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Review

Structure and function of membrane proteins encapsulated in a polymer-bound lipid bilayer☆

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ABSTRACT

New technologies for the purification of stable membrane proteins have emerged in recent years, in particular methods that allow the preparation of membrane proteins with their native lipid environment. Here, we look at the progress achieved with the use of styrene-maleic acid copolymers (SMA) which are able to insert into biological membranes forming nanoparticles containing membrane proteins and lipids. This technology can be applied to membrane proteins from any host source, and, uniquely, allows purification without the protein ever being removed from a lipid bilayer. Not only do these SMA lipid particles (SMALPs) stabilise membrane proteins, allowing structural and functional studies, but they also offer opportunities to understand the local lipid environment of the host membrane. With any new or different method, questions inevitably arise about the integrity of the protein purified: does it retain its activity; its native structure; and ability to perform its function? How do membrane proteins within SMALPS perform in existing assays and lend themselves to analysis by established methods? We outline here recent work on the structure and function of membrane proteins that have been encapsulated like this in a polymer-bound lipid bilayer, and the potential for the future with this approach. This article is part of a Special Issue entitled: Beyond the Structure-Function Horizon of Membrane Proteins edited by Ute Hellmich, Rupak Doshi and Benjamin McIlwain.

1. Introduction

Membrane proteins are encoded by approximately 30% of the human genome and are vitally important for the function of a cell [1]. Transporters and channels control what enters and leaves, whilst receptors are responsible for communication between different cells. Membrane proteins can also have important enzymatic or structural functions. Due to these crucial roles, the dysfunction of numerous membrane proteins is associated with a wide range of human diseases, and they provide potential therapeutic targets for many conditions. However, their location within a membrane lipid bilayer has meant that the study of the structure and function of these very important proteins lags behind that of easier-to-isolate, soluble proteins.

Whilst a biological membrane is a complex and dynamic mixture of many different lipids and proteins, in order to study an individual protein it must be removed from the membrane. Traditionally this has been solved with detergents, which simultaneously solubilise membrane proteins by substituting for their lipids in hydrophobic regions of the protein and presenting a hydrophilic surface to aqueous solutions [2]. However this approach is challenging; finding the right balance between efficient extraction and maintenance of protein structure and function requires a time consuming and costly screening approach, which is highly protein specific. Many proteins lose activity in detergent solution, perhaps due to loss of the lateral pressure usually provided by the membrane [3], because the detergents can also denature stabilising interactions within the protein itself, or because vital lipids required for protein function have been stripped away. The membrane is not simply a hydrophobic 'sea' within which membrane proteins are distributed, but specific interactions occur between certain lipids and proteins, and the function of many membrane proteins is

Abbreviations: SMA, styrene-maleic acid copolymer; SMALP, SMA lipid particle; MSP, membrane scaffolding protein; GPCR, G protein-coupled receptor; ABC, ATP binding cassette; hENT1, human equilibrative nucleoside transporter 1; CzcD, zinc diffusion facilitator; NMR, nuclear magnetic resonance; CHS, cholesteryl hemisuccinate; $A_{2A}R$, adenosine 2a receptor; MT1R, melatonin receptor; TLC, thin layer chromatography; MS, mass spectrometry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; CD, circular dichroism spectroscopy; DDM, dodecylmaltoside; OG, octylglucoside; DM, decylmaltoside; EM, electron microscopy; LCP, lipidic cubic phase; HwBR, bacteriorhodopsin from *Haloquadratum walsbyi*; FRET, Förster resonance energy transfer; DIBMA, di-isobutylene maleic acid copolymer; SMA-SH, SMA with solvent exposed sulphydryls

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highly dependent on the specific lipid environment in which they are located [4–6]. To try to combat this issue following detergent-mediated purification, proteins have been reconstituted back into lipid bilayer systems, in order to reproduce the lipid environment for downstream functional studies. Most frequently this has involved the formation of proteoliposomes. However this can result in high lipid:protein ratios, and the large proteoliposomes scatter light, which can be problematic for some downstream techniques. The development of nanodiscs provided a step change in reconstitution of membrane proteins [7]. In nanodiscs membrane proteins are reconstituted into a small disc of lipid bilayer surrounded by a membrane scaffolding protein (MSP). These particles are small (approx.10 nm diameter) and stable and have been shown to be amenable for numerous downstream techniques [8]. However, they do still require initial solubilisation and purification using detergents.

1.1. SMALPs – what is new and different about them?

A new approach to membrane protein extraction has been developed utilising a styrene-maleic acid (SMA) copolymer instead of conventional detergents. This polymer inserts into membranes and 'cuts it up' to form small discs of bilayer encircled by the polymer, termed SMALPs (SMA lipid particles) (Fig. 1), also known as native nanodiscs and lipodisqs. SMA is amphipathic due to its mixture of hydrophobic styrene and charged, hydrophilic maleic acid moieties. So far, SMA polymers with both 2:1 and 3:1 styrene:maleic acid ratios have been used successfully. Importantly, membrane proteins extracted by this polymer retain their lipid bilayer environment, yet the particles are small (approx.10 nm diameter), stable, soluble and amenable to many downstream techniques [9–13]. SMA encapsulated proteins can be purified using affinity chromatography, meaning you can extract, purify and study your membrane protein of interest without ever removing it from its lipid bilayer environment (Fig. 1).

SMA is not the first foray into using amphipathic polymers for membrane protein stabilisation; this approach was pioneered in the 1990s by Jean-Luc Popot and co-workers [14,15]. However, SMA is the first polymer shown to be capable of directly solubilising biological membranes. In fact, it could be argued that the membrane proteins remain solubilised in their native lipids, whilst SMA allows the lipids to be soluble in aqueous solution.

