than typical reports of 10–12 nm. However, it compares well with the diameter of 9 nm reported for PagP-SMALPs in Knowles et al. [1] and the small-angle neutron scattering studies of lipid-only SMALPs in Jamshad et al. [13]. Surprisingly, we found that LeuT-SMALPs formed from the larger polymers SMA 3000, XZ09008 and SZ25010 gave smaller diameters of ∼5 nm. This is in contrast with previous reports where SMA 3000 was used and diameters of 12 nm were reported [10,15]. Possible explanations for this could include the formation of SMALP–SMALP interactions to form dimers, as has been observed recently [12], methodological and sample preparation differences in DLS and electron microscopy that have previously been shown to give different measurements for the same sample [1,13], or that the polymer can form different size particles depending on the size of the protein encapsulated. It has also been shown using lipids only that if less SMA is used during solubilisation, larger SMALPs can actually be formed, and the size of the particle formed depends on the ratio of SMA/lipid [24,25]. In our study, we have used an excess of SMA polymer at 2.5% (w/v) with membranes at 30 mg/ml (wet pellet weight). We have shown recently that lower concentrations of SMA, down to ∼1% (w/v), are still effective for protein solubilisation under these conditions, but below this solubilisation is less efficient [21]. It will be interesting, in the future, to establish if conditions can be tailored to produce larger SMALPs while still extracting sufficient protein.

Interestingly, we also observed differences in BmrA ligand binding and ZipA Mg^{2+} sensitivity for the same three polymers, SMA 3000, XZ09008 and SZ25010, compared with SMA 2000. BmrA within SMA 2000 SMALPs bound both Hoechst 33342 and doxorubicin in a manner comparable to DDM-solubilised BmrA, and gave parameters similar to previous published studies [17]. However, although BmrA within SMA 3000, XZ09008 and SZ25010 polymers was able to bind the substrate Hoechst 33342, a lower binding affinity was observed. This was not the case for the substrate doxorubicin. It is not totally clear why this difference was observed, but BmrA is a multidrug transporter, and possesses multiple distinct drug-binding sites. Like with its relative P-glycoprotein/ABCB1, it is likely that Hoeschst 33342 and doxorubicin bind to different sites [26]. The different physical properties of the various polymer SMALPs seem to have different effects on the two drug-binding sites.

SMALPs formed from SMA 2000 are known to be sensitive to divalent cations such as Mg^{2+}. It is plausible to think that the two carboxyl groups of a maleic acid chelate Mg^{2+}, possibly inducing strain or a conformational change in the SMA surrounding an SMALP. If this occurs to too many of the maleic acid groups protruding from a single SMALP, it causes the SMA to precipitate. Without the SMA belt surrounding the lipid disc, it becomes unstable and the encapsulated proteins and lipids also precipitate. For ZipA within SMA 2000 SMALPs, this occurs at concentrations exceeding 5 mM $MgCl_2$ (Figure 4C). However, for ZipA in SMA 3000, XZ09008 and SZ25010, this precipitation occurs at lower concentrations (≤1 mM). It is possible that this difference is due to the smaller size discs obtained with these polymers, as tighter wrapping around a smaller disc might be perturbed more significantly by chelating Mg^{2+}.

To summarise, the present study has shown that several commercially available SMA polymers are capable of extracting and purifying membrane proteins. However, our results suggest that SMA 2000 is the best choice in terms of solubilisation efficiency, purification yield, purity and protein function. None of the other polymers tested were able to overcome the current limitations of SMA 2000, such as sensitivity to divalent cations or limited size. In fact, the other polymers were more sensitive to Mg^{2+} and produced smaller diameter discs. Nonetheless, there may be occasions when this would be beneficial. For example, if you wanted to analyse the annular lipids associated with a protein, extraction with an SMA that gave a smaller disc would contain fewer total lipids, and only retain the ones most closely associated with the protein. A structure that is less stable or more easily disrupted may be helpful for reconstituting SMALP-encapsulated proteins into proteoliposomes or other bilayer systems for functional analysis. Alternatively for structural studies, a smaller disc size could be beneficial. For electron microscopy, a smaller belt of lipids would mask the protein less, and for crystallisation trials, fewer lipids and a more easily disrupted structure may be beneficial for forming crystals, whereas for NMR a smaller disc size might be beneficial in giving more rapid tumbling times. Thus, while SMA 2000 remains the first choice for protein purifications, understanding the properties of alternative polymers may allow us to fine-tune the method for specific downstream applications.

Abbreviations

ABC, ATP-binding cassette; BCIP/NBT, 5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt/nitro-blue tetrazolium chloride; BVs, bed volumes; DDM, dodecyl-β-D-maltoside; DLS, dynamic light scattering; IPTG,

isopropyl β-D-1-thiogalactopyranoside; Ni-NTA, nickel nitrilotriacetic acid; OG, octyl-β-D-glucoside; SMA, styrene–maleic acid; SMALPs, SMA lipid particles.

