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Structural biology of endogenous membrane protein assemblies in native nanodiscs

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Abstract

The advent of amphiphilic copolymers enables integral membrane proteins to be solubilized into stable 10–30 nm native nanodiscs to resolve their multisubunit structures, post-translational modifications, endogenous lipid bilayers, and small molecule ligands. This breakthrough has positioned biological membrane:protein assemblies (memteins) as fundamental functional units of cellular membranes. Herein, we review copolymer design strategies and methods for the characterization of transmembrane proteins within native nanodiscs by cryo-electron microscopy (cryo-EM), transmission electron microscopy, nuclear magnetic resonance spectroscopy, electron paramagnetic resonance, X-ray diffraction, surface plasmon resonance, and mass spectrometry.

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Introduction

Structural elucidation and biophysical characterization of integral membrane proteins remains a challenge for structural biologists [1]. Using detergents for extraction disrupts the stable, active, and homogenous states of macromolecular assemblies necessary for high-resolution structural analysis and biophysical characterization. This major obstacle to characterization has stimulated the development of polymer nanotechnologies that extract portions of native membranes to isolate biologically intact membrane protein complexes for structural and screening analyses.

Using amphiphilic copolymers in lieu of detergents enables rapid preparation of virtually any memtein for structure–function studies (Figure 1). Since the discovery of styrene–maleic acid lipid particles (SMALPs) [2], several synthetic copolymers have been designed to improve the solubilization, purification, screening, detection, and resolution of a wide range of membrane systems in affordable and scalable quantities (Figure 2). This builds on the earlier development of membrane scaffold proteins to stabilize transmembrane proteins in nanodiscs but allows greater scalability and direct solubilization of protein:lipid complexes from biological tissues and membranes without use of detergents.