1.2. What is SMA and how does it work?

In many ways it is remarkable that a crude industrial product can

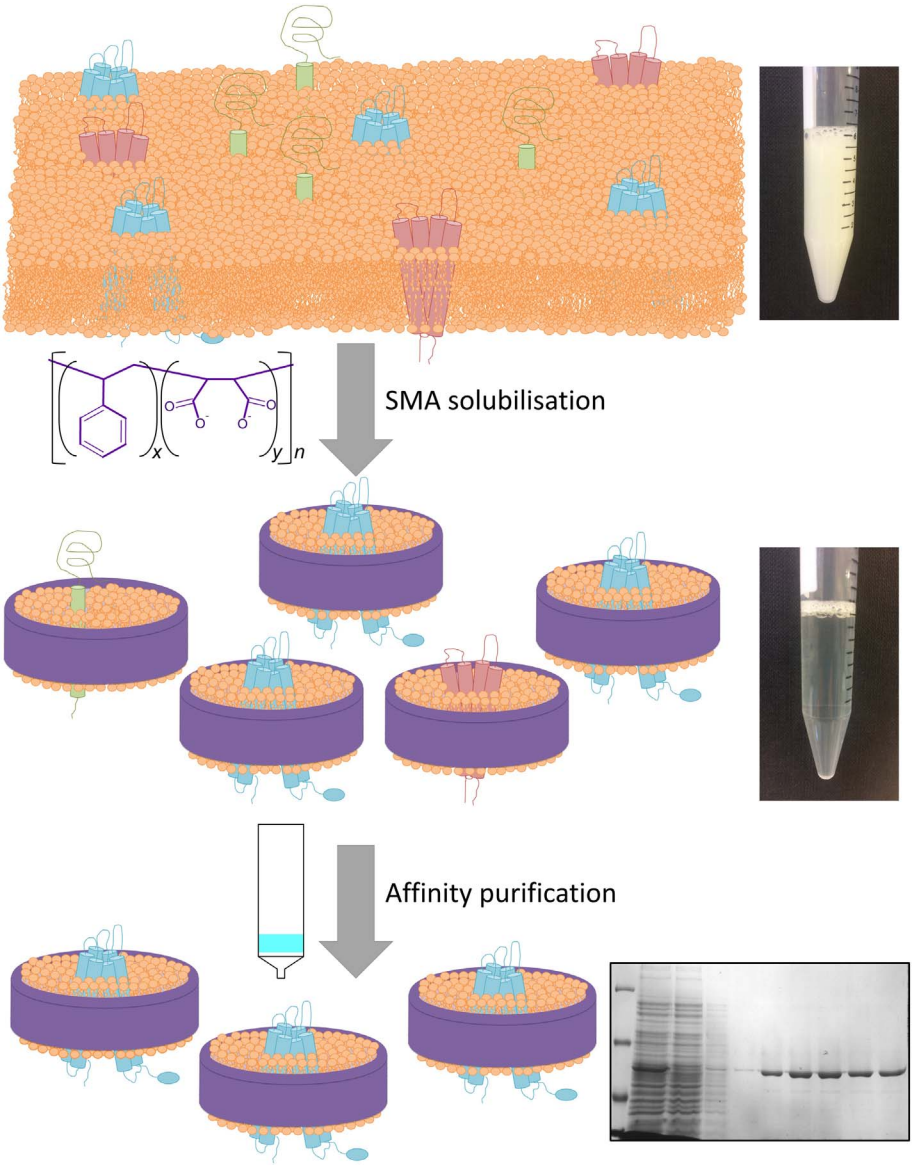

Fig. 1. Schematic of SMA mediated membrane protein solubilisation and purification. SMA is added to membrane preparations where it inserts and forms small, soluble nanoparticles, SMALPs, containing proteins and lipid bilayer surrounded by polymer. The cloudy suspension of membranes turns to a clear solution. SMALPs containing membrane proteins of interest can be purified by affinity chromatography, enabling solubilisation and purification of membrane proteins without ever removing them from a lipid bilayer environment.

facilitate the purification of monodisperse samples of membrane proteins. As a result of its synthesis by free-radical polymerisation [16], the SMA currently used for protein purification is a random copolymer and is fundamentally polydisperse (polydispersity index = 2.8) [17], consisting of polymers of different lengths and compositions. The ratio of styrene:maleic acid in each case (i.e. 2:1, 3:1 etc) refer to an average across a whole batch, rather than indicating strict alternation of -[S-S-M] sections. It is yet to be determined what subset of the total polymer mixture is actually active in forming SMALPs. Likewise, the number of SMA polymers encircling the lipid is controversial. Despite these uncertainties, SMA has already enabled the purification and analysis of a long list of membrane proteins [18].

Aside from its ability to solubilise biological membranes, SMA does not form micelles in the way that they are understood for conventional head-and-tail detergents. This means that the polymer is used only for solubilisation and does not have to be added to buffers for the remaining steps of the purification. This is both convenient and removes many difficulties associated with the presence of micelles (e.g. interference with light scattering methods, excessive concentration of large micelles).

The solubilisation of model lipid vesicles by SMA has been used to start to answer some of the mechanistic questions about how SMA works. Keller and co-workers have demonstrated that thermodynamically, SMALP formation incurs a much lower free energy penalty than solubilisation by conventional detergents. Whilst SMA has a thermodynamic preference for being within SMALPs, the lipids display little preference between SMALPs and vesicular bilayers, thus highlighting their more native-like properties [19,20]. Factors that affect the kinetics of solubilisation have also been investigated. SMA solubilisation is faster when lipid chain lengths are shorter, lateral pressure is low, ionic strength is increased or the temperature is increased, particularly above the melting temperature (Tm) of the lipid bilayer [21]. However the kinetic efficiencies obtained do not correlate with thermodynamic efficiency, in that slow solubilisation of gel-phase bilayers actually requires less SMA than the fast solubilisation of fluid-phase bilayers [19]. Thus there are still many questions remaining to be answered to fully understand the mechanism of SMA solubilisation of lipids. It also remains to be seen if/how the presence of proteins within the lipid bilayer affects this process.

