# Recent Advances on Polymer Lipid Particles (PoLP) in Membrane Protein Research

Advanced article

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**Membrane proteins (MPs) structure elucidation is of crucial importance as they represent a major target for drug design. However, owing to their high hydrophobicity, MPs are challenging proteins to study. The use of polymer lipid nanoparticles (PoLP) has recently allowed a leap forward in the field of MP research. A wide range of polymers have been successfully used for elucidating the structure of MPs. One polymer in particular, styrene maleic acid (SMA) copolymer, is recently catching a large interest owing to its easy use in detergent-free membrane solubilisation and the recent successes in purification and stabilising MPs for further biophysical studies.**


## Introduction

From expression to purification, scientists studying specific membrane proteins (MPs) face multiple hurdles that prevent further success in understanding the function and structure of the MPs. Owing to their hydrophobic nature, MPs are difficult to extract from their native environment: membranes (John Wiley & Sons, Ltd, 2001). The conventional method is to use detergents as their amphipathic nature mimics a lipidic environment. A wide range of detergents have been developed which led to many successful MP structure elucidation. Nevertheless, the identification of the most adequate MP/detergent/buffer combination requires a lot of time and effort. Furthermore, finding the right detergent conditions for the extraction of an MP does not necessarily imply its stability and activity for biochemical and biophysical studies. Most of the time, additional steps of screening to optimise the conditions of those steps are often necessary.

In order to stabilise detergent-solubilised MPs, amphipols and nanodiscs have been developed successfully in the past decades. Both amphipols and nanodiscs warp around the MP to stabilise the purified protein in aqueous solution. One of the main differences between these two molecules is their nature: while amphipols are water-soluble polymers, nanodiscs are protein scaffolds (Parmar *et al*., 2016). Both systems have been used successfully for MP structure determination. However, their main limitation is the requirement of detergent-purified MPs before their reconstitution in those systems.

Recently, a new polymer, compatible with conventional purification strategies and able to solubilise MP from artificial and natural lipidic membranes without detergent requirements, has been introduced. The SMA (styrene maleic acid) copolymer, extensively used in the plastic industry, is a rising asset for many MP biologists. This copolymer produces stable soluble nanoparticles with MP or protein complexes surrounded by their native lipids. Therefore, proteins purified via the styrene maleic acid lipo particles (SMALP) technology are in majority found active as they are still embedded in the same or very similar lipidic environment. The use of SMA is simple and versatile. It can be tailored by varying conditions such as concentration, ionic strength or SMA ratio. However, although the technique represents a great progress in the membrane biology field, some limitations remain for further biochemical and biophysical characterisations.

This article's purpose is to summarise in a comprehensive manner the advantages and limitations of using polymer lipid particles (PoLP) for MP study, with a particular focus on SMA copolymers. It is aimed at nonspecialist readers who would like to start using SMA copolymer, and gives an overview of the current information of SMA copolymers and their use in membrane biology.


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## Styrene maleic acid (SMA) copolymer synthesis

Styrene maleimide acid copolymers (coSMA) were first described in the 1940s by Frank Mayo and Frederick Lewis (Lewis *et al*., 1948; Mayo, 1943, Mayo *et al*., 1948; Mayo and Lewis, 1944) who gave their name to the Mayo–Lewis equation describing the distribution of monomers in a copolymer. The formation of coSMA is first based on the copolymerisation of styrene and maleic acid monomers leading to the generation of anhydride SMA (**Figure 1**). For subsequent applications in membrane biology, anhydride SMA should be submitted to an alkaline hydrolysis (Lee *et al*., 2016b). This reaction leads to the hydrolysis of the maleic acid carbonyl groupments, making the copolymer overall more hydrophilic and soluble in biologically compatible buffers (Dörr *et al*., 2016; Pollock *et al*., 2017). Following this protocol, the hydrolysed SMA is able to solubilise membranes and form water soluble nanodiscs by wrapping around the extracted MPs. The polymer maintains a hydrophobic native lipid environment which stabilises the protein. The first study of a MP purification using SMA was reported (Knowles *et al*., 2009) and then later optimised (Lee *et al*., 2016b).

By modifying the ratio between the styrene to maleic acid moieties, a variety of copolymers can be synthesised with different molecular weights (MWs). The SMA copolymers most commonly used for MP studies are styrene to maleic acid in the ratio of 3:1 and mostly 2:1 with MWs ranging from 5 to 10 kDa (although other ratios are also available commercially) (**Table 1**).

Copolymers are synthesised industrially using a batch procedure which uses a continuous flow of both monomers (Belkhiria *et al*., 1994). In principle, this allows a statistical distribution of styrene and maleic acid units along the copolymer which should reflect the desired ratio 2:1 or 3:1, and so on. Unfortunately, for unclear reasons, these optimum conditions still result in a high polymer polydispersity, that is the synthesis of heterogeneous copolymers varying in size and distribution (Scheidelaar *et al*., 2016). This is quantified via the polydispersity index (PDI), the ratio between the theoretical MW and the average molecular weight (Mn) (**Table 1**). Furthermore, due to a higher amount of styrene group required within the polymer, a polystyrene tail tend to be added at the end of the polymer once the reaction runs out of maleic anhydride.

