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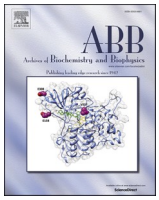

GPCRs in the round: SMA-like copolymers and SMALPs as a platform for investigating GPCRs

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ABSTRACT

G-protein-coupled receptors (GPCRs) are the largest family of membrane proteins, regulate a plethora of physiological responses and are the therapeutic target for 30–40% of clinically-prescribed drugs. They are integral membrane proteins deeply embedded in the plasma membrane where they activate intracellular signalling via coupling to G-proteins and β-arrestin. GPCRs are in intimate association with the bilayer lipids and that lipid environment regulates the signalling functions of GPCRs. This complex lipid 'landscape' is both heterogeneous and dynamic. GPCR function is modulated by bulk membrane properties including membrane fluidity, microdomains, curvature, thickness and asymmetry but GPCRs are also regulated by specific lipid:GPCR binding, including cholesterol and anionic lipids. Understanding the molecular mechanisms whereby GPCR signalling is regulated by lipids is a very active area of research currently. A major advance in membrane protein research in recent years was the application of poly(styrene-*co*-maleic acid) (SMA) copolymers. These spontaneously generate SMA lipid particles (SMALPs) encapsulating membrane protein in a nano-scale disc of cell membrane, thereby removing the historical need for detergent and preserving lipid:GPCR interaction. The focus of this review is how GPCR-SMALPs are increasing our understanding of GPCR structure and function at the molecular level. Furthermore, an increasing number of 'second generation' SMA-like copolymers have been reported recently. These are reviewed from the context of increasing our understanding of GPCR molecular mechanisms. Moreover, their potential as a novel platform for downstream biophysical and structural analyses is assessed and looking ahead, the translational application of SMA-like copolymers to GPCR drug discovery programmes in the future is considered.

1. Introduction

Located in the plasma membrane of cells, G-protein-coupled receptors (GPCRs) detect and bind specific signalling molecules from the extracellular milieu, such as hormones and neurotransmitters, then transduce that signal across the membrane. Generally GPCRs couple to heterotrimeric guanine nucleotide binding proteins (G-proteins) which, together with β-arrestins, mediate the signal transduction process via activation of intracellular signalling cascades. GPCRs are found in organisms throughout the phylogenetic tree including fish, insects, plants, slime-moulds and viruses (but not bacteria) and form the largest class of 'chemical switches' in biology. GPCRs constitute the largest family of membrane proteins in the human genome with 826 members [1]. The diversity in physico-chemical properties of the activating ligands is extreme, ranging from photons to peptides and proteins, from biogenic amines to metabolites and lipids [2]. As a result, GPCRs modulate the majority of physiological responses and are therefore major therapeutic targets, with 30–40% of clinically-approved drugs acting at these receptors [3]. Despite the structural heterogeneity of the activating ligands and the diverse primary sequences exhibited by GPCRs, these receptors all share a common protein architecture with a bundle of seven transmembrane helices at their core. Sequence homologies have been used to divide GPCRs into families [4,5], with three of these families

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Abbreviations

5- HT	5-hydroxytrptamine (serotonin)
7TM	seven transmembrane helices
$A_{2A}R$	adenosine A_{2A} receptor
β_2AR	β_2-adrenergic receptor
bRho	bovine rhodopsin
CGRP	calcitonin gene-related peptide
CHAPSO	3-(3-cholamidopropyl)dimethylammonio-2-hydroxy-1-propane sulfonate
CHS	cholesteryl hemisuccinate
CRAC	cholesterol recognition amino-acid consensus
cryo-EM	cryogenic electron microscopy
DDM	*n*-dodecyl-ß-D-maltoside
DIBMA	poly(diisobutylene-*alt*-maleic acid)
DIBMALP	DIBMA lipid particles
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
FCS	fluorescence correlation spectroscopy
GABA-R	γ-aminobutyric acid receptor
GPCR	G-protein-coupled receptor
GRK	GPCR kinase
HDL	high density lipoprotein
HwBR	*Haloquadratum walsbyi bacteriorhodopsin*
M1R	M1 muscarinic acetylcholine receptor
M2R	M2 muscarinic acetylcholine receptor
Meta I	metarhodopsin I
Meta II	metarhodopsin II
mGluR1-8	metabotropic glutamate receptors
MSP	membrane scaffold protein
NECA	5′ *N*-ethylcarboxamido adenosine
PtdIns(4,5)P_2	phosphatidylinositol 4,5 bisphosphate
SMA	poly(styrene-*co*-maleic acid)
SMALP	SMA lipid particle
SMI	poly(styrene-*co*-(*N*-(3-N′,N'dimethylaminopropyl) maleimide))
SMILP	SMI lipid particle

