European Polymer Journal 110 (2019) 63–68

Contents lists available at ScienceDirect

European Polymer Journal

journal homepage: www.elsevier.com/locate/europolj

Advancing membrane biology with poly(styrene-*co*-maleic acid)-based native nanodiscs

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ARTICLE INFO

Keywords:
Amphipathic polymer
Lipid bilayer
Membrane solubilization
Native nanodiscs
Styrene maleic acid
SMA copolymer
SMALP
Transmembrane protein

ABSTRACT

The elucidation of the structures and interactions of proteins and lipids in intact biological membranes remains largely uncharted territory. However, this information is crucial for understanding how organelles are assembled and how transmembrane machines transduce signals. The challenge of seeing how lipids and proteins engage each other *in vivo* remains difficult but is being aided by a group of amphipathic copolymers that spontaneously fragment native membranes into native nanodiscs. Poly(styrene-*co*-maleic acid) (SMA) copolymers have proven adept at converting membranes, cells and tissues directly into SMA lipid particles (SMALPs), allowing endogenous lipid: protein complexes to be prepared and analyzed. Unlike other amphipathic polymers such as amphipols, SMALP formation requires no conventional detergents, which typically strip lipid molecules from proteins and can destabilize multimers. A collaborative community of hundreds of investigators known as the SMALP network has emerged to develop and apply new technologies and identify new challenges and design potential solutions. In this contribution, we review recent practices and progress, focusing on novel SMA copolymers, modifications, alternatives and mechanisms. In addition, a brief overview will be provided, with reference to the further contributions to this special issue on the SMALP technology.

1. Introduction

Lipid molecules are bound to the surfaces of all membrane proteins, and have profound influences on their structures, stabilities and activities. It is perhaps obvious that removing these bound lipids will compromise these properties. Moreover, once removed by classical 'head and tail' detergents, the biological lipid ligands are essentially impossible to replace as originally bound. This realization led to testing and optimization of the use of amphipathic copolymers for isolation of detergent-free, biologically intact lipid:protein complexes by a research group at the University of Birmingham, UK, starting in 2005. This effort resulted in the report in 2009 that SMA copolymers are able to spontaneously convert membranes into native nanodiscs, as demonstrated with palmitoyl transferase PagP and bacteriorhodopsin proton pump, which retain their respective structure and activity in SMALPs [1]. Since then, the nanodiscs formed by these polymers have been the subject of over 110 publications (Fig. 1), including the characterization of molecular machines and 3D structures of native lipid:protein complexes that were otherwise inaccessible. A collaborative network of academic and industry researchers has formed to propel the field forward, promoting the development and sharing of a variety of polymers (Fig. 2). This has fostered the emergence of consensus methods using hundreds of membrane proteins from many different membranes, cell cultures and tissues, as well as highlighting any bottlenecks. The SMALP community is now expanding the variety of polymers in order to achieve wider applicability ranges (e.g. in terms of pH range, ion resistance, etc.), diverse disc sizes and novel functionalities. While the nanodisc field builds on earlier studies on amphipols [2] and membrane scaffold proteins [3], the latter approaches rely on the application of conventional detergents. SMA and related copolymers are distinguished by their ability to form detergent-free native nanodiscs of membrane proteins that retain bound biological lipids. The polymer formulations

Abbreviations: ATP, adenosine triphosphate; CSTR, continuous stirred tank reactor; DIBMA, poly(diisobutylene-*co*-maleic acid); DMPC, dimyristoyl phosphatidylcholine; EM, electron microscopy; GPCR, G protein-coupled receptor; MA, maleic acid; MI, maleimide; NMR, nuclear magnetic resonance spectroscopy; PC, phosphatidylcholine; RAFT, reversible addition-fragmentation chain transfer; S, styrene; SMA, poly(styrene-*co*-maleic acid); SMAd-A, dehydrated styrene maleic acid ethylenediamine; SMA-EA, styrene maleic acid ethanolamine; SMA-ED, styrene maleic acid ethylenediamine; SMALP, styrene maleic acid lipid particle; SMA-QA, styrene maleimide quaternary ammonium; SMA-SH, SMA with sulfhydrils; SMI, poly(styrene-*co*-maleimide); zSMA, zwitterionic SMA; TM, transmembrane