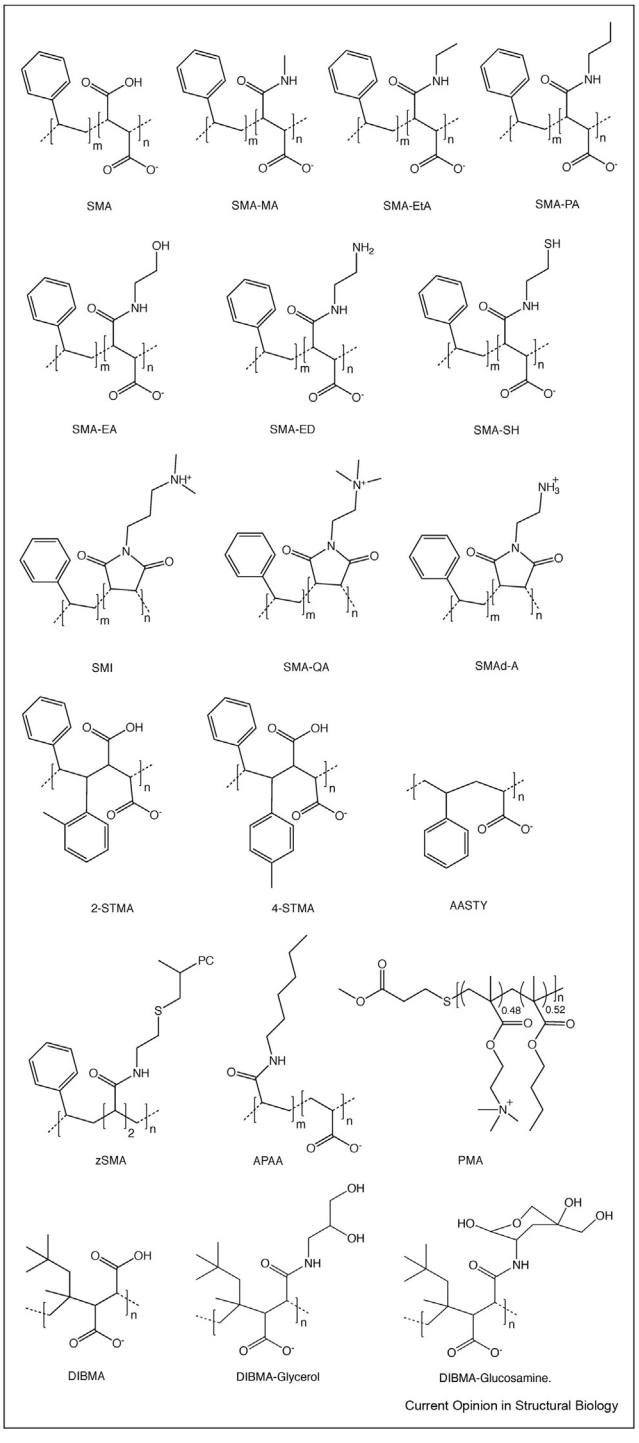
Polymer design strategies

Hydrolyzed poly(styrene-*co*-maleic anhydride) (SMA) solubilizes bacterial, fungal, mammalian, and plant membranes into nanodiscs of ~10 nm that enable purification and analysis of membranes bearing up to 48 transmembrane helices [2–4]. Standardized SMA preparation and membrane solubilization protocols exist [5,6]; however, widespread use has been by limited divalent cation tolerance, a narrow pH range (i.e. pH 7–9), and relatively small (~10 nm) nanodisc sizes.

Derivatization of SMA has resolved some limitations (Figure 1, Table 1). SMA derivatized with ethanolamine (SMA-EA) or ethylenediamine (SMA-ED) provide utility at wider pH ranges and higher divalent cation concentrations [7,8]. The SMAd-A derivative, produced by dehydrating SMA-ED, facilitates solubilization of lipid vesicles below pH 6. SMA derivatives bearing tertiary and quaternary ammonium cations, SMI, SMALP 1100I and SMA-QA, respectively, work well over a wide range of pH and divalent cation concentrations [9,10]. The cysteamine derivative, SMA-SH, enables covalent attachments of fluorescent or affinity tags, which can be used to visualize and purify native complexes [11].

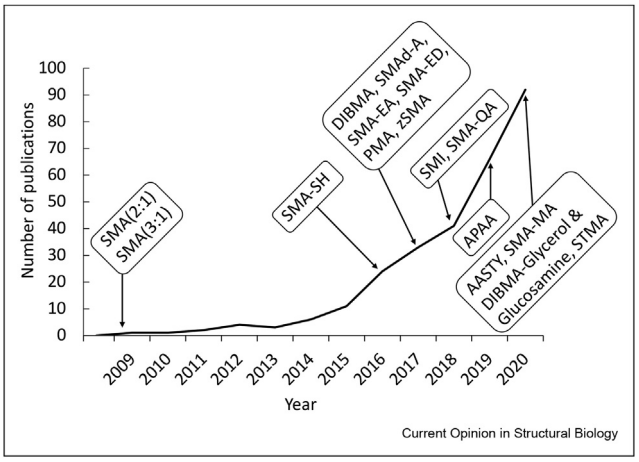
Several SMA derivatives yield larger nanodiscs, such as zwitterionic SMA, a derivative with phosphatidylcholine (PC) groups that solubilizes lipid membranes into ~10–30 nm discs at low pH values [15]. Methylamine (SMA-MA), ethylamine (SMA-EtA), and propylamine (SMA-PA) derivatives solubilize protein multimers over a wide range of pH values [16,17], with SMA-EtA and SMA-MA forming ~25 and 32 nm nanodiscs, respectively.

