

## Review Article

# Membrane protein nanoparticles: the shape of things to come

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The use of styrene–maleic acid (SMA) for the purification of a wide range of membrane proteins (MPs) from both prokaryotic and eukaryotic sources has begun to make an impact in the field of MP biology. This method is growing in popularity as a means to purify and thoroughly investigate the structure and function of MPs and biological membranes. The amphiphilic SMA copolymer can effectively extract MPs directly from a native lipid bilayer to form discs ~10 nm in diameter. The resulting lipid particles, or styrene–maleic acid lipid particles (SMALPs), contain SMA, protein and membrane lipid. MPs purified in SMALPs are able to retain their native structure and, in many cases, functional activity, and growing evidence suggests that MPs purified using SMA have enhanced thermal stability compared with detergent-purified proteins. The SMALP method is versatile and is compatible with a wide range of cell types across taxonomic domains. It can readily be adapted to replace detergent in many protein purification methods, often with only minor changes made to the existing protocol. Moreover, biophysical analysis and structural determination may now be a possibility for many large, unstable MPs. Here, we review recent advances in the area of SMALP purification and how it is affecting the field of MP biology, critically assess recent progress made with this method, address some of the associated technical challenges which may remain unresolved and discuss opportunities for exploiting SMALPs to expand our understanding of structural and functional properties of MPs.

## Introduction

Proteins embedded within lipid-based membranes mediate the interactions between cells and their environment, define the boundaries of intracellular organelles and influence the passage of most molecules into and out of the cells. In short, they are of critical importance. However, the study of membrane proteins (MPs) presents several challenges: they are not typically abundant and can be difficult to stably purify and subsequently characterise. As a result, our understanding of the structure and function of MPs has failed to keep pace with our burgeoning knowledge of soluble proteins, limiting our understanding of fundamental biological processes and affecting our ability to treat diseases.

Detergent purification has been essential in the MP methodological toolkit and has allowed us to answer important questions surrounding the structure and function of key targets [1–3]. Head-and-tail detergents like dodecyl  $\beta$ -D-maltoside (DDM) act as a simple replacement for the lipid bilayer and provide a membrane mimetic. Detergent micelles are dynamic, and over time, detergent molecules will fully replace lipids adjacent to the MP. Without these lipids, many MPs no longer function properly. Indeed, purified MPs are generally considered to be unstable and susceptible to aggregation. There is an increasing understanding that this instability is not an intrinsic quality of MPs, but a result of their removal from the lipid bilayer. Therefore, many recent advances in MP biochemistry

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have focused on preserving stability by developing purification methods that provide better membrane mimetics. These mimetics can be grouped into four classes: next-generation detergents [1], amphipols [4,5], nanodiscs [6] and styrene–maleic acid lipid particles (SMALPs).

While the first three technologies are essential for MP science, they have been thoroughly reviewed elsewhere. The focus of this review is SMALPs, and the evolution of this method to involve new polymers and experimental progress.

The use of the styrene–maleic acid (SMA) copolymer allows the direct isolation of proteins and their local lipids from the surrounding crude membrane (Figure 1). SMA, which results from the hydrolysis of precursor styrene–maleic anhydride (SMAnh), consists of alternating styrene and maleic acid moieties, forming an amphipathic copolymer. The resulting SMA copolymers contain distinct ratios of styrene:maleic acid, depending on the polymerisation reaction that is utilised to create the SMAnh precursor. These differences in the SMA can modulate their properties in MP purification. Isolation of transmembrane proteins using SMA creates monodisperse lipid discs of 10–11 nm in diameter containing the protein of interest as well as its surrounding native lipid bilayer [7–9]. Previous work with SMA has focussed on its application as a drug delivery system for enhanced bioavailability of hydrophobic molecules, as an antiviral treatment and as a tumour-targeting agent due to its enhanced cell permeability and size, which makes it well suited for invading the compromised vasculature of tumours [10–12]. More recently, however, SMA copolymer has been used to extract a variety of  $\alpha$ -helical and  $\beta$ -barrel transmembrane proteins, with much success [7,13,14]. For this application, SMA copolymers with a ratio of either 2 : 1 [7] or 3 : 1 [15] are most often utilised. By capitalising on the properties of SMA as an amphipathic copolymer capable of permeating a cell membrane, we can create SMALPs that contain an intact MP in its native form, with a lipid composition likely to reflect the native membrane environment. In contrast, detergent-purified proteins typically do not retain interactions with lipids or other proteins.