Author Contribution

Alice Rothnie, Tim Knowles and Tim Dafforn conceptualized the paper. Kerrie Morrison, Aneel Akram, Ashlyn Mathews, Zoeya Khan, Jaimin Patel, David Hardy, Chumin Zhou, Charles Moore-Kelly, Roshani Patel, Victor Odiba, Masood Javed, Nikola Chmel and Alice Rothnie performed the experiments. Alice Rothnie and Nikola Chmel analysed the data. Alice Rothnie wrote the paper.

Funding

A.J.R. was the recipient of a Royal Society Research Grant [RG110156]. D.J.H. is supported by a Biotechnology and Biological Sciences Research Council Industrial Case Studentship [BB/L015846/1]. A.A. was the recipient of a Wellcome Trust summer studentship. T.R.D. was supported by Biotechnology and Biological Sciences Research Council grants [BB/MO18261/1, BB/J017310/1, BB/I020349/1, BB/H016309/1].

Acknowledgements

We thank Prof. Jean-Michel Jault (IBCP, Lyon) for providing the BmrA expression construct, Prof. Harald Sitte and Dr Thomas Stockner (Medical University of Vienna) for providing the LeuT expression plasmid and Dr David Roper (Warwick University) for the ZipA expression plasmid. We thank Dr Mike Davies for his help in preparing the polymers. The underlying data for this publication can be found at http://dx.doi.org/10.17036/ff13833e-68c5-449f-82f2-4f07363fc55d.

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

References

1 Knowles, T.J., Finka, R., Smith, C., Lin, Y.-P., Dafforn, T. and Overduin, M. (2009) Membrane proteins solubilized intact in lipid containing nanoparticles bounded by styrene maleic acid copolymer. *J. Am. Chem. Soc.* **131**, 7484–7485 doi:10.1021/ja810046q

2 Lee, S.C., Khalid, S., Pollock, N.L., Knowles, T.J., Edler, K., Rothnie, A.J. et al. (2016) Encapsulated membrane proteins: a simplified system for molecular simulation. *Biochim. Biophys. Acta* **1858**, 2549–2557 PMID: 26946242

3 Dorr, J.M., Scheidelaar, S., Koorengevel, M.C., Dominguez, J.J., Schäfer, M. et al. (2016) The styrene–maleic acid copolymer: a versatile tool in membrane research. *Eur. Biophys. J.* **45**, 3–21 doi:10.1007/s00249-015-1093-y

4 Gulati, S., Jamshad, M., Knowles, T.J., Morrison, K.A., Downing, R., Cant, N. et al. (2014) Detergent-free purification of ABC (ATP-binding-cassette) transporters. *Biochem. J.* **461**, 269–278 doi:10.1042/BJ20131477

5 Dorr, J.M., Koorengevel, M.C., Schäfer, M., Prokofyev, A.V., Scheidelaar, S., van der Cruijsen E.A.W. et al. (2014) Detergent-free isolation, characterization, and functional reconstitution of a tetrameric K^+ channel: the power of native nanodiscs. *Proc. Natl Acad. Sci. USA* **111**, 18607–18612 doi:10.1073/pnas.1416205112

6 Jamshad, M., Charlton, J., Lin, Y.-P., Routledge, S.J., Bawa, Z., Knowles, T.J. et al. (2015) G-protein coupled receptor solubilization and purification for biophysical analysis and functional studies, in the total absence of detergent. *Biosci. Rep.* **35**, 1–10 doi:10.1042/BSR20140171

7 Swainsbury, D.J.K., Scheidelaar, S., van Grondelle, R., Killian J.A. and Jones, M.R. (2014) Bacterial reaction centers purified with styrene maleic acid copolymer retain native membrane functional properties and display enhanced stability. *Angew. Chem. Int. Ed. Engl.* **53**, 11803–11807 doi:10.1002/anie.201406412

8 Prabudiansyah, I., Kusters, I., Caforio, A. and Driessen, A.J.M. (2015) Characterization of the annular lipid shell of the Sec translocon. *Biochim. Biophys. Acta* **1848**(10 Pt A), 2050–2056 doi:10.1016/j.bbamem.2015.06.024

9 Logez, C., Damian, M., Legros, C., Dupré, C., Guéry, M., Mary, S. et al. (2016) Detergent-free isolation of functional G protein-coupled receptors into nanometric lipid particles. *Biochemistry* **55**, 38–48 doi:10.1021/acs.biochem.5b01040

10 Orwick-Rydmark, M., Lovett, J.E., Graziadei, A., Lindholm, L., Hicks, M.R. and Watts, A. (2012) Detergent-free incorporation of a seven-transmembrane receptor protein into nanosized bilayer Lipodisq particles for functional and biophysical studies. *Nano Lett.* **12**, 4687–4692 doi:10.1021/nl3020395