Figure 1



Overview of copolymers used for the solubilization of membrane proteins into native nanodiscs. The amphiphilic copolymers depicted include styrene-*co*-maleic acid (SMA) [2], styrene-*alt*-maleamic acid (SMA-MA) [59], SMA ethanolamine (SMA-EA) [8], SMA ethylenediamine (SMA-ED) [7], SMA with sulfhydrils (SMA-SH) [11], poly(2-methylstilbene-alt-maleic anhydride) (2-STMA), poly(4-methylstilbene-alt-maleic anhydride) (4-STMA) [22], styrene-co-maleimide (SMI) [9], styrene maleimide quaternary ammonium (SMA-QA) [10], dehydrated SMA ethylenediamine copolymer (SMAd-A) [7], acrylic acid-*co*-styrene (AASTY) [18], diisobutylene-*alt*-maleic acid (DIBMA) [12], zwitterionic SMA (zSMA) [60], alkyl polyacrylic acid (APAA) [16] and polymethacrylate (PMA) [15].

Figure 2



The rise of the field of native nanodiscs. The increasing number of publications each year about SMA and related polymers used to analyze membrane proteins is shown, as are the points at which nanodisc-formed copolymers were reported.

Related copolymers that lack aromatic moieties are well suited to membrane protein structure analysis by circular dichroism (CD) methods. Poly(diisobutylene-*co*-maleic acid) (DIBMA) is compatible with higher polycation levels [12], effectively solubilized membranes with G-protein coupled receptor (GPCR) complexes [13*], and recently yielded the YnaI structure by cryo-EM [14]. Polymethacrylates (PMAs) with variations in butyl to acetylcholine sidechain ratios fragment membranes into ~17 nm discs with CD compatibility [15]. A series of alkyl (butyl, pentyl, or hexyl) polyacrylic acid (APAA) polymers solubilize membranes into discs of ~7–17 nm; longer alkyl chains show greater efficacy than shorter ones [16].

While memtein structures can be resolved in SMALPs, the copolymers are obscured due to dispersity in chain length (Đ), comonomer sequence distribution, and composition. Several studies reveal a correlation between polymer parameters and solubilization efficacy. Compositionally defined SMAs reveal that lipid solubilization kinetics depend on copolymer chain length; short copolymers solubilized DMPC vesicles faster than longer copolymers [17]*. Low Đ acrylic acid and styrene copolymers provided low resolution structures of transient receptor channel TRPM4, highlighted by a dependence of membrane solubilization efficacy on lipid composition [18].

Replacing styrene subunits with substituted stilbenes in copolymerizations with maleic anhydride results in strictly alternating stilbene and maleic anhydride (STMA) copolymers with semirigid polymer backbones [19–21]. Well-defined copolymer sequence distribution, composition, and an increase in polymer backbone

Table 1

Comonomer ratios and molecular weights of polymers.

Copolymer	Apolar subunit	Polar subunit	Subunit ratio	M_n[a] (g/mol)	Đ[a]	References
2-STMA	Stilbene	Maleic acid	1:1	4400	1.19	[22]
4-STMA	Stilbene	Maleic acid	1:1	5800	1.54	[22]
AASTY	Styrene	Acrylic acid	1:1	8900		[18]
APAA	Alkyl	Acrylic acid				[16]
DIBMA	Alkyl	Maleic acid	1:1	8500	1.4	[12]
DIBMA glucosamine	Alkyl	Maleamic acid-glucose	1:1			Cube Biotech
DIBMA glycerol	Alkyl	Maleamic acid-propanediol	1:1			Cube Biotech
PMA	Butyl acrylate	Acetylcholine	1:1.1	6900		[15]
SMA2000	Styrene	Maleic acid	2:1	3000	2.5	[2]
SMA3000	Styrene	Maleic acid	3:1	3800	2.5	[2]
SMA-EA	Styrene	Maleamic acid-ethanolamine	1.3:1	1600		[8]
SMA-ED	Styrene	Maleamic acid-ethylene-diamine	1.3:1	1600		[7]
SMA-EtA	Styrene	Maleamic acid-ethylamine	1:1			[59]
SMA-MA	Styrene	Maleamic acid-methylamine	1:1			[59]
SMA-PA	Styrene	Maleamic acid-propylamine	1:1			[59]
SMA-QA	Styrene	Maleimide-ethyl-trimethylammonium	1.3:1	1600		[10]
SMA-SH	Styrene	Maleamic acid-cysteamine	2:1			[11]
SMAd-A	Styrene	Maleimide-ethanolamine	1.3:1	1600		[7]
SMALP 25010	Styrene	Maleic acid	3:1	4000	2.5	[2]
SMALP 30010	Styrene	Maleic acid	2.3:1	2500	2.6	[2]
SMALP 40005	Styrene	Maleic acid	1.2:1	2000	2.5	
SMI	Styrene	Maleimide-propyl-dimethylamine	2:1	2700	2.8	[9]
zSMA1	Styrene	Phosphatidylcholine			1.1	[60]
zSMA2	Styrene	Phosphatidylcholine		35,000	1.17	[60]
zSMA3	Styrene	Phosphatidylcholine		53,000	1.19	[60]