One significant advantage of the SMA purification method is the absence of detergent from the protocol. As a consequence, proteins purified in this way can be extracted along with both their natural lipid-support system and any interacting proteins. Not only does this provide useful information about associated proteins and their potential roles in regulating MPs, but it also offers a means for identifying the endogenous lipid composition surrounding the protein of interest [16–19]. Another advantage is the remarkable stability of proteins that have been purified into SMALPs. It is common for protein SMALPs to remain intact and monodisperse at 4°C for at least a week, and undergo several rounds of freeze/thaw with minimal loss to particle integrity or protein function [20,21].

In 2016, we presented a summary of research using the SMALP technology [22]. We considered its advantages and limitations. As part of that discussion, we explored three potential future directions for the SMALP field: we mentioned expanding our understanding of amphipathic copolymers and asked whether they could be improved, we discussed using SMALPs to solve high-resolution structures of MPs and we considered the value in using SMALPs to better understand the local lipids surrounding an MP. It is a remarkable testament to the utility of this method that progress has already been made in all three areas and that the number of publications pertaining to this technology has tripled since 2016 (Figure 2). In this review, we will return to these predictions. We will also discuss new developments related to SMA and its associated methods, and consider what could and should be next.

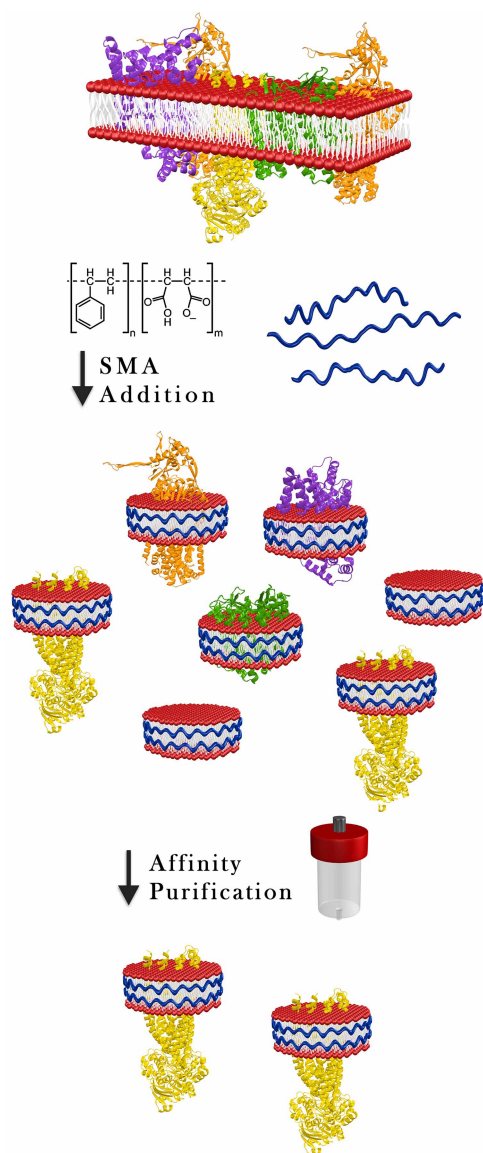
## Latest applications of SMALPs

### Use of SMALPs in high-resolution structure determination

High-resolution structural information is a cornerstone of protein biochemistry. It is critical that new purification methods yield MP samples that facilitate structure determination. Therefore, reports of atomic-resolution structures from X-ray crystallography and cryo-transmission electron microscopy (cryo-EM) are an essential validation of the SMALP method.