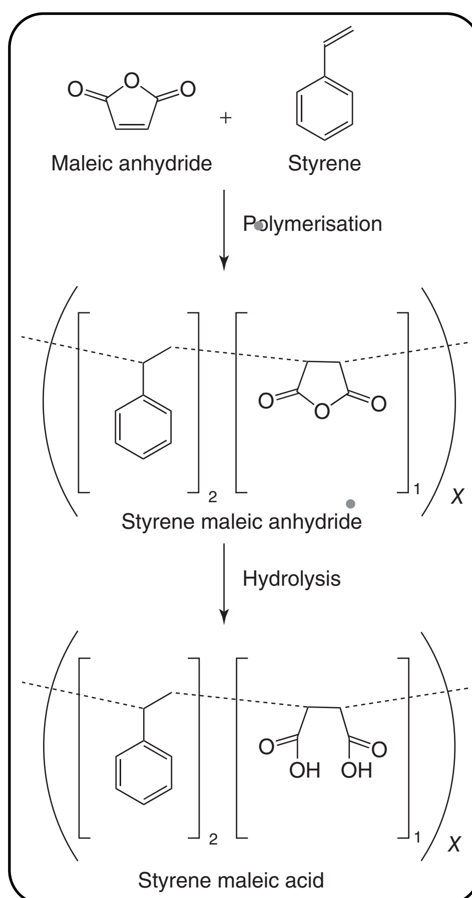


**Figure 1** Schematic of the polymerisation and alkaline hydrolysis events leading to the formation of the styrene maleimide copolymer 2:1.

Such a large variation between the MW and Mn has lead scientists to develop more controlled methods to synthesise copolymers. RAFT (reversible addition–fragmentation chain transfer) polymerisation allows a narrower end product polydispersity. It is a block polymerisation where an excess of styrene monomers is incubated with maleic acids monomers in the presence of a RAFT agent. The resulting block copolymerisation allows the

**Table 1** SMA copolymers commercially available

| Name | SMA ratio | MW (kDa) | Mn (kDa) | Company |
|---|---|---|---|---|
| Xiran® SL 40005 P20 | 1.2:1 | 5 | 2 | Polyscope |
| SMA2000 | 2:1 | 7.5 | 3 | Cray Valley |
| Lipodisq L8920 | 2:1 | n/a | n/a | Sigma |
| Xiran® SL 30010 P20 | 2.3:1 | 6.5 | 2.5 | Polyscope |
| SMA3000 | 3:1 | 9.5 | 3.8 | Cray Valley |
| Lipodisq L9045 | 3:1 | – | – | Sigma |
| Lipodisq L9170 | 3:1 | – | – | Sigma |
| Xiran® SL 25010 P20 | 3:1 | 10 | 4 | Polyscope |


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synthesis of an alternating styrene and maleic acid block (strictly depending on desired ratio) and can be followed by a polystyrene block. This strategy was shown to generate more homogeneous copolymers with low polydispersities (around 1.25–1.35) (Craig *et al*., 2016; Hall *et al*., 2017; Smith *et al*., 2017). However, a recent study suggests that RAFT-produced SMA copolymers were less thermostable and efficient in solubilising membrane *Escherichia coli* BL21 (DE3) membranes than their polydispersed commercially equivalent ones (Hall *et al*., 2017). On the other hand, RAFT-synthesised copolymers are more flexible to functionalisation of SMA particles, paving the way to new avenues of research. Clearly, there is an urgent need for more evidence as to whether the use of RAFT-produced copolymers is proven to be more advantageous for MP characterisation, but early evidence suggest that it might be the case.

## Solubilisation efficiency

One of the major advantages of SMA copolymers in the field of MPs is the lack of need for detergents during the purification stage. In contrast to other types of purification tools such as amphipols and nanodiscs, SMAs have the ability to directly solubilise the membrane, without the requirements for detergents. This is achieved by insertion of the copolymers in the membrane and the wrapping around proteins which in turns destabilise the membrane and lead to soluble nanoparticles called SMALP. Therefore, SMA copolymers were originally described as 'cookie cutters' acting agents. The exact mechanisms by which this happens has been investigated by the field on both artificial membranes and native membranes, and the emerging picture is as shown in **Figure 2** (Scheidelaar *et al*., 2015). The first step contacting the membrane would be mainly driven by hydrophobic interactions and modulated by electrostatic interactions. The second step involves the insertion of the copolymer in the membrane. Finally, the last step leads to destabilisation of the membrane and the formation of the nanoparticle. The data to explain how copolymers influence each step of solubilisation is still scarce, but scientists have concentrated on particular questions: *is the nature/composition and packing of the lipid bilayer important for SMA solubilisation* ? *Is it important for the size of nanoparticle*? *Does the pH or ionic strength influence solubilisation efficiency*? *Does the ratio/length of copolymer affect solubilisation*?