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https://doi.org/10.1016/j.eurpolymj.2018.11.015
Received 19 June 2018; Received in revised form 14 October 2018; Accepted 9 November 2018
Available online 10 November 2018

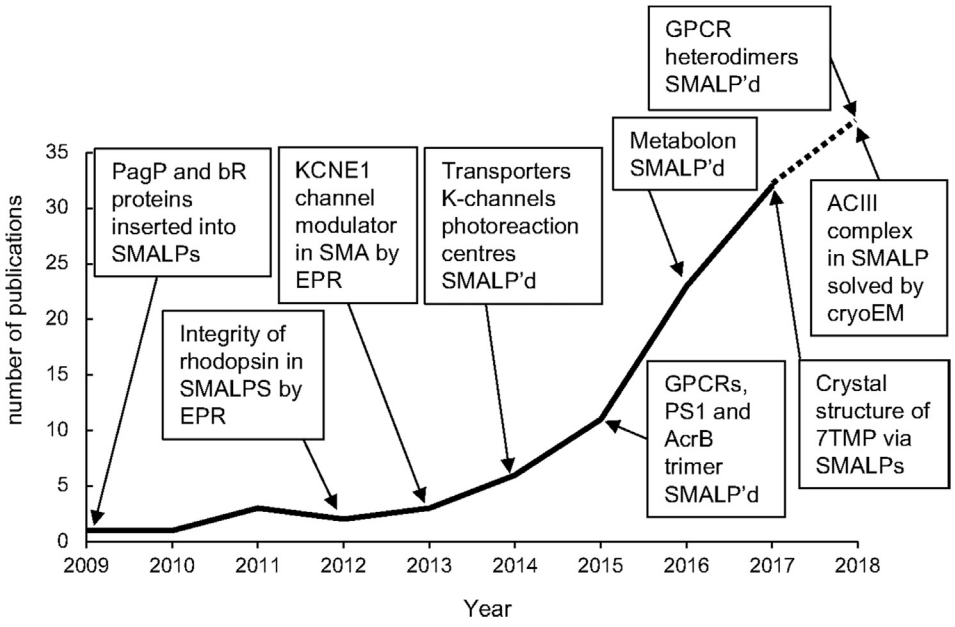

Fig. 1. Development of the SMALP field. The number of publications and patents describing the use of SMA-related polymers in native nanodiscs are shown, and several seminal discoveries in the field are noted for SMA-solubilized proteins including bacteriorhodopsin (bR) [1], Alternative Complex III [7], bacterial reaction centres [29], metabolon [38], a bacterial 7 transmembrane protein (TMP) [16], GPCRs [32,33], photosystem 1 (PS1) [28], and ABC transporters [37]. The dotted line indicates a projection for 2018.

shown thus far to be capable of this unique activity are the specific focus here.

2. Polymers used for native nanodisc formation

A small set of commercially available SMA copolymers are able to spontaneously isolate membrane proteins complexes from lipid bilayers. They contain linear sequences of styrene (S) and maleic acid (MA) residues arranged in statistically defined patterns. The typical ratios of S to MA groups used are between 2:1 and 3:1, which offers a balance of hydrophobic insertion into lipid bilayers and acidic charge density at the critical polymer concentrations needed to fragment membranes into nanodiscs, which typically have 10 nm diameters. The polar groups control the pH-dependent solubility profile, with the optimal range of membrane solubilization activity being between pH values 6 and 9, where the maleic acid residues possess a single negative charge.