11 Sahu, I.D., McCarrick, R.M., Troxel, K.R., Zhang, R., Smith, H.J., Dunagan, M.M. et al. (2013) DEER EPR measurements for membrane protein structures via bifunctional spin labels and lipodisq nanoparticles. *Biochemistry* **52**, 6627–6632 doi:10.1021/bi4009984

12 Postis, V., Rawson, S., Mitchell, J.K., Lee, S.C., Parslow, R.A., Dafforn, T.R. et al. (2015) The use of SMALPs as a novel membrane protein scaffold for structure study by negative stain electron microscopy. *Biochim. Biophys. Acta* **1848**, 496–501 doi:10.1016/j.bbamem.2014.10.018

13 Jamshad, M., Grimard, V., Idini, I., Knowles, T.J., Dowle, M.R., Schofield, N. et al. (2015) Structural analysis of a nanoparticle containing a lipid bilayer used for detergent-free extraction of membrane proteins. *Nano Res.* **8**, 774–789 doi:10.1007/s12274-014-0560-6

14 Bell, A.J., Frankel, L.K. and Bricker, T.M. (2015) High yield non-detergent isolation of photosystem I-light-harvesting chlorophyll II membranes from Spinach Thylakoids: implications for the organization of the PS I antennae in higher plants. *J. Biol. Chem.* **290**, 18429–18437 doi:10.1074/jbc.M115.663872

15 Long, A.R., O'Brien, C.C., Malhotra, K., Schwall, C.T., Albert, A.D., Watts, A. et al. (2013) A detergent-free strategy for the reconstitution of active enzyme complexes from native biological membranes into nanoscale discs. *BMC Biotechnol.* **13**, 41 doi:10.1186/1472-6750-13-41

Biochemical Journal (2016) **473** 4349–4360
DOI: 10.1042/BCJ20160723

16 Li, D., Li, J., Zhuang, Y., Zhang, L., Xiong, Y., Shi, P. et al. (2015) Nano-size uni-lamellar lipodisq improved in situ auto-phosphorylation analysis of *E. coli* tyrosine kinase using (19)F nuclear magnetic resonance. *Protein Cell.* **6**, 229–233 PMID: 25564343
17 Steinfels, E., Orelle, C., Fantino, J.-R., Dalmas, O., Rigaud, J.-L., Denizot, F. et al. (2004) Characterization of YvcC (BmrA), a multidrug ABC transporter constitutively expressed in *Bacillus subtilis*. *Biochemistry* **43**, 7491–7502 doi:10.1021/bi0362018
18 Yamashita, A., Singh, S.K., Kawate, T., Jin, Y. and Gouaux, E. (2005) Crystal structure of a bacterial homologue of Na^+/Cl^--dependent neurotransmitter transporters *Nature* **437**, 215–223 doi:10.1038/nature03978
19 Hale, C.A. and de Boer, P.A.J. (1997) Direct binding of FtsZ to ZipA, an essential component of the septal ring structure that mediates cell division in *E. coli*. *Cell* **88**, 175–185 doi:10.1016/S0092-8674(00)81838-3
20 Lee, S.C., Knowles, T.J., Postis, V.L.G., Jamshad, M., Parslow, R.A., Lin, Y.-p. et al. (2016) A method for detergent-free isolation of membrane proteins in their local lipid environment. *Nat. Protoc.* **11**, 1149–1162 doi:10.1038/nprot.2016.070
21 Rothnie, A.J. (2016) Detergent-free membrane protein purification. *Methods Mol. Biol.* **1432**, 261–267 doi:10.1007/978-1-4939-3637-3_16
22 Rothnie, A., Storm, J., Campbell, J., Linton, K.J., Kerr, I.D. and Callaghan, R. (2004) The topography of transmembrane segment six is altered during the catalytic cycle of P-glycoprotein. *J. Biol. Chem.* **279**, 34913–34921 doi:10.1074/jbc.M405336200
23 Scheidelaar, S., Koorengevel, M.C., Pardo, J.D., Meeldijk, J.D., Breukink, E. and Killian, J.A. (2015) Molecular model for the solubilization of membranes into nanodisks by styrene maleic acid copolymers. *Biophys. J.* **108**, 279–290 doi:10.1016/j.bpj.2014.11.3464
24 Cuevas Arenas, R., Klingler, J., Vargas, C. and Keller, S. (2016) Influence of lipid bilayer properties on nanodisc formation mediated by styrene/maleic acid copolymers. *Nanoscale* **8**, 15016–15026 doi:10.1039/C6NR02089E
25 Vargas, C., Arenas, R.C., Frotscher, E. and Keller, S. (2015) Nanoparticle self-assembly in mixtures of phospholipids with styrene/maleic acid copolymers or fluorinated surfactants. *Nanoscale* **7**, 20685–20696 doi:10.1039/C5NR06353A
26 Martin, C., Berridge, G., Higgins, C.F., Mistry, P., Charlton, P. and Callaghan, R.(2000) Communication between multiple drug binding sites on P-glycoprotein. *Mol. Pharmacol.* **58**, 624–632 PMID:10953057