#### Crystallography using LCP

The first X-ray structure of an MP extracted and purified as a SMALP was reported by Broecker et al. [23]. SMA-purified recombinant microbial rhodopsin (bR), a seven-transmembrane  $\alpha$ -helical MP, was crystallized using the lipidic cubic phase (LCP) method [24] resulting in a structure of 2.0 Å resolution. This comparative study undertook the parallel LCP crystallisation of both SMA- and detergent-purified bR. In the LCP method, the MP spontaneously transfers from the SMALP or detergent micelle into the lipid *meso* phase, where



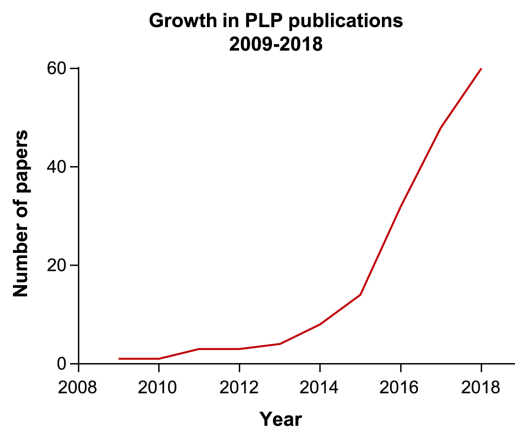
**Figure 1. A schematic representation of the preparation of SMALP MP using SMA.**

When SMA is added to the MP preparation, it inserts into the lipid bilayer forming an SMALP. The SMALP contains MP, the lipid bilayer surrounded SMA polymer. The SMALPs containing the MP of interest can be purified by affinity chromatography. Thus, the MP can be purified within their local lipid environment, which maintains structural integrity and stability.

crystallogenes occurs. The two bR structures were nearly identical: the bR-SMALP was determined to be a resolution of 2.0 Å and the detergent-purified bR resolved to be 2.2 Å. The present paper showed that high-resolution structural determination is possible for MPs purified using the SMALP methodology.

### High-resolution Cryo-EM structures

Despite success using the LCP method, most MPs have remained elusive to crystallography. To date, only the LCP method has been reported as a successful means for crystallising an SMA-purified protein. The advent of the cryo-EM revolution, however, has provided a new path to high-quality, high-resolution structures of MPs [25]. Cryo-EM offers structural biologists a way to visualise MPs in several different orientations by suspending particles in vitreous ice prior to imaging. Recent advances in instrumentation have resulted in a sharp increase in the number of high-resolution MP structures deposited into the Protein Data Bank (PDB).



**Figure 2. Growth of publications describing MPs purified using SMALP technology and its associated derivatives from 2009 to 2018 (year-to-date).**