Two major routes are used to synthesize SMA copolymers, and influence the resulting dispersity in terms of chemical composition and polymer chain length. The most widely used commercial method employs conventional radical copolymerization, as conducted in a continuous stirred tank reactor (CSTR). This technology results in a narrow chemical composition distribution and a chain length dispersity $Đ \sim 2$. Polymerization mediated by reversible addition fragmentation chain transfer (RAFT) methods reduces the dispersity in chain length, but yields a comonomer gradient along the chain with potentially a polystyrene terminal block, depending on comonomer ratios and conversion [4]. Both routes utilize maleic anhydride, a strong electron acceptor which is virtually unable to undergo homopolymerization, but which copolymerizes well with electron rich styrene monomers. Both processes yield copolymers with single maleic anhydride residues separated by variable numbers of styrene residues in a defined average ratio that is dictated by their relative concentrations in the reactor and their respective reactivities. Basic hydrolysis is used to convert the maleic anhydride to maleic acid residues, yielding the charged amphipathic polymer that is highly soluble in aqueous media and that inserts into membranes. They spontaneously form a lipid-containing nanoparticle when a critical concentration of polymer in solution is mixed with a membrane or cell suspension, yielding a clarified emulsion. These charged nanoparticles are stable and soluble in aqueous solutions and can be readily reconstituted after freeze-drying and storage while retaining the activity of the inserted proteins [32].

SMA from two commercial sources are produced by the CSTR method outlined above. Hydrolyzed SMA2000 and SMA3000 copolymers from Cray Valley (USA), which contain styrene to maleic acid molar ratios of 2:1 and 3:1 and a terminal cumene group, were originally most used (Table 1). Polyscope (Netherlands) offers comparable XIRAN 30010 and 25010 reagents, which have styrene to maleic acid molar ratios of 2.3:1 and 3:1, respectively, and molecular weights as stated in Table 1. These are available in ready to use maleic acid solutions as well as maleimide forms that need to be hydrolyzed before use. Thus far the SMA2000 and similar XIRAN reagents are the most generally effective SMA polymers for solubilizing membrane proteins [5], which have ranged from single transmembrane α-helices [6] to oligomeric complexes containing 48 transmembrane helices [7].

Nanodisc diameters of between 10 and 28 nm have been produced using RAFT-SMA copolymers, which show little apparent effect of polymer length on particle size. An optimal ratio of styrene to maleic acid of about 3:1 in RAFT-SMA co-polymers ensures aqueous solubility as well as membrane binding and solubilization, although larger protein assemblies may be more effectively solubilized by RAFT-SMA (2:1) copolymers [8]. The best solubilization by RAFT-SMA is obtained using a steep gradient of styrene and maleic acid subunits in a small (e.g. 3 kDa) copolymer, while a long terminal polystyrene block tends to be counter-productive [4].

3. How SMA copolymers form native nanodiscs

SMA copolymers bind to biological membranes through the hydrophobic insertion of the styrene groups into the lipid bilayer where they pack against the acyl tails while preserving protein activity and stability [1]. At a critical concentration, the build-up of charged groups destabilizes the membrane, causing it to fragment and form nanodiscs that are encircled by a belt of amphipathic polymer molecules. A major advantage of SMA over classical detergents is due to its mild solubilizing activity. That is, it causes minimal disruption to the integrity of protein:lipid complexes while inserting into bilayers, and then forms nanodiscs spontaneously and devoid of detergents [9,10]. SMALPs include the protein-bound lipids, which are preserved by the styrene groups that pack against their lipid acyl chains [11,12]. The pH at which SMA copolymers are non-aggregating and membrane-active as monomers ranges from 7.0 to 9.0. Polymer solubility is generally further enhanced by low salt conditions [13], although if divalent cations are necessary, higher salt may be needed. A 2:1 ratio of styrene to