2. Purification of membrane proteins using SMA

Simplicity is one key advantage of SMA compared to conventional detergents for the purification of membrane proteins. A new purification typically begins with testing a panel of detergents for optimal solubilisation, and considering a similar panel for downstream processes such as purification, functional assays and structural characterisation [22–24]. Factors such as head groups, tail lengths and critical micelle concentration can have different effects on the experiment. By contrast, SMA provides a much more generic solution: proteins are solubilised from membranes, along with a portion of lipid bilayer, as stable nanoparticles [25] that, once purified, are suitable for many structural and functional characterisations. Crucially, once assembled SMALPs are very stable and in contrast to micelle-forming detergents, it is not necessary to supplement buffers with further SMA, which makes many downstream processes technically less challenging than they are with conventional detergents.

A generically-applicable protocol describing the purification method for membrane proteins using SMA was recently published [26]. In this protocol, SMA is used at 2.5% (w/v) to solubilise membranes at 40 mg/ml wet weight, and the experiment is carried out at room temperature. Thereafter, the target protein can be isolated by nickel affinity chromatography including an overnight binding step followed by size exclusion chromatography. This method has allowed successful purification of a large variety of membrane proteins, including G protein-coupled receptors (GPCRs) [27], ATP binding cassette (ABC) proteins [9], ion channels [10] and recently, several membrane protein complexes [28–31]. These reports demonstrate successful solubilisation of membrane proteins from a range of membranes: bacterial, insect, yeast, plant and mammalian, and also from organelle membranes [31,32].

Following solubilisation with SMA, nickel-affinity purification is the most widely used method to isolate specific protein-SMALPs [9,10,26,27,31,33–36]. Some simple modifications to standard nickel affinity have been necessary to adapt it for use with SMALPs - namely, the longer incubation of protein-SMALPs and nickel resins to ensure binding. In addition, low imidazole concentrations (> 10 mM) can elute protein-SMALPs from nickel resins, which restricts the stringency of the washing for proteins with short histidine tags ($\leq His_6$). Whilst this could be a concern regarding the purity of the final sample, in practice it is possible to isolate highly pure protein-SMALPs and in some cases the purity of samples isolated using SMA exceeds that of the same construct in common detergents.

As the SMALP method becomes more widespread, and purifications of more proteins are attempted, new modifications and applications of the method have emerged. These demonstrate the fundamental versatility of protein-SMALPs, and may facilitate, amongst other things, solubilisation of proteins from different membranes, use of different affinity tags, and purification of proteins with differing stabilities. As the details of many of the initial studies have recently been summarised [9,10,18,27], in this section, we will discuss the most recent membrane protein purifications in SMA, including novel methodologies.

2.1. New proteins and purification methods

The human equilibrative nucleoside transporter (hENT1/SLC29A1), an example from a class of membrane proteins not previously studied in SMA, was recently purified from insect cell membranes [37]. This study demonstrated successful use of the FLAG tag for purification of a SMALP-encapsulated protein, with an approximate yield of 0.4 mg hENT1 per litre of insect cell culture, which was about 40% of the total solubilised hENT1. Immunoaffinity chromatography was also used to purify the zinc diffusion facilitator CzcD via a Strep tag and the Streptactin resin [38]. In order to maximise the efficiency of binding of the CzcD-SMALPs to the resin, free SMA was removed after solubilisation by washing the solubilised material in a 50 kDa molecular weight cut off centrifugal filter. This purification yielded 1–4 mg protein per litre of culture, which was subsequently used in an NMR study. A further antibody-based approach has been used to purify small quantities of the SecYEG translocon complex from its native *Escherichia coli* membranes using immunoprecipitation [30].

Another novel purification method demonstrated the use of an ADP-sepharose affinity purification to isolate the dhurrin metabolon once again from its native membrane, in this case those from sorghum plants [28]. This dynamic multisubunit complex was isolated along with its native lipids, which were extracted from the SMALPs and analysed by liquid chromatography-mass spectrometry.

2.2. Modifications to the original SMALP protocol

As well as reporting new purification methods for protein-SMALPs, a number of recent papers, including those discussed above, have described modifications to the original SMALP solubilisation protocol. For example, SMA has been used to solubilise the melatonin receptor (MT1R) from *P. pastoris* membranes, but this yielded less protein than a comparable detergent purification [35]. This corresponded to findings from other authors who showed that yeast membranes may be less susceptible to SMA solubilisation than those from bacteria [21]. In *P. pastoris* membranes, the yield of protein solubilised in SMA was comparable to the yield from detergent only at the elevated temperature of 40 °C, which resulted in unfolding of the target protein [35].

In the case of hENT1, the authors observed that isolation of functional protein required the use of SMA in the solubilisation step at a

lower concentration than previously reported (0.25% vs 2.5%). Moreover, to prevent protein degradation the solubilisation was carried out overnight at 4 °C, as opposed to 1–2 h at room temperature as described in the original protocol. However, in the new protocol the use of protease inhibitors was not mentioned, the inclusion of which may prevent protein degradation and remove the need for this modification. The disadvantage of reducing the SMA concentration and temperature was that it reduced the solubilisation efficiency for the target protein to 20%, however this seems a more than reasonable compromise for obtaining pure samples of a functional membrane protein, and indeed the authors reported a final yield of 0.4 mg protein per litre of insect cell culture, which is adequate for most applications.