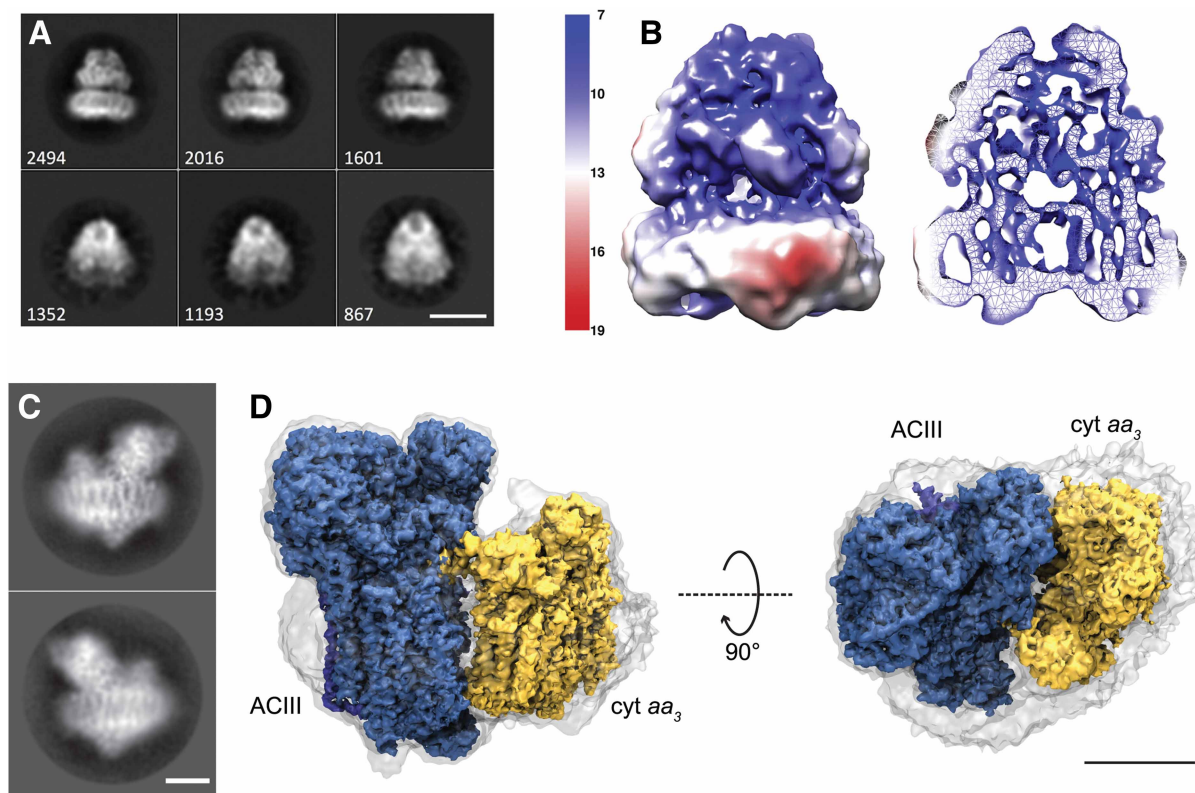
The graph shows the total number of publications by the end of each year. Data were assembled by searching for (SMALP or DIBMA or styrene–maleic acid) and (MP).

Following an early 23 Å structure of the *Escherichia coli* multidrug transporter AcrB in SMALPs [9], Parmar et al. [14] recently published the first subnanometre resolution structure of a protein SMALP. The AcrB–SMALPs cryo-EM map is displayed in Figure 3A,B. This map is consistent with high-resolution crystal structures and other EM-derived maps for AcrB [26,27]. These studies demonstrated the suitability of the SMALP method for structural analysis of MPs. It is interesting to note that when preparing cryo-EM grids, the authors found it essential to blot the grids with ash-free filter paper low in metal ion content; its use proved critical to avoid destabilising the AcrB–SMALPs, which are sensitive to divalent cations.

Very recently, Sun et al. [28] showed that protein SMALPs could be visualised at high resolution using cryo-EM when they published a 3.4 Å resolution structure of a protein SMALP (Figure 3C,D). Alternative complex III (ACIII) was isolated as a functional supercomplex with an aa3-type cytochrome *c* oxidase (cyt aa3) using 3:1 SMA. Collectively, this represents a total mass of 464 kDa and 48-TM spanning  $\alpha$ -helices, the largest SMA-purified protein complex reported to date. The final map of this complex revealed 11 lipid molecules adjacent to the protein and PTMs that were previously indiscernible when traditional detergent purification methods were employed. This work highlights a significant advantage of SMA purification, which is the ability to gather information about the native protein complex, including endogenous lipids and associated binding partners — both soluble and membrane-bound — without the need for cross-linking [28–30].