being of particular importance: the rhodopsin/β-adrenergic receptor family (Family A), the secretin receptor family (Family B) and the metabotropic glutamate receptor family (Family C). Family A is by far the largest family with 719 members, which collectively regulate a wide range of physiological responses. As a consequence, family A is also the most-targeted GPCR family with respect to therapeutic intervention [1]. Family B, which comprises 48 receptors, is further sub-divided into B1 (secretin) and B2 (adhesion) with 15 and 33 members, respectively. Receptors belonging to the B1 subfamily have been targeted by drug-discovery programmes for the treatment of some major diseases currently afflicting society, including type-2 diabetes, obesity, migraine and osteoporosis. Family C encompasses 22 receptors including receptors for γ-aminobutyric acid (GABA-R_{B1} and GABA-R_{B2}) and metabotropic glutamate receptors (mGluR1-8). Intracellular signalling by these receptors requires formation of an obligate dimer.

2. Regulation of GPCR signalling by the membrane bilayer

As integral membrane proteins, GPCRs are deeply embedded within cell membranes and are in intimate association with the bilayer lipids. Moreover, it is well-established that lipids have a critical role in modulating the structure and function of membrane proteins [6–10] and this is particularly true for GPCRs. Mammalian cells produce a plethora of lipids varying in structure and composition, including their acyl chains and head groups. These lipid species vary with cell-type, subcellular localisation, disease state and metabolic state. Furthermore, cell membranes are heterogeneous with respect to a range of lipid-focussed biophysical parameters. Examples include: lipid asymmetry between the bilayer leaflets [11,12]; enrichment of certain lipids surrounding membrane proteins [13]; existence of micro-domains with specific lipid enrichment, such as 'lipid rafts' [14]; variation in membrane thickness caused by differences in the length of lipid acyl chains, packing order of membrane lipids and 'hydrophobic mismatch' between the bilayer thickness and the length of protein transmembrane domains [15]; membrane curvature [16,17] and lipid peroxidation [18]. This complex lipid 'landscape' is both heterogeneous and dynamic. The term 'functional paralipidome' has been proposed to describe the preferred lipid microenvironments that solvate membrane proteins and the resultant membrane properties [19].

In addition to the functional ramifications of bulk membrane lipids on GPCR activation and signalling, specific lipids can directly interact with a GPCR and thereby regulate receptor function. Cholesterol regulates membrane fluidity and is a major component of the mammalian plasma membrane, comprising c.30% of the total lipid content. Many years before the availability of atomic-level structures of GPCRs, it was known that cholesterol could modulate receptor function [20,21]. Cholesterol can exert its effects either at the bulk lipid level to change the physico-chemical properties of the membrane, or as an allosteric modulator via direct interaction with the GPCR protein. These two mechanisms are not mutually exclusive. Evidence of direct interaction of cholesterol with GPCRs was provided by crystal structures. Bound cholesterol was observed in the X-ray crystal structure of the β_2-adrenergic receptor (β_2AR) [22] and in many subsequent GPCR structures, with the cholesterol binding sites corresponding to both leaflets of the bilayer [23–25]. A 'cholesterol consensus motif' was proposed [26] and Cholesterol Recognition Amino-acid Consensus (CRAC) sequences have been identified that are conserved in a large subset of family A GPCRs [27,28].