### Nature of the lipid bilayer

The efficiency of solubilisation by SMA copolymer has been investigated using artificial lipids, with known composition, as

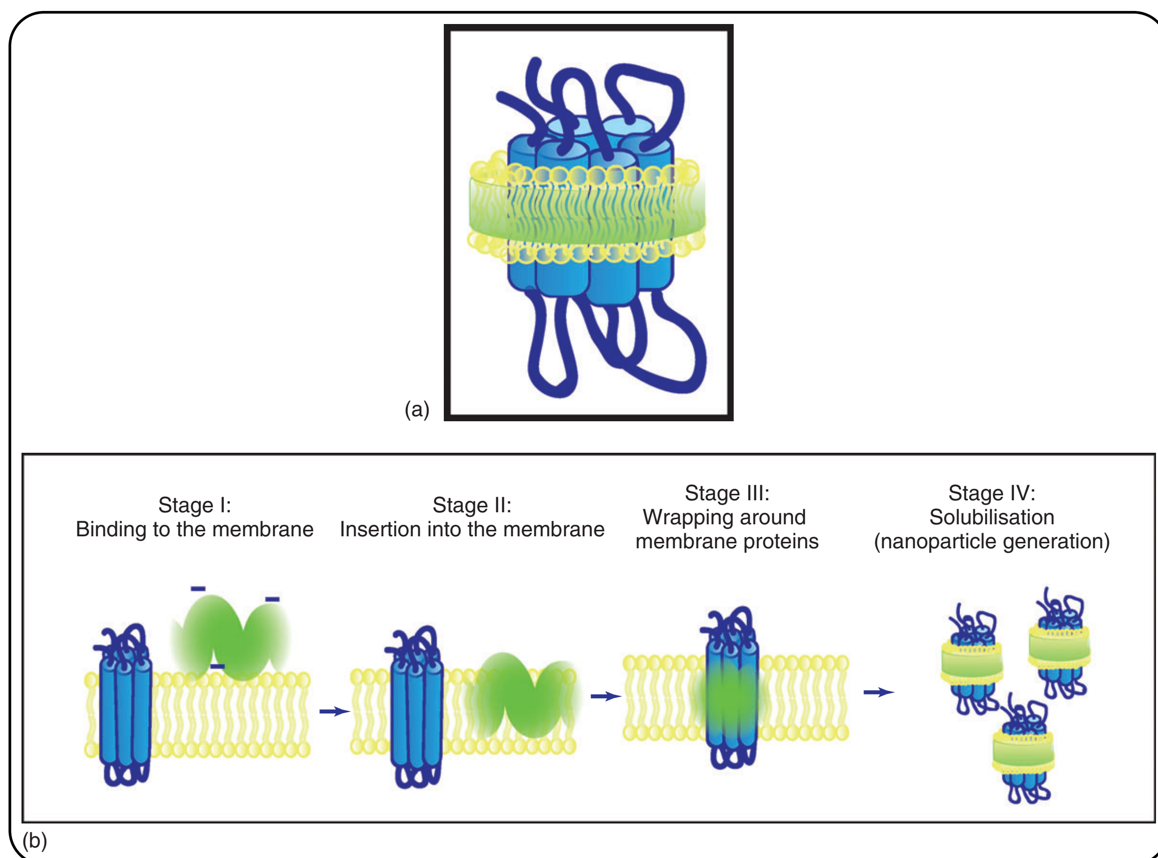


**Figure 2** Solubilisation stages and nanoparticle generation. (a) Representation of a membrane protein in a SMA nanoparticle. (b) Schematic of the putative steps involved in the solubilisation of membranes by SMA copolymer to form nanoparticles.

**Table 2** Membrane proteins purified so far with SMA

| Protein | TM | Protein origin | Membrane origin | Copolymer used | References |
|---|---|---|---|---|---|
| KCNE1 | 1 | Human | *E. coli* | 3:1 | Sahu *et al*. (2017) |
| PBP2/PBP2a | 1 | Bacteria | Native | 2:1 | Paulin *et al*. (2014) |
| ETK tyrosine kinase | 2 | *E. coli* | Native | 3:1 | Li *et al*. (2015) |
| TSPAN7 | 4 | Human | *S. cerevisiae* | 3:1 | Skaar *et al*. (2015) |
| GPCRs | 7 | Human | *P. pastoris* HEK cells | 2:1 | Jamshad *et al*. (2015) |
| Bacteriorhodopsin | 7 | Bacteria | Native | 3:1 | Orwick-Rydmark *et al*. (2012) |
| HwBr bacteriorhodopsin | 7 | Bacteria | Native | 3:1 | Broecker *et al*. (2017) |
| Potassium channel | 8 | | | | Dörr *et al*. (2014) |
| PagP | 8 | Bacteria | Native | 2:1 | Knowles *et al*. (2009) |
| RC photo | 11 | Bacteria | Native | 2:1 | Swainsbury *et al*. (2014) |
| Various ABC transporter | 12 | Human/murine | Insect cells | 2:1 | Gulati *et al*. (2014) |
| SECYEG | 15 | Bacteria | Recombinant *E. coli* | 3:1 | Prabudiansyah *et al*. (2015) |
| GPCR (MT1R, GHS-R1a) | 7 | Yeast | Liposomes and native | 2:1 | Logez *et al*. (2016) |
| Complex IV | cplx | *S. cerevisiae* | *S. cerevisiae* | 3:1 | Long *et al*. (2013) and Smirnova *et al.* (2016) |
| AcrB | cplx | *E. coli* | *E. coli* | 2:1 | Parmar *et al*. (2017) |