## Hydrogen–deuterium exchange-mass spectrometry

Another method of interrogating protein structure and dynamics is hydrogen–deuterium exchange-mass spectrometry (HDX-MS), an approach historically challenging for analysing hydrophobic MPs. HDX-MS, which measures the exchange rate of deuterium in place of the amine hydrogens along the polypeptide backbone, provides valuable insights into higher-order protein folding of solvent-accessible portions of the molecule. This is accomplished by incubating the protein of interest in deuterated buffer for set time intervals before quenching the reaction in low pH buffer and immediately freezing the sample to prevent loss of deuterium atoms (referred to as ‘back-exchange’). Despite several advantages to applying HDX-MS to stable, monodisperse SMALPs as opposed to MPs indiscriminately surrounded by detergent micelles, it has remained a challenge, as exposure to quench buffer causes the sample to immediately and irreversibly aggregate due to the pH sensitivity of SMA. To overcome the hurdle, Reading et al. [31] have outlined a protocol for HDX evaluation that is suitable for use with SMALPs. They prevented total protein aggregation during the quench step by including 0.1% DDM to the buffer and avoided sample incompatibility issues with ESI-MS by filtering the sample through a pre-chilled 0.22  $\mu$ m spin filtration device following trypsin digestion to remove lipid molecules. This overcomes the aforementioned technical issue to provide another sample characterisation method that is compatible with protein SMALPs.



**Figure 3. Cryo-EM structures of two proteins in SMALPs.**

(A) AcrB-SMALP from *E. coli* [14]. Representative 2D classes for the AcrB single-particle cryo-EM dataset side views of AcrB-SMALP and high angle views shown on the top and bottom row, respectively. The corresponding particle number in each class is shown on the bottom left. The white scale bar represents 100 Å. (B) The AcrB-SMALP 8.8 Å single-particle reconstruction coloured by local resolution and shown as a surface (left) and slice through (right). (C) Two representative 2D class average images of the ACIII in a supercomplex with an aa<sub>3</sub>-type cytochrome c oxidase (cyt aa<sub>3</sub>) from *Flavobacterium johnsonia*, in an SMALP nanodisc [28]. (D) Side (left) and top (right) views of the ACIII-cyt aa<sub>3</sub> supercomplex cryo-EM map. The transparent surface indicates the boundary of the nanodisc. Scale bars, 50 Å.

## Exploiting SMALPs for lipidomics work

Retention of local lipids is arguably the most unique aspect of SMALPs, when compared with other membrane mimetic systems. Lipids have been detected in SMALPs in several studies and lipid preferences for many proteins have been characterised for the first time using SMALPs [32,33]. A study of *Rhodobacter sphaeroides* proteins solubilised by SMA showed that they retained significant numbers of lipids [34]. However, they also related the solubilisation efficiency of *R. sphaeroides* MPs to their local lipid environment. This finding supports a similar observation that some membrane regions display resistance to solubilisation by SMA [35]. This highlights the fact that to use SMA to probe the lipidomics of MPs, we first need to develop a detailed understanding of the ability of SMA to solubilise proteins from different membrane types and regions. Similarly, the tendency of protein SMALPs to retain, lose, gain or exchange lipids must be better understood or we risk misinterpreting data on lipid preferences of proteins [36–39]. Nonetheless, it is clear that SMALPs provide a tool to address questions about the lipid preferences of MPs, which otherwise lack practical approaches.

## Mass spectrometry of membrane proteins

As a more general application, MS is a powerful technique for the detection of both proteins and lipids. Obtaining intact masses of MPs and their complexes is challenging, as their hydrophobic regions are difficult to isolate and ionise [40]. However, some methods have been reported for MS using MP-detergent complexes [41,42], that may be adaptable to SMALPs [31]. Significantly, such studies are also beginning to probe the

lipids that associate with MPs; SMALPs are an excellent platform for such studies, since they isolate MPs along with their local lipids.

## Understanding and developing the polymer family

Fundamentals of the formation of SMALPs have been investigated largely using lipid-only discs [36–39]. This has recently been thoroughly reviewed [43] and a couple of key points emerge. Firstly, SMA is an effective but mild solubiliser of model lipid bilayers [44]. However, SMALPs may be more dynamic than we initially assumed, and there is evidence that lipids exchange between discs [37,39]. Secondly, this work has also shown that the size of the discs can be, to some extent, manipulated by adjusting the ratio of polymer to lipid during disc formation [45]. This modification may allow us to accommodate larger proteins within SMALPs. However, it is important to reiterate that much of this work has been done using lipid-only SMALP discs, and their behaviour may be altered by the presence of an MP.