SMALPS, styrene–maleic acid lipid particles; SMA, styrene-*co*-maleic anhydride; zSMA, zwitterionic SMA; AASTY, acrylic acid and styrene; STMA, stilbene and maleic anhydride.
[a] Determined by gel permeation chromatography (GPC) on polymers before hydrolysis and derivatization.

rigidity confers several advantages to STMA over SMA, including an increase in useable pH range, as well as size and homogeneity of nanodiscs [22*]. Short hydrolyzed methyl-substituted STMA copolymers solubilize biological membranes into homogenous bilayer discs of ~20 nm and extract *Escherichia coli* membrane protein PagP in sizeable yields. Pursuing well-defined copolymers will enable a critical investigation on how copolymer parameters influence efficacy of lipid solubilization and protein extraction.

Visualizing memteins in native nanodiscs

Proteins in bacterial, yeast, insect, and mammalian cell membranes have been solubilized with SMA and related copolymers for structural and biophysical analysis (Table 2). High-resolution structures illuminate orientations of multisubunit proteins in asymmetric lipid bilayers along with post-translational modifications and cofactors. These advances represent major steps towards enabling single-particle cryo-EM of diverse memteins. Furthermore, these advances highlight the need for techniques to purify and monitor large assemblies and densely packed membranes [23].

Resolution of SMALP 30010-solubilized HEK293 cells by single-particle cryo-EM revealed an acid-sensing ion channel [24**] with a set of N-terminal residues, including a re-entrant loop not visible in detergent micelle preparations. The need to further enhance resolution motivates the development of homogeneous and rigidified copolymers such as STMA [22] to visualize such elements.

Comparing agonist-bound open, closed, and desensitized states of ion channels is uniquely feasible in SMALPs. The cryo-EM structures of zebrafish glycine receptor (GlyR) at a 2.9 Å resolution revealed the positions of the bound glycine agonist, inhibitory γ-aminobutyric acid (GABA) neurotransmitter, and taurine [25**]. This range of physiologically relevant states could only be obtained and visualized when native lipids are retained. Similarly, SMALP solubilized homopentameric α1 GlyR shows glycine and taurine binding induce conformational transitions, which support these agonists have distinct effects on channel gating.

Two human neuronal and cardiac voltage-gated potassium (Kv) channels, solubilized from mammalian COS-1 cells with SMA3000, are resolvable with intact pores within four-fold symmetric tetramers [26]. The regulatory and pore forming subunits of the KCNH5 and KCNQ1 channels are more stable and easier to

Table 2

Memtein structures characterized in native nanodiscs. The solubilized proteins are listed along with their identified post-translational modifications (PTMs), associated lipids unless not determined (nd) and ligands, size in kilodaltons, source material for extraction with various copolymers for analysis by a variety of structural and biophysical methods.