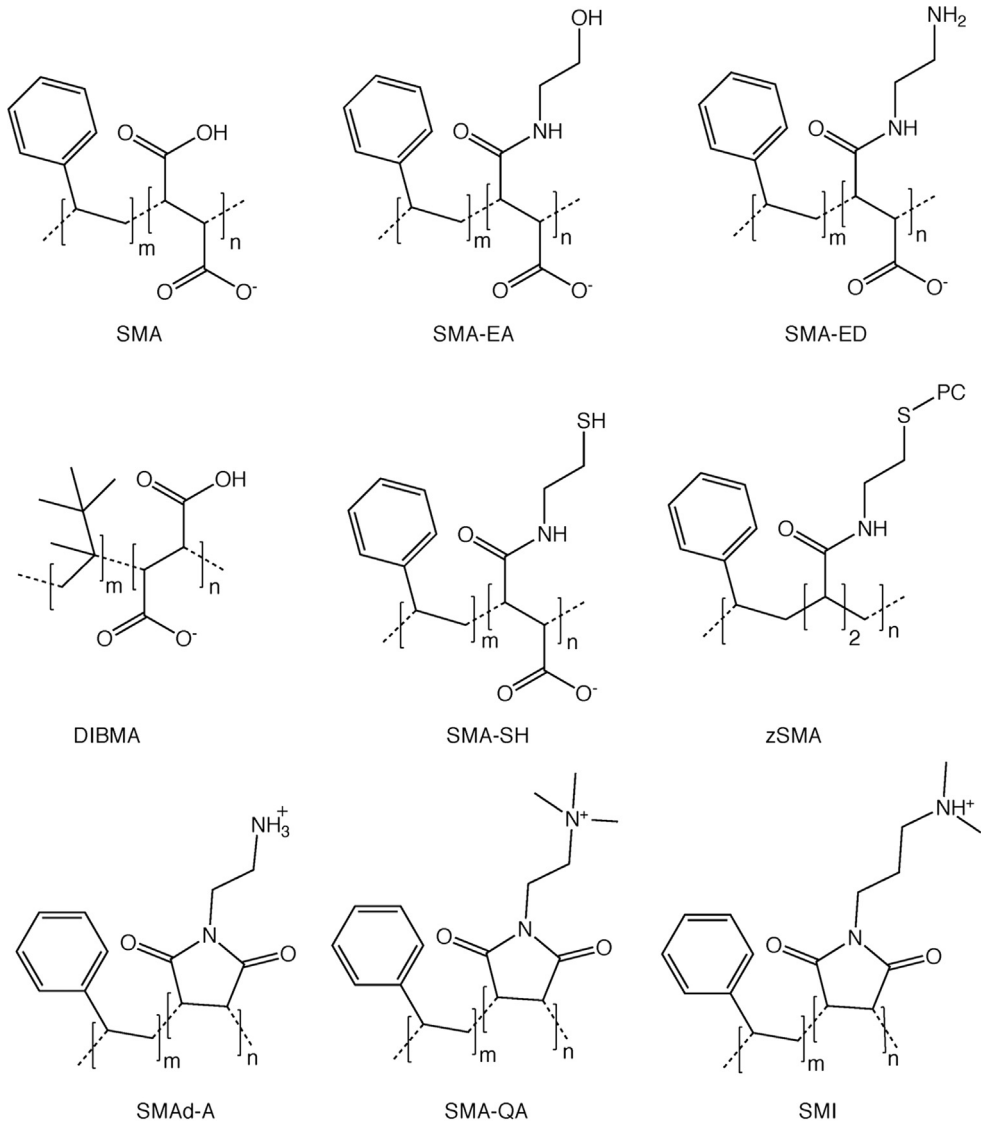

Fig. 2. SMA-related copolymers which form native nanodiscs. Structures of synthetic copolymers that have been developed for converting biological membranes into nanodiscs including SMA [1], SMA-EA [20], SMA-ED, SMAd-A [22], SMA-QA [23], SMI [24], SMA-SH [25], zSMA [26] and DIBMA [27].

maleic acid appears optimal in most cases as this perturbs the lipid bilayer structure less than, for example, a more hydrophobic 3:1 ratio [12]. An evaluation of different SMA copolymer activities showed that those with molecular weights in the 10 kDa range and 2:1 or 3:1 ratios of styrene to maleic acid were best able to solubilize monomeric, dimeric, trimeric and tetrameric forms of the *Rhodobacter sphaeroides* reaction centre [14]. Adding lipids to the membranes or using longer chain SMA can increase the yields of large protein oligomers in

Table 1
Amphipathic polymers capable of direct membrane solubilization.