While the SMALP technology already has a strong track-record for MP purification, it is not without limitations, principally its sensitivity to divalent cations (e.g.  $Mg^{2+}$ ) and pH (SMA is insoluble below pH 6.5) [46]. These properties, which are the result of the negatively charged, outward-facing maleic acid moiety, complicate certain analyses such as  $MgCl_2$ -dependent ATPase assays.

Another challenge associated with this method has been the light-absorbing properties of SMA. Its styrene group absorbs at  $\sim 260$  nm, which partially overlaps with UV absorption by proteins. While purified protein SMALPs contain only the SMA associated with each nanodisc, enough may be present to interfere with UV absorption and light-scattering assays (e.g. differential scanning fluorimetry, static light scattering and protein quantification).

## Alternative and functionalised polymers

The limitations of SMA have prompted the rapid discovery and the development of additional disc-forming polymers. Both the hydrophilic and hydrophobic moieties of SMA can be varied to make new copolymers. In addition, the maleic acid side chains can be chemically altered to adjust their properties, without sacrificing the ability of the polymers to form lipid discs. The properties of a selection of the new and alternative polymers are summarised in Table 1.

To date, there are limited publications describing the application of these new polymers to MPs, and it will be interesting to see how this develops in the future.

### Diisobutylene maleic acid

Similar to SMA, diisobutylene maleic acid (DIBMA) is a maleic acid-containing copolymer capable of solubilising MPs into lipid discs known as DIBMALPs [47]. In this case, the hydrophobic region of the polymer consists of aliphatic diisobutylene and therefore lacks the aromatic styrene moiety found in SMA. Hence, DIBMA

**Table 1** Selected alternative and functionalised amphipathic polymers, showing structures, size of nanodiscs and tolerance to divalent cations

	SMA	DIBMA	SMI	SMA-QA
Chemical structure				
Optimum pH for nanodisc preparation	$\geq 6.5$	7.3, 8.4	5–7.8	2.5–10
Size of nanodiscs (nm)	10–13	18	6–11	30
Tolerance to divalent cations (mM)	5	35	$>100$	$\leq 200$
Key references	[7,15,46]	[37,47]	[36,37]	[47,48]

is compatible with optical spectroscopy permitting routine characterisation of the sample [47]. Lipid exchange rate studies using fluorescence resonance energy transfer (FRET) indicate that DIBMALPS may retain lipid bilayers better than SMALPs, which presents an exciting opportunity to investigate the lipids associated with different proteins [37]. Surprisingly, DIBMA is reported to be more tolerant to divalent cations than SMA (despite the presence of the maleic acid moiety), remaining soluble in up to 35 mM CaCl<sub>2</sub> and >20 mM MgCl<sub>2</sub> for lipid-only DIBMA particles. Improved cation tolerance could be critical for functional characterisation of proteins that rely on magnesium or calcium binding for their function. However, it should be noted that the insensitivity to divalent cations has not yet been reported on DIBMALPs containing an MP [47].

### Styrene maleic imide

Styrene maleic imide (SMI), a positively charged polymer comprised of alternating styrene and maleimide moieties, is also capable of solubilising MPs into lipid discs of ~11 nm in diameter [36,37]. As is the case with SMA, the presence of the styrene head group complicates analysis involving optical spectroscopy. However, unlike SMA, it can tolerate divalent cations at high concentrations, and its solubility range is pH 5–7.8. Biophysical characterisations of lipid-only SMI-lipid particles indicate that its lipid content is low, but that SMI is capable of solubilising proteins across a broad range of molecular mass [36].

### Styrene maleimide quaternary ammonium

Ravula et al. [48] describe the synthesis of a pH-resistant form of SMA that is effective at solubilising proteins into discs >20 nm in diameter and within a pH range of 2.5–10: styrene maleimide quaternary ammonium (SMA-QA). The resulting lipid discs exhibit ‘ultra-stability’ even in the presence of divalent metal ions.