To isolate functional hENT1, it was also necessary to supplement the solubilisation mixture with cholesteryl hemisuccinate (CHS) or asolectin lipids. Supplementation with CHS is commonly used in detergent solubilisation to stabilise membrane proteins [39–43], but proteins in SMALPs typically retain membrane lipids, so it is surprising that CHS was necessary to maintain protein function. Indeed the opposite observation was made for the GPCR adenosine 2A receptor ($A_{2A}R$) purified from *P. pastoris* membranes [27,40]: CHS supplementation was necessary for detergent-purified samples, but not those prepared using SMA. Doubtless as more proteins are isolated in SMALPs, a consensus on when and why this supplementation is necessary will eventually emerge.

2.3. Identification of lipids

An aspect of the protein solubilised by SMA is the consistent co-purification of lipids with the encapsulated membrane protein. Capitalising on this opportunity, several of the papers described above, as well as earlier publications, have described extraction and analysis of the co-purifying lipids, with the intention of identifying which, if any, membrane lipids are of particular importance to the structure and function of each protein, and indeed whether significant lipid selectivity exits. Such evidence is currently sparse.

Methods employed for analysis of lipids from SMALPs include thin layer chromatography (TLC) [10,29,31,32,44], gas chromatography [10,28], phosphorous assays [25,36,38], and mass spectrometry (MS), both liquid chromatography-MS and electrospray ionisation mass spectrometry [29,37]. These methods offer different levels of detail about the lipids contained in the SMALPs, from quantification of total phospholipid or sterol content and identification of phospholipid type by headgroup, to detailed analysis of the chain lengths and saturations. Several studies report an estimation of the number of lipids present in the nanoparticle, but to date this shows considerable variability.

Lipid-only SMALPs have been estimated by theoretical calculations to contain 140–180 lipids total (70–90 per leaflet) [45,46]. Phosphorus assays for lipid content within purified SMALP samples give values of 11 lipids per SMALP for the beta-barrel protein PagP [25], 32 lipids per dimer of the 34 kDa protein CzcD [38], and 40 lipids per SMALP for AcrB [36]. These estimates suggest that the nanoparticles are only sparsely populated with lipid. This is perhaps not surprising for AcrB, which with 36 transmembrane helices is the largest protein encapsulated to date, but might be considered low for the smaller proteins. In contrast it was estimated that purified SMALPs containing the bacterial reaction centre contained 150 lipid molecules per reaction centre [44]. These differences may arise due to variability in the size of the SMALP formed, or technical differences in sample preparation or analysis, but the large variability suggests more data are needed to be certain of these methods.

Arguably more interesting than the total number of lipids co-extracted with a membrane protein are the characteristics of those lipids, and whether or not a given protein associates with specific lipids. Analysis of lipids that co-purified with the dhurrin metabolon indicated that phosphatidyl choline (PC), phosphatidyl ethanolamine (PE) and phosphatidyl glycerol (PG) were enriched in the dhurrin metabolon-SMALPs compared to the bulk phospholipids from sorghum membranes [10,28]. This corresponded to results from functional assays of the detergent-purified metabolon subunits, which were more active when reconstituted into anionic phospholipid-enriched liposomes. This indicates a selectivity of the dhurrin-metabolon for anionic lipids.

The SecYEG complex displayed a preference for anionic lipids based on an analysis of the lipids contained within SecYEG-SMALPs [29]. In this case, lipids were extracted from SecYEG-SMALPs using organic solvents [47], and detected by TLC and electrospray ionisation-MS. The enrichment of PG and cardiolipin (CL) were 40% and 90% higher respectively. The ion channel KcsA extracted and purified with SMALPs also had higher preference for anionic lipids, particularly PG and CL [48]. This might hint that SMA itself had a preference for negatively charged lipids, but in fact in all these cases, the proportions of lipids in the total SMA-solubilised fraction (i.e. the soluble material prior to purification) matched those of the bulk membranes. This indicates that in fact, SMA does not selectively extract certain lipids.

Mass spectrometry offers the opportunity to derive more information than just the phospholipid head groups from the lipids extracted from SMALPs. For example, hENT1-SMALPs showed an enrichment of long chain saturated and monounsaturated PC species but an exclusion of polyunsaturated PE species compared to the bulk *Sf*9 insect cell membrane from which it was extracted [37].

Overall, these data do appear to suggest that particular proteins and lipids have preferential interactions. On the other hand, recent publications have reported that the lipids contained by SMALPs are capable of exchange, either between SMALPs and immobilised bilayers [49], or by rapid lipid exchange between nanodiscs [50]. Both of these studies were undertaken using model membranes and lipid-only SMALPs and may not apply to protein-SMALPs. Nonetheless this is a question that must be settled before the findings from lipidomic studies of SMALPs can be regarded as definitive.

3. Stability, structure & function

3.1. Enhanced stability of membrane proteins

One of the most common problems experienced when working with extracted and purified membrane proteins is their inherent lack of solubility in aqueous environments. As previously mentioned, detergents render membrane proteins not only soluble but also, in many cases, unstable, leading to an irreversible conformational change and/or aggregation. This destroys the structure, function and solubility of the protein, making it impossible to carry out further biochemical and biophysical studies.

SMA solubilisation offers a new outlook on membrane protein instability. SMALPs remove many of the difficulties previously encountered in concentrating detergent-purified membrane proteins, where concomitant concentration of detergents often occurs, which can lead to decreased stability of the protein [51]. Due to the presence of lipids, it might be expected that SMA-extracted membrane proteins demonstrate an enhanced stability compared to detergent-purified proteins. Perhaps more accurately, SMALP-encapsulated proteins are able to demonstrate their inherent stability. This hypothesis has been investigated for several different proteins using a range of approaches, including gel electrophoresis [34], light scattering [34], fluorescence [9,48], absorbance [44], and circular dichroism spectroscopies [10,25,27], as well as binding assays using radiolabeled substrates [27,37].