well as native membranes. A wide variety of membrane types (cell membrane or organelles) from bacteria, yeast, insect cells or human cells have been successfully solubilised with SMA (**Table 2**). Altogether, the results suggest that the lipidic composition of the membranes does not affect the efficiency of solubilisation by 2.5% SMA polymer with a ratio above 2:1 (Swainsbury *et al*., 2017). Nevertheless, membranes containing a very high content in anionic lipids are expected to be more resistant to solubilisation due to electrostatic repulsion with the SMA carbonyl groups that would hinder the interaction with the bilayer. Moreover, there is clear evidence that the lipid packing and phospholipid order play an important role in SMA solubilisation efficiency. Indeed, two papers have reported that SMA solubilisation is inefficient on densely packed membranes (Bell *et al*., 2015; Swainsbury *et al*., 2017). Efficient solubilisation, however, can be restored after addition of lipids to reduce the protein:lipid ratio (Swainsbury *et al*., 2017), demonstrating that the large size of the protein complexes to purify was not the limiting factor for efficient solubilisation. Therefore, avoiding densely packed membranes and high overexpression of proteins in heterologous systems is preferable for SMA solubilisation.

## pH and ionic strength

pH and ionic strength are two factors that have been shown to influence SMA solubilisation. Most solubilisation with SMA copolymers are performed at pH close to neutrality, as SMAs dissociate at pH lower than 6 (see the section on ‘limitations’). However, pH adjustments are sometimes required to maintain protein function. Therefore, SMA pH sensitivity range has been tested for solubilisation efficiencies on artificial or native membranes with an SMA concentration varying from 0.1% to 2.5%. The general conclusion is that solubilisation is slower but overall more efficient at higher pH such as 8.3 than at 7.4 and 6.4 (Grethen *et al*., 2017). Increasing the pH above 8.3 results in a strong decrease in solubilisation efficiency (Scheidelaar *et al*., 2016). This suggests that the most functional pH range for efficient soluble SMA is between 6.4 and 8.3, although these can vary slightly depending on the polymer ratio (Scheidelaar *et al*., 2016). Similarly, low ionic strength (below or around 50 mM NaCl) leads to poor solubilisation, while increasing the ionic strength seems to improve solubilisation efficiencies (Grethen *et al*., 2017; Scheidelaar *et al*., 2015, 2016). Unfortunately, for some copolymers with higher hydrophobic content (high ratio styrene:maleic acid), increasing the salt concentration can induce polymer aggregation at physiological pH (Scheidelaar *et al*., 2016). Therefore, typically a safe range of 150–300 mM NaCl is used for efficient SMA solubilisation, although higher concentration with low MW copolymers also gives successful results (Parmar *et al*., 2017).

## Nature of SMA (ratio, size and concentration)

Various SMA copolymers have been tested for their efficiency in solubilising membranes. The first aspect to consider is the ratio of styrene to maleic acid. Various ratios ranging from 1.4:1 to 4.5:1 were studied on both artificial and native membranes (Craig *et al*., 2016; Logez *et al*., 2016; Morrison *et al*., 2016; Scheidelaar *et al*., 2016; Swainsbury *et al*., 2017). Reports have demonstrated that copolymers with a ratio of 2:1, 3:1 and 4:1 all have similar solubilisation efficiencies at physiological pH. SMA 4:1, however, suffers from a restricted pH range as it aggregates close to neutral pH (Scheidelaar *et al*., 2016). SMA 2:1 and SMA 3:1 are therefore favoured for membrane solubilisation assays. In addition, the length of the copolymers is also of utmost importance when it comes to solubilisation of membranes. Indeed, shorter chains leading to MWs ranging between 7.5 and 10 kDa were found to be the most efficient, with higher MW being very poor at solubilisation, even with a 2:1 or 3:1 ratio (Morrison *et al*., 2016). SMA concentration does not appear to be crucial as it is always added in excess (usually 2.5% w/v). Nevertheless, in some cases,

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increasing concentration of SMA could help solubilise recalcitrant membranes (Swainsbury *et al*., 2017).

### Polystyrene tail

As previously mentioned, owing to a synthesis artefact, industrially produced SMAs have a polystyrene tail. It was originally suggested that this extra polystyrene tail would stimulate membrane solubilisation due to increased carbonyl groups numbers. However, different authors have suggested that this very tail might actually have a detrimental effect on this process by partially destabilising lipids in membranes (Hall *et al*., 2017; Smith *et al*., 2017). In agreement with this, RAFT copolymers with similar ratio tend to have a better solubilisation efficiency with shorter tails. On the other hand, reports have shown that bigger styrene tails contribute to increased nanoparticle size, therefore allowing incorporation of bigger proteins.

### Conclusion on solubilisation

In conclusion, SMA 2:1 and 3:1 copolymers with short chains (7.5–10 kDa) appear to be the most efficient for membrane solubilisation, with SMA 2:1 being more popular because of slightly better efficiencies (Morrison *et al*., 2016). As a consensus, an optimum trial solubilisation condition to use would be a buffer with a pH ranging from 6.4 to 8.3, a NaCl concentration between 150 and 300 mM and a final concentration of 2.5% SMA 2:1 with short chain. Nevertheless, varying these conditions (e.g. increasing the SMA/lipid ratios, raising the salt concentration to 500 mM or adding some lipids to lower the ratio protein:lipid) has been shown to help in some cases (Gulati *et al*., 2014; Scheidelaar *et al*., 2015) and should therefore be tailored to the membrane and the size of the protein of interest. Thanks to these conditions, protein complexes that usually fall apart in detergent (Prabudiansyah *et al*., 2015) or large homotrimers up to 340 kDa (Parmar *et al*., 2017) have been purified and shown to be active in SMALP.