### Thiolated styrene–maleic acid

Functionalisation of SMA to include a thiol group has also been reported, resulting in a lipid-solubilising derivative called thiolated styrene–maleic acid (SMA-SH) [49,50]. Using a fluorescent label attached to the SMA-SH, FRET experiments have demonstrated that polymer, as well as lipids, can be rapidly exchanged between discs. This is another key insight into the dynamic nature of SMALPs [49]. The production of polymers that can be conjugated with a range of functional groups will also offer new possibilities for how protein SMALPs can be studied [49].

## Perspectives

Head-and-tail detergents, MSPs, APols, SMA and other amphipathic polymers share many similarities. By some measures, SMA combines the best features of the other systems, but we are a long way from understanding both its full capabilities and its full limitations. The publication of high-resolution structural data from both crystallography and cryo-EM are an enormous milestone and provide a high-profile vindication of the SMALP method. Meanwhile, its proven utility in biophysical and functional work is growing as more publications appear. The next challenge is to build on the existing success to contribute further insight into MP structure and function.

Because the capacity to extract the local lipid environment surrounding a protein is a unique aspect of SMALPs, it provides an opportunity to study the lipids associated with MPs. However, our understanding of the dynamics of SMALPs is still developing and until we understand the fundamentals, we may struggle to interpret lipidomic data. On a similar note, reintegration of proteins from SMALPs into a bulk lipid bilayer would be a significant milestone and would open up the possibility of isolating proteins for transport assays.

To maximise the potential of SMALPs, there is a need to catalogue successes as well as failures with SMA (and similar polymers). Early successes have been dominated by more abundant bacterial proteins, arguably the lower-hanging fruit. As we turn to SMALPs to purify less abundant, and potentially less stable, proteins, it is essential to document outcomes, both positive and negative, to deepen our understanding of the potential of this method.

Another consideration is how we can use SMALPs mostly effectively. The generic applicability of 2:1 SMA has frequently been discussed as an advantage of this method [51] but as more polymers are developed, we may be moving back towards the screening approach that has become *de rigueur* for detergents. Indeed, important advances have been reported using both the 3:1 and 2:1 S:M polymer variants and each may be appropriate for different proteins and studies. An alternative to screening would be to adopt a funnel approach, whereby the most widely applicable polymer (2:1 SMA) is tried first and others are used if needed for specific

applications and proteins. Once again, extensive and honest cataloguing of results will increasingly allow patterns to emerge from the data that can guide these strategies.

Among the unknowns, it is critical to remember that SMA has already provided a generic, cost-effective and successful method for isolating a wide range of MPs, including several considered to be highly challenging [20,23,52]. While the fundamental chemistry of SMA presents certain challenges when it comes to biophysical and biochemical characterisation of protein SMALPs, the rapid accumulation of data and publications are indisputable proof of its utility.

As more lipid disc-forming polymers are discovered and synthesised, it may be useful to consider them as a family of polymer/lipid particles, rather than just SMALPs. However, the remarkable ability of SMA to directly and efficiently solubilise and stabilise MPs bodes well for its ongoing potential to deepen our understanding of MP. As MP purification continues to evolve and the limits of our capabilities become apparent, we must address these limitations by continuing to innovate new technologies and by using them judiciously.

### Abbreviations

ACIII, alternative complex III; bR, rhodopsin; cyt aa3, aa3-type cytochrome c oxidase; cryo-EM, cryo-transmission electron microscopy; DDM, dodecyl  $\beta$ -D-maltoside; DIBMA, diisobutylene maleic acid; FRET, fluorescence resonance energy transfer; HDX-MS, hydrogen–deuterium exchange-mass spectrometry; LCP, lipidic cubic phase; MPs, membrane proteins; SMA, styrene–maleic acid; SMA-SH, thiolated styrene–maleic acid; SMA-QA, styrene maleimide quaternary ammonium; SMALPs, styrene–maleic acid lipid particles; SMANh, styrene–maleic anhydride; SMI, styrene maleic imide.

### Author Contribution

K.S., N.L.P. and S.C.L. contributed equally to preparing the manuscript.

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### Competing Interests

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

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