Proteins	PTMs	Lipids	Ligands	kDa	Source	Copolymer	Method	Resolution	References
AcrB homotrimer	–	PE	–	344	*E. coli*	SMA2000	cryoEM	3.0 Å	[30,31]
ActA, ActB, ActC, ActD, ActE, ActF	Triacyl cysteine	PE	Heme, cyt *aa*3	464	*Flavobacterium johnsoniae*	SMA3000, SMALP 25010	cryoEM	3.4 Å	[4]
ASIC1a channel	–	nd	–	180	HEK293	SMALP 30010	cryoEM	2.8 Å	[24]
Cytochrome *b*5	–	DMPC	–	16	*E. coli*	SMA-EA SMA-QA	NMR		[8,38]
CzcD homodimer	–	nd	Zn^{2+}	68	*E. coli*	SMALP 25010 SMALP 30010	NMR		[42]
Daptomycin tetramer, octamer	Decanoic acid	DMPC, DMPG	Ca^{2+}	6, 13	Synthetic	SMA-MA	NMR		[45]
ENT1 transporter	–	PC, PE	NBMPR	50	Insect cells	SMALP 30010	MS		[51]
FtsA	–	PE, PG, CL	–	45	*E. coli*	SMA2000	MS		[53]
GlpG protease	–	PE, PG, CL	–	35	*E. coli*	DIBMA, SMA2000	MS, TLC		[26,48]
GlyR homopentamer	–	nd	GABA, Gly, taurine	383	oocyte, HEK293, insect cells	SMALP 30010 SMA3000	cryoEM, MST	2.9 Å	[25,61]
KCNE1 channel	–	POPC, POPG	–	15	Liposomes	SMA3000	EPR		[27]
KCNH5, KCNQ1 homotetramers	–	nd	KCNE1	358	COS-1 cells	SMA3000	EM		[26]
KimA homodimer	–	nd	K^+ (3)	136	*E. coli*	SMALP 30010	cryoEM	3.7 Å	[28]
microbial rhodopsin homotrimer	–	Mono-olein	Trans-retinal	88	*E. coli*	SMALP 25010	LCP-XRD	2.0 Å	[39]
Pf1 coat protein	–	DMPC, DMPG	–	5	*E. coli*	SMALP 25010	ssNMR		[43]
PgpB phosphatase	–	PE, PG, CL	–	29	*E. coli*	SMA2000	MS		[53]
P-glycoprotein (ABCB1) monomer	–	nd	ATP, doxo-rubicin, verapamil	141	Insect cells	SMA2000	cryoEM	35 Å	[32]
PTH1R GPCR	–	nd	Antibody	53	HEK293	SMA2000	HDX-MS		[56]
Rhodopsin as four sets of trimers	–	nd	Trans-retinal	310	*E. coli*	SMA2000	AFM		[40]
TRPM4 tetramer	–	nd	–	537	HEK293	AASTY	cryoEM	18 Å	[18]
Wsc1 sensor	–		–	130	*S. cerevisiae*	SMA3000	cryoEM	18 Å	[41]
YnaI channel	–	PE/PC	LPC	40	*E. coli*	DIBMA	cryoEM	3.0 Å	[14]
ZipA monomer and ZipA:FtsZ complex	–	PE, PG, CL	FtsZ	36, 76	*E. coli*	SMA2000 SMI	cryoEM, SANS, SAXS,MS	16 Å	[9,46,53]

NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; XRD, X-ray diffraction; MS, mass spectrometry; SMALPS, styrene–maleic acid lipid particles; SMA, (styrene-co-maleic anhydride); PC, phosphatidylcholine; AASTY, acrylic acid and styrene; LCP, lipidic cubic phases; TLC, thin layer chromatography.

concentrate without aggregation in SMALPs than in detergent. SMA3000 was used to solubilize another Kv channel protein, KCNE1, from liposomes into nanodiscs for electron paramagnetic resonance studies. SMALPs improve signal-to-noise over liposomes, revealing the restricted local dynamics of spin labeled residues within the transmembrane region as compared with those outside the bilayer [27]. SMALP 30010 solubilization of the *Bacillus subtilis* KimA protein as a K^+/H^+ symporter has enabled cryo-EM structural resolution of 3.7 Å [28].