## SMALP size and stability

Once solubilisation is complete, the copolymer self-assembles in lipid particles called SMALP (SMA lipoparticles). The MP of interest, still in its native lipidic environment (Logez *et al*., 2016; Smirnova *et al*., 2016), is surrounded by the SMA copolymer. These particles have been shown to be more thermostable compared to the same isolated protein in detergent micelles. It was reported that SMALP (lipid-only or protein-containing particles) were stable over more than 7 days at various temperatures (from 4 to 37 °C), while detergent-purified proteins tend to degrade after a couple of days at 4 °C (Gulati *et al*., 2014; Logez *et al*., 2016; Morrison *et al*., 2016; Routledge *et al*., 2015; Tanaka *et al*., 2015). This property is an immense asset in the field of MPs, allowing more time for further biochemical and biophysical tests.

The size of formed SMALPs has also been investigated by two complementary methods, dynamic light scattering (DLS) and size exclusion chromatography. While DLS could provide more accurate sizes than size exclusion chromatography, care should be taken as partial aggregation of the SMA nanoparticles (e.g. at acidic pH) could be mistaken for an increase in average diameter. Therefore, the homogeneity should be verified by exclusion chromatography. Although commercial SMA polymers usually give a wider polydispersity than the RAFT SMA, the accepted average diameter of SMALP is about 10–12 nm. Nevertheless, there is a wide range of nanoparticle diameter size being reported in various studies, probably due to the differences in systems being studied. Factors affecting size include the type of membrane – native (containing proteins) or artificial (entirely lipidic), the concentration of SMA used, the ratio lipid:polymer and the salt concentration.

By controlling the size of the styrene chain using RAFT polymers, Craig *et al*. have shown that the styrene tail does not influence the size of the SMA nanoparticle. In contrast, they showed that the ratio styrene:maleic acid within the copolymers has an influence on the size of the nanoparticles. When using artificial lipids POPC/POPG at a ratio of 1.25:1 (SMA:lipid), SMA 3:1 gives an average of 10 nm particles, while 2:1 and 4:1 give, respectively, an average size of 20 and 30 nm. Complementary to these experiments, a range of SMA:lipid ratios have been tested, ranging from 0.25:1 to 2.5:1, on artificial lipids, either POPC/POPG or DPMC (Hall *et al*., 2017; Tanaka *et al*., 2015; Zhang *et al*., 2015). In all the cases, results suggest that increasing the SMA to lipid ratio lead to a decrease in the diameter size of nanoparticles from 190 down to 8 nm.

When a MP, isolated from native biological membranes, is present in the disc, most reports disclose 5–25-nm diameter nanoparticles, resulting from the solubilisation of membranes using 2–2.5% SMA and 150–500 mM NaCl (Broecker *et al*., 2017; Gulati *et al*., 2014; Logez *et al*., 2016; Morrison *et al*., 2016; Smirnova *et al*., 2016). Similarly to their use with artificial lipids, copolymers with different monomer ratios give different nanodiscs sizes. SMA 3:1 leads to smaller discs than SMA 2:1 (Morrison *et al*., 2016).

One important factor, the SMA:protein ratio, is unfortunately generally omitted in 'materials and methods'. Similar sizes nanoparticles (10–15 nm) have been reported for the purification of similar sizes of proteins (cytochrome *c* oxidase 200 kDa and PgP 140 kDa) using ratios of SMA:protein between 1:1 and 2.5:1 on either yeast or insect cell membranes (Gulati *et al*., 2014; Smirnova *et al*., 2016). However, there is a recurrent question that the field is eager to answer: are the SMALP adjustable to the size of the protein? Until recently, it was suggested that SMA copolymers form unique-size nanodiscs based on the inability of SMA to solubilise specific membranes containing large protein complexes (Lee *et al*., 2016a). However, membrane packing, rather than the size of the protein complex to purify, appears to be problematic, as the addition of lipids allows better solubilisation levels (Swainsbury *et al*., 2017). Moreover, cryo-EM experiments on AcrB purified in SMALP have demonstrated that SMA copolymers make close contact with the protein, nicely wrapping around the trimer (Parmar *et al*., 2017). This demonstrates that SMA can be used to purify large protein complexes (314 kDa for AcrB) by adjusting their size (Swainsbury *et al*., 2017). Furthermore, it has now been shown that SMALP nanodiscs are highly dynamic, constantly exchanging lipids and polymer once formed (Cuevas Arenas *et al*., 2017; Schmidt and Sturgis, 2017; Vargas *et al*., 2015). SMALP nanodiscs therefore have the benefit to be

tuneable by their environment by rapidly exchanging material, a characteristic that has not been explored until now.