The effects of cholesterol on GPCR function are manifold and include regulation of ligand binding and signalling (reviewed in Refs. [24,29]). Mutual allosteric regulation between the orthosteric ligand binding site and a cholesterol binding site has been reported [30]. However, the mechanism by which ligand binding is directly modulated by cholesterol can be receptor-specific. For example, a specific cholesterol molecule directly shapes the ligand binding pocket of the 5-HT_{1A} receptor to create high affinity binding but is absent in 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1E} receptors [31]. In addition, cholesterol has been observed at the interface between GPCR dimers [32].

Regulation of GPCR signalling by lipids is not restricted to cholesterol. Phosphatidylglycerol and phosphatidylethanolamine were identified as allosteric regulators of the β_2-AR, favouring active and inactive conformational states respectively [33]. Furthermore, phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P_2), a minor component of the plasma membrane, has been identified as an allosteric modulator that stabilises the active receptor conformation and enhances G-protein selectivity. PtdIns(4,5)P_2 present at the GPCR:G-protein interface forms bridging contacts between the receptor and G-protein thereby stabilising the complex [34,35]. Interestingly, PtdIns(4,5)P_2 was also found to facilitate formation of a functional complex between the β_2AR and GRK5, one of the family of GPCR kinases that phosphorylates the agonist-occupied receptor to terminate G-protein-mediated signalling and initiate arrestin-mediated signalling [36]. Anionic phospholipids can affect the conformational plasticity of GPCRs, with phosphatidylserine altering the equilibrium between MI and MII conformations of rhodopsin, resulting in an increase in the active MII conformation [37]. Furthermore, it has recently been shown for the $A_{2A}R$ that anionic lipids interact with positively charged residues on the receptor's intracellular face, impacting on the $A_{2A}R$ conformational equilibria and thereby

stabilising a pre-activated conformation primed to couple to G-protein [38].

3. Extracting GPCRs from the plasma membrane: solutions and problems

GPCRs have been extensively studied in the plasma membrane of cells, where they are subjected to all of the lipid interactions cited previously in this review. However, for a full understanding of the receptor protein it is necessary to study the purified protein. Historically, this universally required the use of detergents to disrupt the lipid bilayer. Although detergents are effective at molecular dispersal and share some physico-chemical properties with phospholipids, they are poor mimetics of the plasma membrane. Not only do they strip away the annular lipid in close association with the receptor, but solubilisation also removes the lateral pressure inherent in the plasma membrane. To compensate for the loss of native cholesterol, cholesteryl hemi-succinate (CHS) is frequently added to purification buffers for GPCRs. Nevertheless, the detrimental effects of detergent would be expected to be particularly disruptive to conformationally-dynamic membrane proteins like GPCRs. These receptor proteins populate a wide range of conformational states and undergo extensive rearrangements of their helical bundle during activation. This includes changes in rotation, elongation, tilt, side-chain rotamer plus a characteristic outward translation of TM6 by as much as 19 Å [39]. As predicted, solubilisation of GPCRs by detergent resulted in their instability and progressive loss of function in the continued presence of detergent. As a result, extensive modifications to the GPCR structure were routinely required to obtain crystal structures [40]. Effects of the detergent could also be subtle; thus the detergents digitonin and 3-(3-cholamidopropyl)dimethylammonio-2-hydroxy-1-propane sulfonate (CHAPSO) preserved high affinity binding of the non-selective antagonists *N*-methyl scopolamine and atropine to the M1 and M2 subtypes of the muscarinic acetylcholine receptor (M1R and M2R respectively) but changed the affinity of the selective antagonist pirenzepine [41,42].