Compound	Subunits	Subunit Ratio	Polar Sidechain	M_w (g/mol)	M_n (g/mol)	Reaction Method
SMA2000	S, MA	2:1	–	7500	3000	CSTR
SMA3000	S, MA	3:1	–	9500	3800	CSTR
SZ25010	S, MA	3:1	–	10,000	4000	CSTR
SZ30010	S, MA	2.3:1	–	6500	2500	CSTR
SZ40005	S, MA	1.2:1	–	5000	2000	CSTR
SMA-SH	S, MA	2:1	cysteamine	7500		
SMA-EA	S, MA	1.3:1	ethanolamine		1600	CSTR
SMA-ED	S, MA	1.3:1	ethylene-diamine		1600	CSTR
zSMA1	S, PC		phosphatidyl choline	12,451		RAFT
zSMA2	S, PC		phosphatidyl choline	21,576		RAFT
zSMA3	S, PC		phosphatidyl choline	43,708		RAFT
SMA-QA	S, MI	1.3:1	dehydrated SMA-ED		1600	CSTR
SMAd-A	S, MI	1.3:1	dehydrated SMA-ED		1600	CSTR
SMI	S, MI	2:1	dimethylamino propyl			CSTR
DIBMA	S, MA	1:1	diisobutylene	12,000		

nanodiscs, with diameters of between 50 and 100 nm being observed by transmission electron microscopy (EM), although this is influenced by the type of protein and polymer type studied. SMALPs can incorporate essentially any molecules found within the native membrane environment, and specific lipids contained can be identified by thin layer chromatography or by mass spectrometry. Lipid molecules can exchange rapidly between particles through collisions between nanodiscs [15]. Due to their dynamic properties, lipids can be introduced or removed from a SMALP relatively readily, and proteins can also be transferred back into a liposome from a SMALP [16].

There is a remarkable lack of specificity of SMA for the variety of membrane and lipid types tested so far, presumably due to the non-specific insertion of the styrene moieties into the various bilayers tested. There is a consistent preference of SMA for fluid phase bilayers and lipids with short or unsaturated acyl chains [17], as opposed to lipids which form ordered bilayers and resist penetration of hydrophobic bulk into the tightly packed bilayer [18]. Consequently, SMA differentially solubilizes plasma and intracellular membranes which differ in their degrees of order, as visualized by live cell fluorescence microscopy [19]. When XIRAN 30010 is added to mammalian cells, the plasma membrane is perforated first and then the inner membranes, causing the release of fluorescent proteins contained within their organelles following the release of those present in the cytosol. This suggests that SMA copolymers bind first to liquid disordered sections of plasma membrane, penetrating through and allowing cytosolic molecules to leak out. Shortly thereafter they bind and perforate the intracellular membranes. Controlling the polymer length, concentration, temperature and addition of dynamic lipids such as dimyristoyl phosphatidylcholine (DMPC) provide handles for controlled liberation of membrane proteins from ordered or disordered bilayers into SMALPs [1].