## SMA variants

Before alkaline hydrolysis leading to functional SMA copolymers, SMA anhydrides can be submitted to specific chemical reactions leading to the generation of functional groupments. A few SMA variants have been developed and are described below:

### SMA-SH

The production of SMA-SH involves a few steps, one of which is the incubation of anhydride SMA with cysteamine, resulting in the addition of sulfhydril group on the maleic acid. Incubation with increasing concentration of cysteamine leads to an increased number of sulfhydril group per copolymer (Lindhoud *et al*., 2016). SMA-SH have been prepared from both SMA 2:1 and SMA 3:1 and shown to solubilise native purple membrane (PM) from wild-type *Halobacterium salinarum* R1 or *E. coli* total lipids (Lindhoud *et al*., 2016; Schmidt and Sturgis, 2017). In both the cases, SMA-SH were shown to be as efficient as their SMA counterpart in solubilising membranes/liposomes. In addition, SMA-SH and SMA analysed by DLS form similar sizes nanoparticles. These SMA-SH can be further functionalised by attaching various types of thiol-reactive molecules. This approach has been taken by two groups by attaching fluorescent dyes used in FRET experiments but could also be used for drug delivery (Lindhoud *et al*., 2016; Schmidt and Sturgis, 2017).

### SMA–EA, SMAd–A and SMA–QA

A similar approach was used to create three SMA variants starting from a low size SMA copolymer 1.3:1 (1.6 kDa) SMA–ED (zwitterionic), SMAd–A (positively charged above neutral pH) and SMA–QA (Ravula *et al*., 2017a,b). All SMA variants can solubilise DMPC MLVs and form nanoparticles. The size of nanoparticles can be modulated with the lipid to polymer ratio. Large discs (30–60 nm depending on the variant) will be generated with ratios below 1:1, while smaller discs similar to SMA discs (around 10 nm) can be generated with bigger ratios (above 1:3) (Ravula *et al*., 2017b,c). SMA–ED and SMAd–A have interesting pH sensitivities: while SMAd–A is stable at acidic but not basic pH, SMA–ED is stable across the whole pH range but not between pH 5 and 7. Although this increased stability at acidic pH would not be relevant for the MP field which mostly uses pH above 6.5, this is an attracting feature for other nanodisc applications. SMA–QA appears to be completely tolerant to pH and divalent cations. Interestingly, both SMA–ED and SMA–QA have been shown to have magnetic-alignment properties, suggesting promising applications (Ramadugu *et al*., 2017; Ravula *et al*., 2017a).

### zSMA

zSMA is a RAFT-produced SMA with different repeat units giving rise to different sizes zSMA (Fiori *et al*., 2017a). The size of zSMA is controlled at the synthesis stage, and the synthesis results in copolymers with a low PDI (between 1.08 and 1.19). zSMA can solubilise *E. coli* membranes in a similar efficiency than SMA. The size of the nanodiscs formed by zSMA is increasing with the size of the copolymer but still in the range of those found for SMA. Most importantly, the authors have shown that zSMA are almost insensitive to acidic pH and divalent cations (Fiori *et al*., 2017a). This is extremely promising, and more evidence is now expected on the behaviour of zSMA during examples of MPs purification.

## SMA limitations and other polymers

### Drawbacks using SMA

It is clear that the use of SMA for the purification of MPs has many advantages in terms of solubilisation efficiencies and stability of nanodiscs. Two major drawbacks, however, exist. The first drawback of the use of styrene maleimide polymers is their sensitivity to divalent cations. Above a concentration of 10 mM $Mg^{2+}$, SMALP start to unravel, and the nanodiscs disassemble because of the polymer precipitation, although this can be an interesting feature by allowing to assemble and disassemble the nanoparticles on demand. For example, a protein could be further reconstituted in other systems for functionality studies. It can also represent a major issue when the functionality of proteins of interest needs cations. An example is ABC transporters which require $Mg^{2+}$ for their ATPase activities. The second major downside is the limited use of optical spectroscopic studies of MPs isolated in SMALP. Indeed, SMA copolymers scaffold presents a strong UV absorption spectrum (Oluwole *et al*., 2017). In the following sections, we describe other types of polymers available or being developed to overcome these issues.

### Amphipols

Amphipols are a class of polymers that have been used for the past 20 years. They represent co- or terpolymers synthesised by random distribution of octylamine or isopropylamine forming amide bonds along a chain of low MW polyacrylic acid precursors (Tribet *et al*., 1996). Amphipols are anionic amphiphilic polymers which have been developed to stabilise MPs after detergent removal (Popot, 2014). The first one to be described and the most commonly used is A8-35, but its sensitivity to acidic conditions and to the presence of calcium ions has limited its use. Since then, many more variants and functionalised polymers have been developed with decreased pH sensitivity such as non-ionic amphipols (Sharma *et al*., 2012; Zoonens and Popot, 2014). Amphipols have been instrumental in studying MPs because of their compatibility with various downstream biochemical and biophysical applications (Calabrese *et al*., 2015; Kleinschmidt and Popot, 2014; Zoonens and Popot, 2014). Successful MP structures have been elucidated via CryoEM and crystallisation (Polovinkin *et al*., 2014; Schulz *et al*., 2017; Zhang *et al*., 2017). In contrast to SMA, however, amphipols do not solubilise native membranes (with exceptions (Arunmanee *et al*., 2014)) and therefore need a first solubilisation step with detergents. In addition, only limited number of native lipids remain after exchange of detergents with amphipols (Kleinschmidt and Popot, 2014;


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Sharma *et al*., 2012). Therefore, despite the numerous successes of amphipols, the field is now privileging more lipidic nanoparticles such as SMAs.