Several strategies have been adopted to ameliorate the detrimental effects of detergent exposure [43,44]. Nanodiscs utilise a series of engineered helical membrane scaffolding proteins (MSPs) originally derived from the apolipoprotein-A1 component of human high-density lipoprotein (HDL) particles but developed subsequently to facilitate their use for studying membrane proteins. The MSP self-assembles with lipids to form a discoidal lipid bilayer stabilised by a belt of MSP. Detergent-solubilised membrane proteins can be reconstituted into these nanodiscs to provide a versatile platform that has been used extensively to study a wide range of membrane proteins in defined lipid environments, plus a plethora of other applications [for a recent review see Sligar and Denisov 2021 [45]]. These MSP-nanodiscs have been employed to study GPCRs. For example, the cryo-electron microscopy (cryo-EM) structure of the M2R in complex with β-arrestin 1 was obtained using a reconstituted nanodisc stabilised by MSP1E3D1 [46]. It is beyond question that MSP-nanodiscs are a useful tool for studying membrane proteins, providing a defined lipid bilayer environment that is clearly superior to a detergent micelle. Nevertheless, significant problems remain; i) the membrane protein has to be solubilised by detergent before it can be reconstituted into nanodiscs, so all of the disadvantages of detergent exposure remain unaddressed, ii) the structured complexity and nuances of the native membrane are lost and replaced by a relatively simplistic lipid environment and iii), the stabilising belt is a protein, so can interfere with biophysical characterisation of the encapsulated protein of interest.

4. Preserving the lipid environment with SMA and SMALPs

Membrane protein solubilisation was revolutionised by poly(styrene-*co*-maleic acid) (SMA) which incorporates into membranes and spontaneously forms nano-scale SMA **l**ipid **p**articles (SMALPs) encapsulating membrane proteins [47]. Such polymer-stabilised lipid particles are sometimes referred to as 'native nanodiscs'. Earlier studies had established that SMA could produce nano-particles containing a synthetic lipid bilayer [48] but it was the application of SMA to membrane protein research that was a 'game changer' with the resultant rapid increase in SMA-based publications year on year [49]. SMA extracts proteins directly from the native cell membrane, with all its compositional complexity cited earlier, without detergent or intermediate steps. Once formed, SMALPs do not require the presence of free polymer for stability, in contrast to detergent-solubilised proteins for which there is an absolute requirement for detergent in all subsequent buffers.

Biophysical analysis revealed a disc of the lipid bilayer stabilised by a belt of SMA, with the styrene rings of the polymer intercalated between the lipid acyl chains and the maleic acid likely to interact with the lipid headgroups [50]. The molecular process of SMALP formation is not fully defined but seems to proceed through three stages following addition of polymers; i) membrane binding, ii) membrane insertion and destabilisation iii), lipid particle (SMALP) disc formation [51,52] (Fig. 1). The initial binding is driven by hydrophobic interactions between the styrene moieties and the lipid acyl chains, these are sufficiently strong to overcome repulsion between the negatively-charged maleic acid in the polymer and the anionic head-groups of the bilayer lipids. Deeper penetration of polymer into the membrane hydrophobic core depends on the lipid packing. Packing defects, such as those generated by co-existence of gel-phase and liquid crystal-phase at the transition temperature, aid this process. Subsequent membrane destabilisation has been proposed to involve formation of pores prior to complete SMALP formation [52–54]. The SMALP generated has a diameter of c.10 nm. An important ramification of this mechanism is that the encapsulated protein of interest remains surrounded by its native lipid environment throughout the process, from cell to SMALP. This is a unique advantage of using amphipathic copolymers such as SMA, that is not replicated with any of the other strategies for isolating membrane proteins, including MSP-based nanodiscs.