The structure of the Alternative Complex III (ACIII) in SMALPs was solved at a resolution of 3.4 Å [4]. Positions of the 6 protein subunits including their 48 transmembrane helices as well as an associated cytochrome *c* oxidase, a triacylated cysteine, haem groups, iron–sulfur clusters and biological phospholipids are revealed. Detergents such as dodecyl maltoside destabilize and reduce the activity of such complexes; the preserved membrane environment in SMALPs allows measuring femtosecond kinetics of electron transfer in photosystems by laser spectroscopy [29].

The multidrug exporter AcrB solubilized from *E. coli* with SMA2000 was resolved by cryoEM to 3.0 Å. This revealed an asymmetric 344 kDa homotrimer and an asymmetric bilayer with over 31 visible lipid molecules forming hexagonal patterns in the inner leaflet and an ordered disarray in the outer leaflet. After SMALP solubilization, wild-type and mutant AcrB proteins from *E. coli* [30**] and *Salmonella typhimurium* [31] were evident by cryoEM, revealing consistent structures. The multidrug transporter P-glycoprotein is visible as a functional monomer postextraction from insect cells by using SMA2000 [32]. That some lipids and ligands remain unresolved suggests a need for more homogeneous and rigid polymers.

Several conformational states of the mechanosensitive channel YnaI are evident using polymers to solubilize the bilayer-bound structure. The 278 kDa heptamer was expressed in *E. coli* and transferred from liposomes into nanodiscs using DIBMA to resolve its structure by cryoEM to 3.0 Å, which is higher than the resolution obtainable in amphipols [14**]. The protein is surrounded by a bilayer of lipid molecules, revealing the contact between a PE headgroup and Lys residue involved in sensing. The addition of lysoPC induces formation of open-like and intermediate states from the closed form. Comparison of four DIBMA-solubilized conformations reveals differences in transmembrane helix positions, bend angles and pore size which explain transduction of bilayer deformation forces across asymmetric membranes.

Native GPCRs can be directly stabilized from mammalian cell membranes in their active forms with DIBMA, PMA, or SMA as demonstrated with the β2 adrenergic [33], neurotensin [34], and adenosine A2A (ARAR) receptors [35], respectively. The melatonin and ghrelin receptors retain activity based on a fluorescent GTPγS assay after solubilization from mammalian cells with SMA [36]. The growth hormone secretagogue receptor and dopamine receptor form heterotetrameric assemblies that are fully active after solubilization in SMALPs [37]. Similarly the cannabinoid receptor is recognized by conformationally specific antibodies in surface plasmon resonance (SPR) assays after solubilization with SMA2000 [38]. This approach can be used to identify variants with improved binding affinity or stability, although there remains a need to conveniently immobilize protein-containing nanodiscs on surfaces for screening campaigns. The trimeric structure of bacteriorhodopsin from *Haloquadratum walsbyi*, solubilized with SMALP 30010, moved into lipidic cubic phases, and resolved by X-ray diffraction to 2.0 Å, exposing the positions of bound mono-olein and *trans*-retinal molecules [39]. Atomic force microscopy shows four sets of microbial rhodopsin trimers in 54-nm nanodiscs extracted from *E. coli* by using SMA2000 [40]. The cell wall sensor Wsc1 was purified directly from yeast using SMA3000, revealing the orientations of extracellular, transmembrane and cytosolic domains as well as attached green fluorescent protein [41]. Such studies suggest broad applicability of SMALP technology to large memteins from diverse hosts.