### DIBMA: diisobutylene/maleic acid

DIBMA is an alternating copolymer between diisobutylene and maleic acid of small MW (12 kDa) and also known under the commercial name Sokalan CP9. In contrast to SMA, DIBMA is less sensitive to cations and shows minimal absorption in UV ranges (Oluwole *et al*., 2017). Until now, DIBMA has been mainly tested for solubilisation on artificial membranes. At the same polymer:lipid ratio, discs formed with DIBMA are slightly larger than SMA (e.g. at a ratio of 0.2, discs diameter are 18 nm for DIBMA compared to 13 nm with SMA 2:1) (Oluwole *et al*., 2017). Solubilisation at similar mass ratio is reported to be less efficient than for SMA 2:1 but more efficient than for SMA 3:1 with DMPC, while in POPC it is less efficient than both SMA copolymers (Grethen *et al*., 2017). Results in artificial membranes, however, do not always reflect results in native membrane, and therefore more evidence needs to be reported on the efficiency of using DIBMA on native membrane. Until now, only one attempt has been made in solubilising *E. coli* Bl21 (DE3) membranes with DIBMA, which shows that this polymer solubilises preferentially larger proteins (Grethen *et al*., 2017). Further evidence is lacking to show that proteins can be purified functionally in DIBMA nanodiscs.

### Polymethacrylate

Polymethacrylate is another polymer alternative to SMA. It was designed to generate an amphipathic based on the same properties as SMA. Polymethacrylate polymer is generated by random copolymerisation of hydrophobic butyl methacrylate and cationic methacroylcholine chloride. The chain length can be adjusted by varying the concentration of a chain transfer agent (methyl 3-mercaptopropionate) (Yasuhara *et al*., 2017). Polymers with moderate hydrophobicity were able to solubilise DMPC LUVs and form nanodiscs of about 17 nm diameter. Owing to the absence of styrene units interfering with UV absorption, polymethacrylate polymers can be used in CD experiments. As a proof of concept, the authors have demonstrated that addition of polymethacrylate polymers interferes with the formation of amyloid fibres, demonstrating that this new polymer can interact with proteins and be used in biophysical studies (Yasuhara *et al*., 2017). It would be interesting to test polymethacrylate's ability to solubilise native MP and to further characterise the polymer for its sensitivity to other factors such as pH, divalent ions and ionic strength.

### Other polymers

A few other polymer-based methodologies have been reported. Polydimethylsiloxane (PMDS)-based triblock copolymers have been used to study a variety of MPs. They form vesicles of 160 nm instead of nanodiscs and are viscous at room temperature (Kumar *et al*., 2007). Polybutadiene and polystyrene block copolymers (PDB) have also been used efficiently for few MPs (Chawla *et al*., 2016). However, the presence of the styrene group implies similar issues as for SMALPs. Finally, recently copolymer HPBD-P4MVP28 has been described for the purification of MsbA, giving raise to nanodiscs of about 11 nm. However, the nanodisc formation necessitates prior solubilisation of membranes or lipids with detergents and addition of MSP to stabilise the nanodiscs (Fiori *et al*., 2017b). Therefore, amongst the many newly designed copolymers, none of them yet bring together all the expected qualities of a polymer for the study of MPs.

## Use with biophysics methods

Membrane solubilisation and formation of stable nanodisc is evidently not the ultimate goal of SMA use in membrane biology. Being able to proceed to protein purification and characterisation via biophysical techniques is the next step.

Unless the protein of interest can be further purified through a functional test, the addition of a tag on the protein will be required to positively select the nanodiscs containing the MP. Until now, all reports have used nickel affinity purifications after generation of the nanodiscs. This technique has been shown to be compatible with SMA, despite the presence of coordinated divalent ions $Ni^{2+}$ or $Co^{2+}$ and appears to give very pure results with minimum contamination (Lee *et al*., 2016b). Some, however, experienced reduced binding and had to use larger His tags (Broecker *et al*., 2017). This could partly be due to shielding of the tag and could possibly be overcome by adding a spacer between the protein and the tag to make it more accessible. In theory, other purification systems should also be compatible with SMA nanodiscs, such as GST or myc-tag, but to our knowledge, none has been reported yet. Noticeably, a GFP-tagged human tetraspanning was purified in SMALP from yeast *Saccharomyces cerevisiae* membranes, but the GFP tag was used to visualise the protein through purification rather than as a purification system (Skaar *et al*., 2015).

Protein-purified SMALPs are compatible with most of the further downstream applications (**Figure 3**). FRET experiments have been used on SMA variants functionalised by the addition of a fluorescent dye. Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR), differential scanning calorimetry (DSC), small angle neutron scattering (SANS), electron microscopy (EM) and nuclear magnetic resonance spectroscopy (NMR) have all been shown to be compatible with SMALP nanodiscs (Jamshad *et al*., 2014; Sahu *et al*., 2013, 2017). Recently, AcrB, a multidrug efflux pump from *E. coli*, represented the first subnanometre single particle being solved by cryoEM (8.8 Å) after solubilisation and purification in SMALP nanodiscs (Parmar *et al*., 2017). Finally, the first crystal structure obtained by LCP with an $\alpha$-helical microbial rhodopsin (7TM) was reported by Broecker *et al*. (2017). The authors state that no difference was seen between purified proteins isolated via the classical detergents techniques or via SMALP, thus validating the use of SMA copolymers for the elucidation of MP crystal structures.