The lipid packing in discs formed by SMA copolymers may not be fully representative of the plasma membrane [55]. Characterisation of the transition temperatures of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) revealed that a SMALP formed from 2:1 SMA (styrene:maleic acid ratio) was membrane-like, whereas the transition temperature was reduced by 10 °C in a 3:1 SMALP, indicating bilayer perturbation. Given that membrane fluidity will impact on the conformational plasticity of GPCRs, 2:1 SMA was judged to be more appropriate for isolating GPCRs. Consequently, we used 2:1 SMA to isolate the adenosine A_{2A} receptor ($A_{2A}R$). This was the very first report of purification of a GPCR in the complete absence of detergent at any stage [56] and was then followed by reports of SMA being used for other GPCRs [57–62]. Given that the $A_{2A}R$ had not been exposed to the detrimental effects of detergent solubilisation cited previously and had remained encapsulated in membrane lipid within the SMALP throughout the purification, we hypothesised that $A_{2A}R$-SMALP would be more stable than $A_{2A}R$ solubilised by *n*-dodecyl-ß-D-maltoside (DDM), a commonly used detergent for GPCRs. A direct comparison between $A_{2A}R$-SMALP and $A_{2A}R$-DDM revealed that the $A_{2A}R$-SMALP was indeed more thermostable than $A_{2A}R$-DDM, as assessed by retention of binding capability for the ligand [^{3}H]ZM241385 with increasing temperature. $A_{2A}R$-SMALP also exhibited superior stability to $A_{2A}R$-DDM over a series of challenges to conformational integrity, including storage at 4 °C, storage at 37 °C and repeated freeze/thaw cycles. Furthermore, $A_{2A}R$-SMALP retained ligand binding (70%) following lyophilisation and rehydration [43,56]. In addition, the non-proteinaceous nature of the stabilising SMA copolymer enabled biophysical interrogation of the encapsulated GPCR by circular dichroism. The repertoire of techniques for interrogating GPCRs following nano-encapsulation has recently been extended to include fluorescence correlation spectroscopy (FCS) to characterise the binding capability. FCS is a quantitative, real-time technique with single molecule sensitivity that is particularly suited to

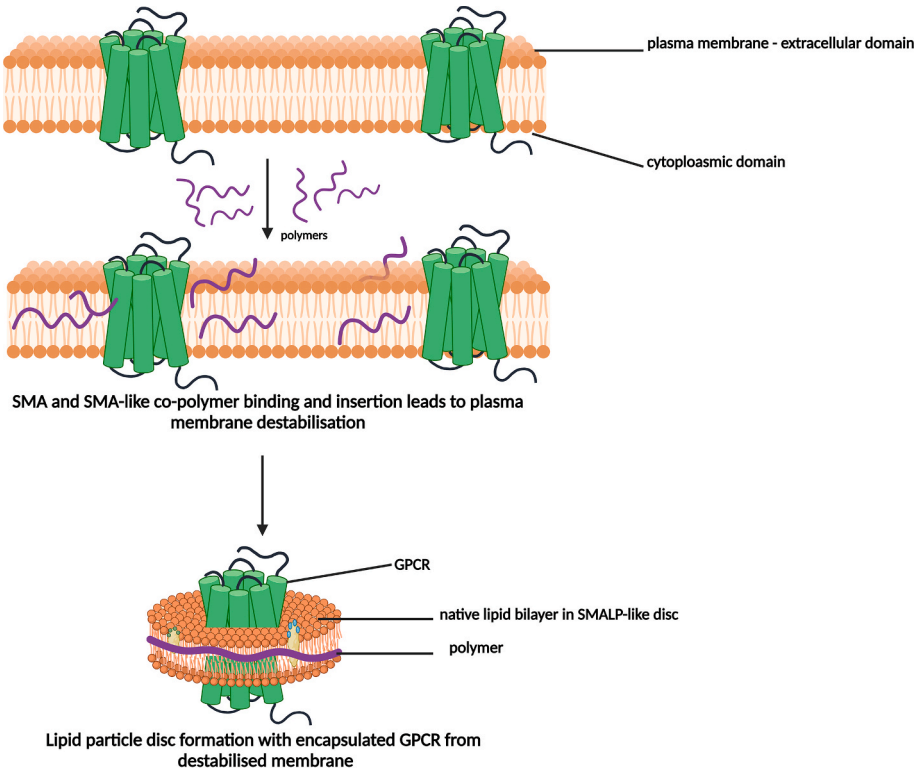

Fig. 1. Schematic representation of SMALP-like disc formation.
SMALP-like disc formation occurs spontaneously. Polymers initially bind to the membrane, then insert into the lipid bilayer which leads to membrane destabilisation and subsequent formation of the lipid particle disc (see text for details). The diameter of the GPCR helical bundle at the level of the membrane is c.4 nm and the diameter of SMALP, SMILP and DIBMALP 'SMALP-like' discs is c.10 nm (SMALP, SMILP) and c.20 nm (DIBMALP).

investigating low concentrations of particles. Using A_{2A}R-SMALP and the fluorescent ligand CA200645, solution-based FCS applied to a GPCR-SMALP was reported for the first time, demonstrating its utility for the development of down-stream applications as an investigation platform [63].