4. Development of alternative polymers for membrane analysis

The growth of the SMALP field has led to the design of new derivatives to enhance polymer functionality and performance range (Fig. 2) under a wider pH range and cation concentrations, while allowing various disk sizes and stability to be produced. The maleic anhydride residue of SMA can be readily modified, allowing a variety of groups to be added. Attaching ethanolamine to a short SMA (1.3:1) polymer with an M_n of 1.6 kDa yields a “SMA-EA” derivative which solubilizes vesicles under wider pH, temperature, salt and polyvalent cation ranges, yielding nanodiscs of between 10 and 50 nm in diameter [20,21]. The solution state nuclear magnetic resonance (NMR) spectra of ^{15}N labelled 16 kDa cytochrome *b5* protein in SMA-EA nanodiscs are well-resolved and indicative of the expected folded state. The addition of lanthanide ions such as Yb^{3+} aligns large SMA-EA discs in magnetic fields, allowing tilt angles of transmembrane helices of contained proteins to be estimated by solid state NMR methods. Attachment of ethylenediamine to the same short SMA copolymer yields a zwitterionic form, SMA-ED, which precipitates when uncharged between pH 5–7, but solubilizes multilamellar vesicles outside this range [22]. Dehydration of this polymer to yield the maleimide derivative, which has been termed SMAd-A, effectively solubilizes DMPC vesicles below pH 6, i.e. where the amine is protonated and therefore positively charged. Both of these ethylenediamine-modified forms tolerate high salt as well as divalent cation up to concentrations of 200 mM. These reports extend the conditions for SMALP formation, and show that maleic acid derivatization does not compromise lipid bilayer binding or nanodisc formation.

Positively charged derivatives of SMA are also capable of nanodisc formation. A poly(styrene-co-(*N*-(2-trimethylammonium ethyl)maleimide))-type copolymer with a quaternary ammonium functional group named SMA-QA has been synthesized starting from 1.6 kDa SMA (1.3:1). It converts vesicles into discs with diameters ranging from 30 and 10 nm as the ratio of DMPC to SMA-QA is altered from 1:0.25 to 1:1.5 (w/w), and works at pH values from 2 to 10 and in the presence of metal ion concentrations up to 200 mM [23]. A positively charged sidechain has been incorporated to form poly(styrene-*co*-(*N*-(3-*N*’,*N*’-dimethylaminopropyl)-maleimide)) (SMI)-type coploymers to produce “SMILP” nanodiscs at pH values under 7.8 [24]. Membrane discs with diameters of 6 nm are formed and retain stability up to 80 °C, and show little binding of divalent cations at concentrations exceeding 100 mM due to the presence of the *N*,*N*-dimethylaminopropyl polar groups. The *E. coli* cell division protein ZipA purifies well in SMILPs although yields are lower than in the case of SMALPs, and the human adenosine A_{2A} receptor in SMILPs retains the expected ligand binding activity. Thus, both of these cationic SMI copolymers expand the solution conditions and size ranges of nanodisc formation, allowing a larger diversity of transmembrane machines to be accommodated.

The experimental versatility of SMALPs can be enhanced by incorporating thiol groups into the polar sidechains, as in the SMA-SH polymer, *i.e.* 1-amino-2-mercapto-ethane modified SMA, allowing fluorescent dyes or molecular tags to be attached [25]. Affinity purification and fluorescence detection of SMALPs is then facilitated by addition of biotin tags or Alexa Fluor 488 to the polymer, for example, thus providing ways to isolate and detect nanodiscs containing untagged or endogenous membrane proteins.

A phosphatidylcholine (PC) lipid-headgroup containing set of zwitterionic “zSMA” derivatives have been made by RAFT methods [26]. Although devoid of maleic acid moieties, they solubilize membranes into nanoparticles with diameters ranging from 10 to 30 nm in a manner that correlates with polymer length. As these copolymers lack carboxylic acid groups, they are compatible with a wide range of conditions including low pH buffers and polyvalent cation solutions, and are thus suitable for solubilization and analysis of a wide variety of proteins including those that require divalent cations or acidic conditions.

A novel alternative to SMA that offers aliphatic rather than aromatic sidechains was found to mediate mild extraction of membrane proteins, and appears to be most useful for labile protein complexes and prefers larger assemblies [27]. Known in the field as DIBMA and commercially available from BASF as Sokalan CP9, it offers alternating diisobutylene and maleic acid residues, and forms nanodiscs with diameters of 12 to 29 nm. The advantages of DIBMA copolymers include their transparency in the UV and circular dichroism spectra, and tolerance to higher concentrations of divalent cations.