## Conclusion

The MP field is quickly evolving, and the development of polymers is paving the way to new discoveries. Although various

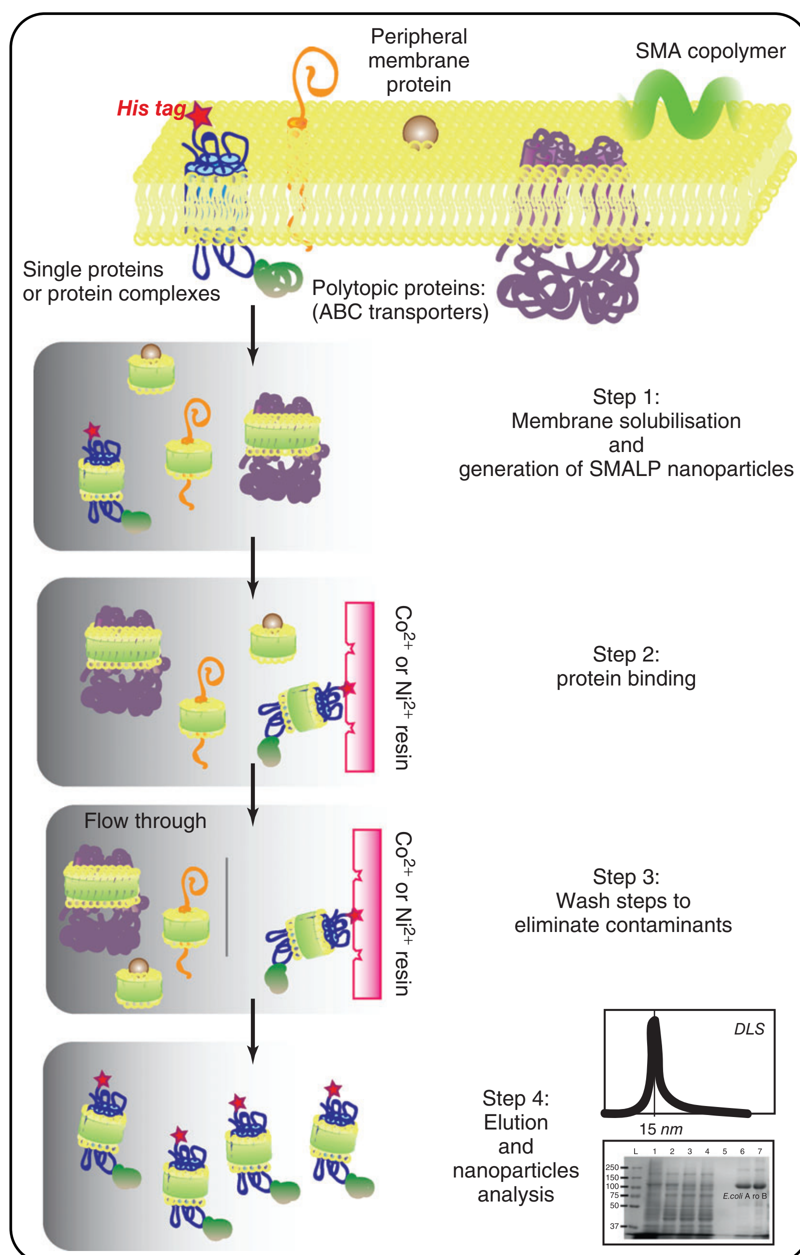


**Figure 3** Purification of membrane protein with SMA copolymer solubilisation. Schematic explaining the various steps of SMA solubilisation of membranes, nanoparticle generation, purifcation via a His-tag (red star) on the protein of interest and further particle analysis.

polymers exist, the SMA copolymer is emerging as very promising. Its ability to directly solubilise most artificial and native membranes, together with its properties of generating soluble and stable nanoparticles containing one or more (complex) MPs, makes SMA one of the most efficient polymers available so far. Limitations, including acidic pH and divalent cations sensitivity, are being addressed by scientists, resulting in promising SMA variants or new polymers emerging regularly. Furthermore, functionalisation by chemical addition of new groups opens up new opportunities for the study of the purified MP. Although more evidence and improvement is still required, SMA copolymers appear to set up a new area for MPs research.

## List of Abbreviations

| | |
|---|---|
| DLS | dynamic light scattering |
| MP | membrane protein |


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SMA styrene maleic acid
SMALP styrene maleic acid lipo particles
PoLP polymer lipid particle

## Glossary

***Detergent*** A substance that helps solubilise membranes and membrane proteins in hydrophilic buffers.

***Membrane*** Selective barrier made of lipids and proteins which allow nonselective passage of some molecules and selective passage of other molecules, depending on their properties.

***Membrane protein*** Proteins that are located in the membrane.

***Polymer*** A large molecule, or macromolecule, composed of many repeated subunits.

***Solubilisation*** The process of making a substance more soluble, especially in water.

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