5. The next generation: SMA-like copolymers and SMALP-like discs

Although SMA has many advantages for investigating GPCR structure and function, it has defined limitations resulting from its chemical composition. SMALPs are sensitive to pH and precipitate in acidic conditions, should low pH be required. More importantly for GPCRs, maleic acid in the copolymer can chelate divalent cations commonly present in biological buffers, including Mg^{2+} and Ca^{2+}, which destabilise SMALPs resulting in precipitation. The styrene ring in the copolymer absorbs in the far-UV spectrum, which could be a complication for some spectroscopic analyses of the encapsulated protein. These practical limitations can restrict the utility of SMALPs and have been the incentive to develop 'second-generation' SMA-like polymers. These include poly(styrene-*co*-(*N*-(3-*N*′,*N*′-dimethylaminopropyl)maleimide)) (SMI) which lacks the maleic acid of SMA and has improved tolerance to divalent cations and altered pH sensitivity characteristics [64]. SMI has been successfully employed to solubilise the A_{2A}R, V_{1a} vasopressin receptor and bovine rhodopsin (bRho) [64,65].

Another alternative to SMA is poly(diisobutylene-*alt*-maleic acid) (DIBMA) in which the aromatic styrene ring of SMA is replaced by aliphatic diisobutylene but the maleic acid remains. From an application perspective, the absence of an aromatic ring in the copolymer, makes DIBMALPs compatible with spectroscopy in the far-UV, plus they were found to be tolerant of divalent cation concentrations in the low millimolar range [66]. DIBMALPs have a diameter in the 20–30 nm range, which is larger than SMALPs (diameter c.10 nm), and they have been used to encapsulate a range of membrane proteins including the GPCRs; A_{2A}R, β_2AR, rhodopsin and the calcitonin gene-related peptide (CGRP) receptor [65,67,68]. Although there were detailed analyses addressing the biophysical characteristics of the various SMALP-like discs enclosing a bilayer of defined lipid composition, very little information was available on the effects different copolymers had on the encapsulated protein of interest. The copolymers SMA, SMI and DIBMA are structurally-related with respect to the two backbone components; SMA and SMI share a styrene ring whereas SMA and DIBMA each possess maleic acid (Fig. 2). Given that these three structurally-related copolymers had been used to encapsulate GPCRs, we investigated the comparative effects of these copolymers on the activation of an encapsulated GPCR. bRho was used as the encapsulated GPCR. The reasons for this choice include; the photoactivation proceeds from dark-adapted inactive rhodopsin through a series of structurally-defined intermediates, each with its own characteristic absorbance, to the fully-activated conformation metarhodopsin II (Meta II), the photoreactive chromophore 11-*cis* retinal is covalently bound and on activation by a photon of light rapidly converts to the full agonist all-*trans* retinal to drive the conversion to the active Meta II conformation, the last step of which (conversion of Meta I to Meta II) corresponds to the large movement at the bottom of TM6 which is characteristic of GPCR activation. The bRho was entirely wild-type without any structural modification, such as mutation or introduction of tags, and was extracted directly from rod outer segment membranes from bovine eyes. Thermostability of encapsulated bRho was dictated by the copolymer employed, with DIBMA and SMI endowing greater thermostability than SMA. However, the greatest influence of the copolymer was on the photoactivation of bRho, which proceeded as far as Meta I but did not progress to Meta II with either SMA or SMI. In contrast, photoactivation of bRho in a DIBMALP progressed beyond Meta I to the active