Thermostability, which is the ability to resist chemical or physical structural changes at increasing temperatures, has been most frequently studied and it can be used as an indicator of the overall stability of a membrane protein [52,53]. Circular dichroism spectroscopy (CD) uses the differential absorption of right-handed and left-handed circularly polarised light measured over various wavelengths (which occurs when a molecule contains chiral groups) to examine the secondary structural

composition of proteins and monitor unfolding. SMALP discs on their own have negligible CD absorbance, and unlike proteoliposomes do not scatter light significantly [25]. Using modern spectrometers, CD signal can be monitored as temperature increases to monitor protein unfolding [54].

Using CD, it was observed that the folded structure of transmembrane protein PagP was maintained within SMALPs, and displayed high thermostability at maximum experimental temperatures of 90 °C compared to proteins extracted via the detergent octyl glucoside (OG) [25]. CD analysis of the GPCR $A_{2A}R$ encapsulated in SMALPs showed secondary structural data consistent with solved crystal structures of this protein. Upon heating from 25 °C to 95 °C, $A_{2A}R$ protein gradually lost its secondary structure, leading to thermal denaturation of the protein, yet even at 95 °C the $A_{2A}R$ signal had not completely transformed to that of a random coil. This indicates that proteins in SMALPs are highly resistant to thermal denaturation [27]. Similarly the SMALP-extracted and purified ion channel KcsA was shown using CD spectroscopy to have markedly increased stability at high temperatures in comparison in the detergent dodecylmaltoside (DDM) [10].

Fluorescence spectroscopy has also been used to study unfolding of membrane proteins upon heating. Monitoring the spectral shifts of native tryptophan residues as they become more solvent exposed reiterated the observation that KcsA within SMALPs was more thermostable than when detergent (DDM) solubilised [48]. In addition, an assay which labelled cysteine residues with fluorophores as the protein denatures [42,52] showed that the ABC transporter P-glycoprotein within SMALPs had a > 10 °C increase in apparent melting temperature compared to detergent (OG) purified protein [9].

Arguably the most important aspect of stability is functional stability. Using radioligand binding assays, functional thermostability has been measured for both the GPCR $A_{2A}R$ [27] and hENT1 [37]. The $A_{2A}R$ within SMALPs showed a marked increase in thermostability compared to DDM solubilised protein [27]. In addition the SMA solubilised hENT1 showed an increase in apparent melting temperature of 6–7 °C compared to solubilisation in detergents DDM or DM (decyl maltoside), and was in fact more similar to that in native cells [37].

Other aspects of SMALP-encapsulated protein stability that have been investigated relate to storage conditions. How long a sample is stable under typical storage conditions such as 4 °C or room temperature can be important for several downstream techniques. Freezing of biological compounds is commonly used in various industries to maintain their quality and stability, increase shelf life, reduce degradation over time and prevent microbial growth. However freezing and thawing proteins through multiple cycles can cause complex structural denaturation. Using radioligand binding, the GPCR $A_{2A}R$ solubilised in SMA was shown to have a 7-fold increase in half-life at the physiological temperature of 37 °C, compared to $A_{2A}R$ in the detergent DDM. Moreover the stability of $A_{2A}R$-SMALP at 4 °C was similar to that of native membranes with a half-life of > 16 days, in comparison to $A_{2A}R$ in DDM which had a half-life of 1.8 + 0.3 days [27]. Using a characteristic absorbance, SMA-purified bacterial reaction centres were also shown to have increased stability over time compared to detergent-purified samples at both 40 °C and 70 °C [44]. Finally $A_{2A}R$-SMALP showed greater resistance to repeated freeze-thaw cycles, and maintained its binding efficiency even after five freeze-thaw cycles on the same sample, whereas $A_{2A}R$ solubilised in DDM lost its ligand binding capacity after only a single freeze-thaw cycle [27].

3.2. Structural studies of membrane proteins within SMALPs

Structural studies typically require stable, monodisperse, highly pure protein samples at a high concentration. To achieve this sample quality for membrane proteins has historically been a significant technical challenge. However the enhanced stability of membrane proteins within SMALPs, coupled with the ease of concentrating these samples, makes this system ideal for downstream structural studies. In fact, SMALPs are compatible with both negative stain and cryo-electron microscopies [9,36]; X-ray crystallography [33] and solid state NMR [38,45].

For many years, X-ray crystallography has been the gold-standard technique for high resolution protein structure determination. However it is hindered by the unpredictability of crystal formation especially for membrane protein samples, due in part to their large membrane-spanning hydrophobic domains. Many membrane proteins have been crystallized from detergent micelles but many targets remain difficult to purify and concentrate sufficiently for crystallization, and for others there is evidence that they are not stable in detergents over the timescales necessary for crystal growth [55]. With mounting evidence that protein-SMALPs retain native lipids, functional properties and thermal stability, there has been great optimism that this could translate to solving reliable structures of membrane proteins at high resolutions. However, it remained unclear whether SMA could be removed from SMALPs in order to allow the close contact between proteins needed for crystallization, without irreversibly destabilising the proteins.

Recently Broecker and co-workers neatly side-stepped this issue by reconstituting an α-helical seven transmembrane bacteriorhodopsin (29 kDa) from *Haloquadratum walsbyi* (HwBR) into the lipidic cubic phase (LCP) directly from SMALPs [33]. Crystals formed and a structure