5. Resolving protein:lipid complex structures in SMALPs

Many biologically and pharmacologically important membrane assemblies have been solubilized and characterized in SMALPs. The Alternative Complex III solubilizes intact in SMA3000 as well as in XIRAN 25010, allowing the structure of this 464 kDa complex to be resolved at 3.4 Å resolution by cryo-electron microscopy (EM). This 9 nm × 13 nm assembly comprises a 6 protein complex including 48 transmembrane helices, as well as accessory cytochrome *c* oxidase, haem groups, iron-sulfur clusters, native phospholipid ligands and acylated residues mediating photosynthetic electron transport [7]. Light-harvesting chlorophyll and photosystem complexes from thylakoids form large assemblies that transduce excitation energy after purification with SMA3000 [28], while the photoreaction center of *Rhodobacter sphaeroides* purifies with SMA2000 with its lipid environment intact within elliptical 12–15 nm discs, allowing the captured proteins to be recognized with gold nanoparticles [29].

The first crystal structure of a SMA-solubilized bacterial protein with seven transmembrane helices was solved at a resolution of 2.0 Å [16]. Voltage-gated potassium ion channel proteins prepared within 14–30 nm nanodiscs [30] can be used to identify structural locations of amino acid residues inside and outside the bilayer using spin labels [31]. The superfamily of G protein-coupled receptors (GPCRs) offers drug discovery targets that can be solubilized with SMA (2:1) into

native nanodiscs with native ligand binding activity and stability that withstand repeated freeze–thaw and storage cycles [32]. The hetero-tetrameric complex of 2 GPCRs that recognize dopamine and the growth hormone secretagogue has been purified using SMA (2:1) and recruits two G protein trimers in a specific conformation [33]. A group of 15 human tetraspanins expressed in *S. cerevisiae* and purified in SMALPs confirms that such multimeric proteins form complex networks in membranes [34]. These studies demonstrate the utility of SMALP technology to solubilize challenging multimeric complexes directly from membranes.

Multimeric complexes are proving uniquely accessible in SMA-based nanodiscs. An active nucleoside transporter produced in insect cells could be solubilized using a low concentration of 0.25% XIRAN 30010 at low temperature with cholesteryl hemisuccinate as a stabilizer [35]. A trimeric multidrug transporter purified using SMA(2:1) reveals about 40 lipid molecules embracing the 36 TM helical bundle [36]. Eukaryotic ATP-binding-cassette transporters solubilized using SMA display higher activity, purity and stability than in conventional detergents, and with molecular envelopes that are evident by cryoEM [37]. The metabolic machine that synthesizes dhurrin comprises a soluble glycotransferase and three membrane-anchored proteins that can be solubilized intact with associated lipids from microsome membranes using SMA2000 [38]. Multi-subunit cytochrome *c* oxidase protein complexes can be extracted with bound phospholipids from mitochondrial membrane into native nanodiscs using SMA (3:1). The cytochrome *c* oxidase protein complexes in the nanodiscs bind respiratory supercomplex factors, exhibiting the expected O_2-reduction activity although excess SMA can reversibly inhibit the catalytic activity [39,40]. The translocon complex that secretes proteins through bacterial inner membranes is enriched in cardiolipin and phosphatidylglycerol lipids when solubilized from *E. coli* membranes using SMA (2:1) and (3:1) copolymers, with subunits that can be recognized by specific antibodies [41,42]. A dimeric 34 kDa bacterial zinc diffusion facilitator solubilized with XIRAN 25010 and 30010 into 10–15 nm nanodiscs retains a shell of bound lipid around the helical dimer, and displays resolvable NMR signals for its cytoplasmic domain [43]. These examples show how large assemblies of multi-protein, multi-lipid complexes can now be studied in isolation, providing opportunities to resolve and exploit their previously unknown mechanisms.