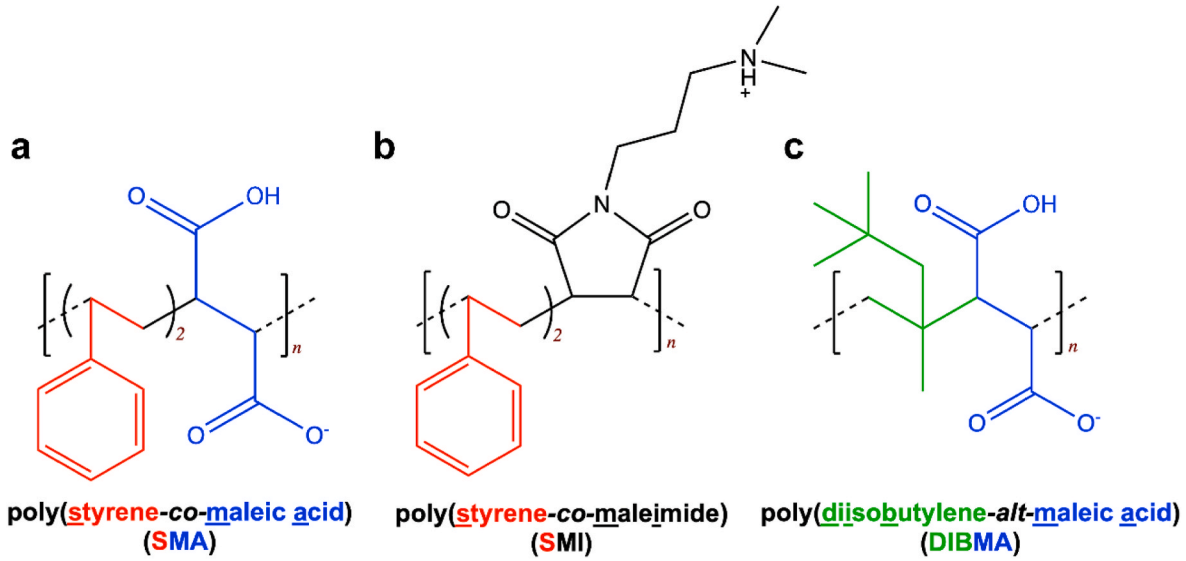

Fig. 2. Structural comparison of copolymers used to solubilise GPCRs. (a) SMA, (b) SMI and (c) DIBMA.

conformation Meta II [65]. The chemical moiety that differentiates DIBMA from SMA and SMI, is that the latter two copolymers possess an aromatic styrene ring which is absent in the DIBMA. One possibility is that the styrene ring of SMA/SMI could be interacting directly with the bRho to restrict conformational change. This could include π-π stacking with an aromatic sidechain on a transmembrane domain constraining receptor movement or possibly interaction of the styrene with a cholesterol-binding site on the GPCR, cited earlier. Alternatively, it is known that this phenyl ring in the copolymer stabilises the enclosed bilayer by inserting between the lipid acyl chains and that this can affect the lipid packing and transition temperature [66]. Consistent with this, a comparison of the lipid dynamics in lipodiscs formed by SMA or DIBMA copolymers showed that the lipids were dynamically more constrained in SMALPs than in DIBMALPs [69]. There are also differences in lipid transfer, which is slower among DIBMALPs than among SMALPs [70]. It is noteworthy that, using 3:1 SMA rather than 2:1 SMA, the active state of bRho was observed when low SMA/rhodopsin molar ratios were used but not with high SMA/rhodopsin molar ratios [71]. Overall, there appears to be some restriction to the conformational plasticity of GPCRs encapsulated in a SMALP or SMILP that prevents the full activation of bRho to Meta II observed with DIBMALP. This restriction was also observed for the human $A_{2A}R$ in a SMALP in which the agonist NECA generated only a small conformational change [62]. Likewise, the conformational freedom of the photoreceptor/transducer complex (*Np*SRII$_2$/*Np*HtrII$_2$) from *Natronomonas pharaonis* was also restricted in a SMALP [72] but *Np*SRII was fully activated in DIBMALP [73].