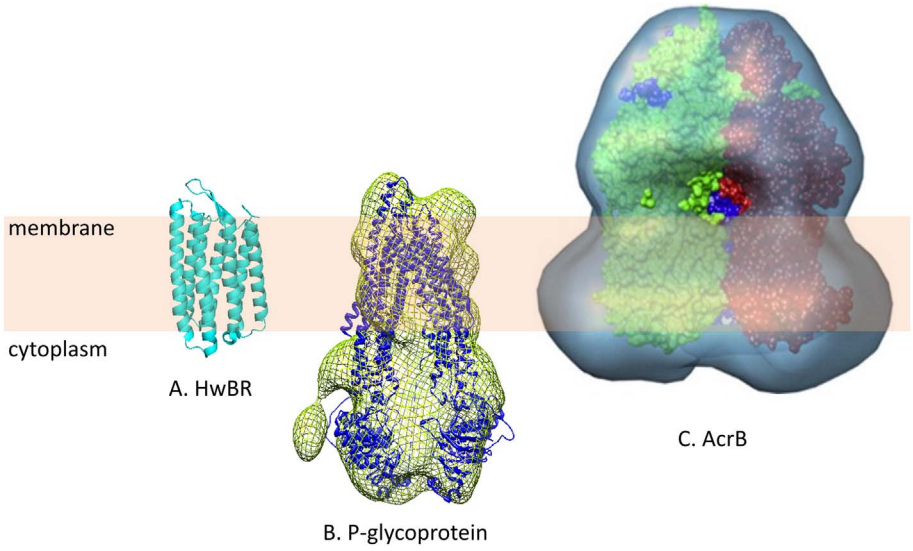

Fig. 2. Membrane protein structures determined using SMA solubilisation and purification. A; Structure of HwBR determined by x-ray crystallography using HwBR solubilised and purified in SMA before incorporation into lipidic cubic phase to generate crystals (PDB ID: 5ITC). Crystals diffracted to a resolution better than 2 Å. This structure was originally published in Broecker et al. (2017) Structure 25; 384–392 [33]. B. Refined envelope of SMA solubilised P-glycoprotein/ABCB1 obtained using single particle cryo-EM (yellow net) with the crystal structure (PDB ID: 3G5U) docked within it (blue ribbon). The refined structure was estimated to be at approximately 35 Å resolution. This figure was originally published in Gulati S et al. (2014) Biochem J. 461; 269–278 [9]. C; Surface view of the reconstruction from negative stain single particle EM of SMA solubilised AcrB, with the crystal structure (PDB ID: 1IWG) fitted into it. Resolution of 23 Å. This figure was originally published in Postis V et al. (2015) BBA Biomembranes 1848; 496–501 [36].

of 2.0 Å resolution was generated (Fig. 2A) that is identical to the 2.2 Å detergent based structure. They report that crystal packing was not affected by the SMA polymer as long as the polymer concentration was low. Bacterial lipids were not observed in the crystal structure but had been replaced by the monoolein used in the lipidic cubic phase for packing. The authors hypothesise that lipids could be retained by the proteins, binding them tightly, but this remains to be proven. Nonetheless, it is possible to obtain a membrane protein structure for a protein sample that consistently retained a lipid environment, and is a very exciting prospect for the future.

Equally excitingly, electron microscopy (EM) has lately "come of age" as a method for high-resolution protein structure determination [56,57]. As a solution-based method, EM does not require crystal formation, relying instead on reconstruction of three-dimensional structures from two-dimensional images of single protein particles. In this case too, SMALPs have demonstrated their utility: single particle cryo-EM was utilised to obtain the structure of the ABC transporter P-glycoprotein/ABCB1 (150 kDa) within SMALPs (Fig. 2B) [9]. Whilst the structure was at low resolution (35 Å), it correlated well with the structures solved by crystallography [58,59] and previous EM [60]. Similarly the structure of the bacterial multidrug efflux pump AcrB, extracted and purified within SMALPs has been determined to 23 Å using negative stain single particle EM (Fig. 2C) [36]. The structure showed a clear 3-fold symmetry as expected from this large (≈ 300 kDa) trimeric protein, and the known crystal structure correlated well with the EM envelope.

Whilst these structures obtained within SMALPs to date are low resolution, they prove that SMALP-encapsulated proteins are amenable to single particle EM studies. Recent technological advances in cryo-electron microscopy, such as direct electron detectors and algorithms to correct for beam-induced motion mean that high resolution structures are now achievable. This was highlighted by the recent 3.4 Å structure of the TRPV1 ion channel, so detailed that side-chains of amino acids were resolved [61]. The existing EM data from proteins in SMALPs suggest that there is great promise for the future.

Solution NMR is not expected to be compatible with SMALPs, due to the size of the disc limiting the tumbling speed of the proteins. Although it has been shown that alterations in the polymer structure can lead to the formation of smaller nanoparticles [34], these remain to be tested. However, a recent study demonstrated that SMALP encapsulated proteins are amenable to atomic-resolution studies using magic-angle-spinning solid-state NMR [38]. Using the zinc diffusion facilitator CzcD (34 kDa), the authors demonstrated that SMA solubilised and purified protein could be utilised to generate NMR spectra, with favourable line widths and potential for high resolution. Spectral overlap has so far prevented the residues from being fully assigned, perhaps due to the size of the protein. Even so, this is another promising area for the future.

3.3. Functional studies on SMALP encapsulated membrane proteins

With any new or different method, questions rightly arise about the integrity of the protein purified: does it retain its activity; its structure; and ability to perform its function? How do membrane proteins within SMALPs perform in existing assays and lend themselves to analysis by well-tested methods?

The first SMALP encapsulated protein reported was PagP, an outer membrane beta-barrel with phospholipase activity. Its function in SMALPs was measured using NMR by detection of a ^{13}C-lysoPC product from ^{13}C-labelled PC [25]. NMR was also utilised to monitor the function of a bacterial tyrosine kinase, ETK, within SMALPs. ^{19}F NMR spectra from an incorporated unnatural amino acid difluorotyrosine was used to measure autophosphorylation [11].

Another early report described purification and functional analysis of the GPCR $A_{2A}R$ using radioligand binding assays. The pharmacology of $A_{2A}R$ was comparable when encapsulated in SMALPs and in the membrane [27]. More recently, purification of the ghrelin receptor and melatonin receptors reiterated that receptors retained their normal ligand-binding ability [35]. This study used a fluorescence-based assay to show that both proteins were capable of activating their cognate G protein, and moreover, that they could recruit arrestin in response to agonist binding (and conversely that this did not happen in the presence of an antagonist).