Recent progress is focusing on understanding the fundamental behavior of native nanodiscs, and developing high-value applications that can drive continued progress, as reported in this issue. The contribution by Bartholomäus Danielczak and Sandro Keller shows how disc size, polymer charge density and the surrounding ionic strength affect the rates of transfer of lipid molecules between nanodiscs *via* diffusion or collisions [44]. Barry Bruce's group explores how varying pH, ionic strength, concentration and temperature affects the range of shapes and sizes of SMALPs based on small angle X-ray scattering analysis, with coulombic repulsion between particles being a critical factor [45]. He and his collaborators have also systematically evaluated a large range of SMA types and conditions for extraction of intact photosynthetic systems from thylakoid membranes, identifying those best suited for energy conversion [46]. A team at Amgen report how a class A GPCR, a cannabinoid receptor 1 that is involved in neurotransmitter release, retains its native fold, enhanced thermostability and binding activity using conformationally-specific antibody, and demonstrates how SMALPs can be used in flow cytometry-based studies [47]. As new generations of polymers emerge [21] to address limitations of the original SMA series such as cation sensitivity in low salt solutions [5], further targets will become amenable to structure-function analysis by a broader range of methods. Together this indicates that the field is beginning to mature in terms of structure-function based exploration of the diversity of polymers and conditions, allowing SMALPs to begin to be deployed to address industry applications including for drug discovery and energy production.

6. Conclusions

The use of amphipathic polymers such as SMA has transformed the field of membrane biology over the past 15 years, allowing otherwise inaccessible cellular machines to be resolved within native nanodiscs. New polymers have been designed by modifying the hydrophilic groups, allowing a broader array of targets to be tackled under a range of pH, salt and cation concentrations. The ability to control nanodisc size and behaviour through modification of the balance of charge, hydrophobicity and polymer length is allowing larger complexes to be studied intact. Future challenges include generating discs with homogeneous size distribution and continued improvements in the polymer properties to minimize undesirable interactions with proteins and enhance resolution. Integration of affinity and fluorescent tags into the polymers will increasingly allow the isolation and precise tracking of a wider assortment of membrane assemblies from endogenous sources as well as opening up imaging and sensor applications. As new polymers emerge, the determination of high-resolution structures including labile heteromultimers in their native states will become more common and complex. The discovery of novel ligands including lipid modulators and drug molecules for previously intractable membrane-associated states in SMALPs remains a major goal that will drive the field's expansion. Lipidomic and proteomic analysis of SMA-solubilized organelles and cells will provide increasingly accurate insights into the interactions and regulatory mechanisms through which membranes are built and trafficked to create dynamic organizations within cells. Eventually the assemblies that form tissues such as cell-cell adhesion patches and cell-cell signaling machines may become resolvable using smart polymers as molecular scissors that gently remove the layers of dynamic lipids while leaving the remainder intact.

Acknowledgments

This project has been supported by NSERC RGPIN-2018-04994, Campus Alberta Innovation Program (RCP-12-002C) and Alberta Prion Research Institute/Alberta Innovates Bio Solutions (201600018) grants awarded to M.O. and TMIC funders the Canada Foundation for Innovation (CFI) and Genome Canada. We thank the SMALP network members including Stefan Scheidelaar for discussions and for sharing polymers and methods. B.K. acknowledges support by the South African Research Chairs Initiative of the Department of Science and Technology (DST) and National Research Foundation (NRF) of South Africa (Grant No 46855).

Author Contributions

M.O. wrote the manuscript. B.K edited the manuscript.

Conflicts of Interest

M.O. is a co-inventor of patent WO2011004158A1 entitled "Solubilisation of Membrane Proteins" which is owned by the University of Birmingham, and co-directs the SMALP network along with B.K.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eurpolymj.2018.11.015.

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