It is possible that the larger diameter of the DIBMALP compared to the SMALP and SMILP, contributes to its ability to support transition to Meta II, as computational simulations of lipid dynamics in empty 'lipid only' discs indicated a stiffening of the lipids due to a confinement effect of the disc but this stiffening was lower when the disc diameter was increased from 9.8 nm to 18.4 nm [74]. SMALP-like discs may provide new opportunities for GPCR drug discovery [75]. On first inspection, this restriction of conformational plasticity appears to be a potential problem for GPCR-SMALP and GPCR-SMILP but it could actually provide an opportunity. It is well-established that GPCR signalling is extremely complex with receptors populating a spectrum of conformational states. Individual GPCRs signal through multiple intracellular cascades and 'biased agonists' selectively activate one signalling pathway over another. Full agonists and partial agonist can stabilise different conformations, so activated GPCRs can sample a wide spectrum of distinct active receptor conformations with different efficacies for different signalling systems [76–78]. The precise receptor conformation stabilised by a particular ligand will dictate that ligand's pharmacological profile. Allosteric ligands bind to sites that are discrete to the classical (orthosteric) binding site and add another level of conformational complexity [79]. Consequently, using copolymers that prevent full activation of a GPCR, might facilitate structural studies on conformational intermediates or drug discovery programmes where stabilisation of an inactive, or partially-active, intermediate receptor conformation is required.

6. Future perspectives

Novel SMA-like copolymers will be developed. These advances will be directed increasingly by an ever-deeper understanding of the molecular mechanisms surrounding the process of extracting encapsulated membrane proteins by copolymers. In addition, it is reasonable to envisage copolymers being designed for specific down-stream tasks; be that structure determination, drug discovery assay platforms, conformation-specific antibody discovery or lipidomics. This will probably include generating SMALP-like discs of different defined diameters. Such 'tuneable' lipid particle discs may find utility in solving atomic level structures of GPCRs and their signalling complexes using cryo-EM. In addition, it has already been demonstrated that SMALPs can be used to obtain crystal structures. Bacteriorhodopsin from the microorganism *Haloquadratum walsbyi* (HwBR) has 7 TMs but is a proton-pump not a GPCR. Nevertheless, as a high-resolution crystal structure (2.0 Å) was solved after the HwBR was transferred from SMALP into monoolein lipidic cubic phase for growth of crystals, it shows the potential for solving GPCR structures in the future [80].

Some copolymers have already been synthesised with specific down-stream applications in mind. For example, sulfhydryl-containing SMA-SH which has potential for further derivatisation with affinity tags or fluorescent groups via the sulphydryl group as well as for immobilisation of encapsulated GPCRs on a chip [81]. In addition, fluorescent SMA which has potential for use in fluorescence-based analyses was reported recently. The aromatic fluorophore-containing monomers were copolymerised at low levels (0.01%) with styrene and maleic anhydride used in SMA synthesis [82]. It needs to be emphasised that the fluorescently labelled copolymers in the study of Neville et al. deviate from the commonly used SMA copolymers in terms of molar mass distribution as well as their monomer sequence along the polymer backbone. Despite these differences, it is very encouraging that the number of tools based on the SMALP technology is increasing. Current and emerging SMA-like polymers will provide a spectrum of useful attributes, effectively

constituting a 'toolkit' for studying GPCRs. So, the future is bright for this very active research field around copolymer encapsulation of GPCRs.

CRediT authorship contribution statement

Hoor Ayub: Writing – review & editing, Writing – original draft, Conceptualization. **Rebecca J. Murray:** Writing – review & editing, Writing – original draft, Conceptualization. **Gestél C. Kuyler:** Writing – review & editing, Writing – original draft, Conceptualization. **Farhaan Napier-Khwaja:** Writing – review & editing, Writing – original draft, Conceptualization. **Joseph Gunner:** Writing – review & editing, Writing – original draft, Conceptualization. **Tim R. Dafforn:** Writing – review & editing, Writing – original draft, Conceptualization. **Bert Klumperman:** Writing – review & editing, Writing – original draft, Conceptualization. **David R. Poyner:** Writing – review & editing, Writing – original draft, Conceptualization. **Mark Wheatley:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

The authors have no conflicting interests to declare that are relevant to the content of this article. GCK and BK are directors of Nanosene (Pty) Ltd, a company that commercializes amphiphilic copolymers for the isolation of membrane proteins.

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