A Förster resonance energy transfer (FRET) assay was used to monitor the activity of SecYEG encapsulated in SMALPs [29]. A substrate of the Sec translocon, proOmpA-DhfR-Atto532, was added to SecYEG-Cy5-SMALPs along with ATP. As OmpA entered the translocon, the fluorescence signal increased. In the presence of a non-hydrolysable analogue of ATP, this was not observed.

The activity of SMALP-encapsulated complex IV (cytochrome *c* oxidase) purified from yeast mitochondria was monitored using standard spectroscopic methods [31]. The complex IV-SMALPs displayed characteristic absorption peaks at 445 and 605 nm due to the haem groups and catalysed the reduction of oxygen to water. Furthermore, addition of DDM to the purified cytcO-SMALPs actually resulted in higher activity, which has previously been shown to result from detergent-mediated loss of regulatory subunits from the complex. This suggests that SMA-encapsulation may solubilise complex IV from the membrane in a more physiologically relevant state that standard detergent preparations, and even challenges the idea that samples with higher activity are necessarily most physiologically relevant.

Conversely, the oxygen reduction assay revealed that adding high concentrations of SMA to equine heart mitochondria inhibited the oxidase activity of complex IV, perhaps due to SMA preventing binding of cytochrome *c* to complex IV. However, this effect was reversible, and was not observed in the purified complex IV samples, most likely because they contained very low concentrations of SMA (little or no free SMA remains beyond the solubilisation step).

Fluorescence-based assays were also used to measure the ligand-binding ability of the ABC proteins P-glycoprotein/ABCB1 and BmrA. Unfortunately SMA is not compatible with standard assays to measure the rate of ATP hydrolysis, which is a typical measure for ABC transporter activity (this is discussed further in the next section). Nonetheless, the function of SMA-encapsulated P-glycoprotein or BmrA was measured by monitoring fluorescence quenching upon binding of nucleotides, inhibitors and transport substrates. These all showed binding affinities comparable to previous reports for detergent solubilised and/or reconstituted proteins [9,34].

Single channel activity has been recorded for the KcsA ion channel reconstituted from SMALPs into an *E. coli* planar lipid bilayer [10]. After reconstitution, the protein retained its characteristic conductivity, both in terms of the amplitude of the signal and open probability of the channel. The channel could also be reversibly blocked with tetraethylammonium. In short, the channel activity of the SMALP-purified KcsA was identical to that purified by standard protocols (i.e. detergent followed by reconstitution into lipids).

To conclude, in the majority of these cases standard activity assays required at most minor modifications in order to be used with protein-SMALPs. In addition, where a comparison is possible, the SMALP-encapsulated proteins typically displayed normal function compared to the same proteins in detergents, liposomes and even native membranes. Some limitations remain, such as the difficulty of detecting ATPase activity, and the effect that excess SMA has on the function of some proteins, but overall it can be concluded that for many proteins SMA does not have a deleterious effect on function, and considered with the enhancements to protein stability, may facilitate functional studies that have previously been impossible. This is especially true for delicate or dynamic protein complexes, which may be more easily isolated using SMA-encapsulation.

4. Current limitations & future directions

It is clear that the SMALP approach offers many advantages over conventional detergents for membrane protein solubilisation and purification. However as with any new technology there are still many parameters to determine and some limitations to be overcome.

4.1. Known limitations

One issue is the sensitivity of the SMALP nanoparticle structure to divalent cations such as magnesium and calcium [34,62]. It is thought that the two carboxyls of the maleic acid groups chelate Mg^{2+}, possibly inducing strain or a conformational change in the polymer surrounding the SMALP, and 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 wrapped around the protein/lipid disc, it is no longer water soluble, leading to precipitation of the protein. For SMALPs formed with the 2:1 SMA polymer, this occurs when the magnesium concentration exceeds approximately 4 mM, whereas for SMALPs formed from the 3:1 SMA polymers the threshold for magnesium is even lower (< 1 mM) [34]. For proteins that require magnesium binding as a co-factor for nucleotide binding or hydrolysis, such as ATPases and G proteins this is particularly problematic. Moreover, it is likely that the SMA sequesters the Mg^{2+} at lower concentrations making it unavailable to the protein, so a simple solution of restricting the concentration of magnesium below the threshold may not be effective.

Similarly, SMA and SMALPs are sensitive to low pH. The pH needs to be retained above pH 7 (typically pH 8 is used), otherwise the SMA precipitates out of solution. For some proteins which require a more acidic environment for optimal function, this can be problematic.

Another potential limitation of the SMALP system is the size of the disc formed. Typically reported to have a diameter of 10–12 nm, the discs are too small for some proteins and complexes with large membrane spanning domains (since these domains of the protein reside in the disc, this characteristic, rather than the absolute molecular weight, is likely to be the limiting factor). However Bell *et al.* exploited this size limitation to produce a membrane preparation highly enriched in their target membrane complex, photosystem I-light harvesting chlorophyll II, which is too large itself for incorporation into a SMALP, by using SMA to solubilise and remove most other smaller membrane components [63]. There are also some reports that the size of the discs formed are not as rigid as previously thought [28,64], and have the potential to be tuned according to the ratio of polymer to lipid [19,20] or the ratio of styrene:maleic acid within the polymer [65].