Both solution and solid state nuclear magnetic resonance (NMR) can be used to obtain structural restraints for proteins inside SMALPs. After solubilization from *E coli* with SMALP 30010 or 25,010, the zinc diffusion facilitator CzcD retains a dimeric structure with ~70 associated lipid molecules with assignable amide and methyl group peaks in ^{13}C, ^{15}N resolved solid state NMR spectra [42]. The coat protein of the Pf1 bacteriophage solubilized by SMALP 25010 similarly reveals stable helices and sharper solid state NMR signals than those in bicelles or peptide-based nanodiscs [43]. The cytochrome *b*5 protein solubilized by SMA-EA or SMA-QA displays stable transmembrane helices in ^{15}N-resolved TROSY NMR spectra, although SMA-QA required 500 mM salt to suppress aggregation [8,44]. Daptomycin, a cyclic peptide antibiotic, creates pores that permeabilize bacterial membranes. SMA-MA phosphatidylglycerol (PG) nanodiscs monitored by Förster resonance energy transfer and NMR reveal calcium-dependent pore formation of tetramers and octamers [45]. The SMA-solubilized ZipA complex with FtsZ is visible by small angle X-ray scattering, small angle neutron scattering, and cryo-EM [46]. The compatibility of SMALPs with multiple analytical methods provides validation of proposed structures and conformational changes, particularly of labile states.

Measuring memtein interactions in SMALPs

Several methods can be used to measure molecules binding to memteins in nanodiscs. A quantitative NMR assay can resolve phospholipids in SMALPs, such as the CLIC1 channel, by virtue of their distinct $^{1}H-^{31}P$ signals [47]. The cardiolipin, PG and phosphatidylethanolamine (PE) lipids associated with the rhomboid protease GlpG in nanodiscs formed by DIBMA or SMA2000 can be identified by thin layer chromatography (TLC) or native mass spectrometry (MS) analysis [48,49]. The lipid complexes of nascent chains of membrane proteins emerging from stalled ribosomes can be solubilized with DIBMA and identified by TLC [50]. MS analysis pinpoints specific PC and PE lipids in the SMALP 30010-solubilized equilibrative nucleoside transporter-1, which retains ligand binding activity [51]. Transmission electron microscopy reveals the morphology of infectious prions solubilized from brain homogenate with SMA-MA [52]. MS analysis of SMA2000 extracted ZipA, PgpB and FtsA proteins from *E. coli* membranes shows distinct preferences for monounsaturated PE and PG lipids, lipid chain lengths and degrees of unsaturation [53]. Fractionating solubilized T cell membranes into SMALPs allowed GPI-anchored proteins and Src family kinases to be identified by immunoblotting and MS was used to reveal the enrichment of associated PC, PE PI and PG lipids [54], suggesting potential for further proteomic applications.

Real-time quantification of lipidomes in nanodiscs from living organisms expressing mutations or subjected to chemical agents enables the measuring of dynamic effects on organelle-specific pathways, as demonstrated by *in vivo* extraction of lipid profiles from cuticles of *C. elegans* strains extracted with SMA [55]. SMALP solubilized-parathyroid receptor-1 enables SPR to measure specific antibody interactions and hydrogen–deuterium exchange MS to identify the helical site of interaction in the extracellular domain of the GPCR [56]. Fluorescence correlation spectroscopy can monitor specific ligand interactions in nanodiscs, as shown with the human A2AR [57] and ATP binding cassette transporter ABCG2 [58] solubilized from *Pichia* and HEK293 cells, respectively, with SMA2000. Harnessing SMALPs with this array of complementary assays allows native membrane protein–ligand complexes to be cross-validated. Structure-aided drug discovery with memteins facilitates protocols that cannot be achieved by recombinant methods. Continued development of new polymers will enhance our ability to resolve and functionally characterize inaccessible assemblies that reside in native membranes.

Conflict of interest statement

MO is a director of the SMALP network and has awarded and filed patents on related polymers and methods.

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