Although the enhanced stability of membrane proteins within SMALPs is one of the major advantages of this approach, particularly in relation to structural biology, it raises the question of whether proteins could be 'held too tightly' for conformational changes to occur. It was shown with lipid only SMALPs that the lipid environment within SMALPs retains a very similar phase transition temperature to those in a larger bilayer, suggesting it is a good mimic of the natural environment [46]. However the lipids at the periphery of the disc which interact directly with the styrene moieties of the polymer are considered to be more ordered [46]. For larger membrane proteins where the number of lipids surrounding the protein will be more limited, and which will be nearer to this ordered outer layer of lipids, might this be a problem? For example for ABC transporters which typically have 12 transmembrane helices, and are known to undergo large scale conformational changes within the transmembrane domains in response to nucleotide binding and hydrolysis in their cytosolic domains, is there enough space or flexibility within the SMALP to accommodate these movements? To date it has not been possible to answer that question since the ATP hydrolysis required to drive these conformational changes requires magnesium binding. However, it is an important area to investigate in the future. Novel polymers which address the magnesium sensitivity may be the answer. Alternatively, production of larger disc sizes such that there is more fluid lipid bilayer surrounding the protein may help.

Finally, whilst the small disc structure is perfect for binding assays since both sides of the membrane are freely available, and are also compatible with spectroscopic techniques, they are not amenable to measuring movement of substrates across a bilayer, either by a transporter or ion channel, where defined, separate compartments are required. Can the SMA be removed and the encapsulated proteins reconstituted into proteoliposomes or other lipid bilayer systems? It has previously been shown that the ion channel KcsA purified within SMALPs can be added to a planar bilayer system and insert into the membrane to enable single channel conductivity measurements [10]. However it was not clear what happened to the SMA, whether it intercalated into the bilayer, remained associated with the protein or not. Also this style of assay requires only a single SMALP to reconstitute into the bilayer, and the question remained whether larger numbers could be reconstituted in bulk into proteoliposomes. Studies using lipids only with SMA suggest that the formation of SMALPs from liposomes is a reversible process and under the right conditions it should be possible to reconstitute SMALPs into liposomes [19]. Most importantly the recent incorporation of SMALP purified protein into lipidic cubic phase showed that it is indeed possible to transfer large amounts of protein from SMALPs into another bilayer system [33], and this is highly promising for the future.

4.2. Exploring SMA variants and novel polymers

Modifications to the polymer structure may provide solutions to the current limitations. To begin with, there are already many styrene-maleic acid polymers with varying sizes and ratios of styrene:maleic acid. These display variations in their ability to solubilise membranes, their sensitivity to ionic strength and pH, and the size of discs they form [34,66]. Strictly alternating SMA polymers also exist (called RAFT polymers after the polymerisation process used to produce them) [16,65,67]. These provide a well-defined comparison to the industrial SMAs that are currently in use.

In terms of the pH sensitivity of SMA, a partial solution already exists in the form of styrene maleic imide (SMI), a polymer soluble below pH 6 [68], that like SMA can solubilise lipids [69,70].

It has previously been demonstrated that SMA can be modified by reaction with cysteamine which replaces one of the maleic acid carboxyl groups with an amide (SMA-SH), creating a modified SMA polymer which is still capable of solubilising membranes and forming discs [71]. With fewer carboxyl groups, this modified polymer may be less able to chelate the divalent cation and thus less sensitive to magnesium, however this remains to be determined. Interestingly, it was recently reported that a di-isobutylene maleic acid (DIBMA) copolymer, which contains a linear carbon chain instead of the cyclic styrene group, is also capable of solubilising membranes. Discs formed from DIBMA are tolerant to much higher concentrations of calcium or magnesium, in excess of 25 mM [62]. This is particularly interesting since this polymer still contains the maleic acid groups, but perhaps other features of the polymer structure mean that binding divalent cations does not induce the same strain as seen with SMA.

4.3. Future directions

The future directions for the field are wide-ranging, falling into two broad areas. The first is continuing to exploit the ability of SMA to solubilise and stabilise membrane proteins for study. The second is to explore the properties of SMA, to better understand how it works and possibly to use this mechanistic insight to design new polymers, or to refine the ones currently available.

In terms of protein purification, we have many examples of initial successes, for instance the first purifications from several classes of membrane proteins, functional purification of GPCRs, the first high-resolution structure determination of a protein purified using SMA, and

lipidomic profile from protein-SMALPs. It is a proven method for many proteins and expression systems. We now need time to amass more data and critically assess whether the potential of SMA delivers in some or all areas. Developments in polymer chemistry and scaling-up the productions of polymers that are well-characterised in terms of composition and length will most likely play a part in this and allow us to better understand the mechanism of SMALP formation. However, these developments may also represent a change in the philosophy of purification of membrane proteins with SMA. At present it is a cheap and plentiful chemical that does a difficult job with remarkable simplicity and success. Will pursuing alternative polymers open up new chemical space and enable us to reach all membrane proteins, or will it lead us to dead ends, or a situation analogous with traditional detergents, in which a range of SMAs (or other polymers) are screened?

5. Conclusion

The encapsulation of membrane proteins in SMALPs is beginning to find wide applications as a research tool. SMA provides an attractive alternative to conventional detergents for membrane protein purification, and offers significant advantages, principally enhanced protein stability. The method is compatible with many established purification methods and biophysical characterisations, and there is increasing evidence that many functional and structural analyses are also compatible with protein-SMALPs. Indeed, for some proteins these analyses have been facilitated by SMA when they were previously challenging or impossible. Particularly notable in the success of this method for structural studies is that it is applicable to methods such as cryo-EM and X-ray crystallography. Indeed the recent crystal structure is the first demonstration that atomic level diffraction data can be achieved from a membrane protein crystallized from a SMALP. Furthermore the opportunities to study the interactions between membrane proteins and their native lipids in pure samples is currently unmatched by other methods. Of course, this method remains relatively new and frustratingly there are many questions that we cannot yet answer. Nonetheless the SMALP encapsulation method provides many promising opportunities and possibilities for the future of membrane protein research.

Author contributions

N.L.P., S.C.L., J.H.P., A.A.G., A.J.R wrote the manuscript.

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