Teaser PoLiPa technology (a.k.a. SMALPs) enables purification of GPCRs while they remain in their natural lipid environment, thus avoiding many of the challenges associated with current techniques and, therefore, increasing access to modern drug discovery techniques

Releasing the technical 'shackles' on GPCR drug discovery: opportunities enabled by detergent-free polymer lipid particle (PoLiPa) purification

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G-protein-coupled receptor (GPCR) drug research is presently hindered by the technical challenges associated with generating purified receptors. Consequently, the application of critical modern discovery technologies has been limited, and the vast untapped opportunity for new GPCR-directed medicines is not being realised. A simple but transformative solution is to purify receptors without removing them from their native phospholipid environment by using polymer lipid particle (PoLiPa) technology, with reagents such as styrene-maleic acid co-polymer (SMA). Compared with contemporary detergent-based and stabilising mutagenesis methods, the PoLiPa approach is simple and generic and, therefore, offers huge advantages, with the potential to revolutionise GPCR research by facilitating the availability of the purified receptors that are required for structural biology, biophysical, and panning technologies.

Introduction

GPCRs are hugely important drug targets, being involved in almost all physiological processes and implicated in all major disease areas. As a result, GPCRs are targeted by 25–30% of marketed drugs [1]. GPCRs continue to be actively pursued in drug discovery because there remains great potential to develop improved drugs for existing therapeutically validated targets, as well as against those disease-relevant receptors for which there are currently no drugs.

Despite this potential, access to the tools required to identify and develop new chemical or biological entities is often limiting progress in drug research. Drug discovery against soluble targets has been greatly accelerated by modern biophysical, structural biology, and panning technologies, which have ultimately led to better drugs for patients [2]. These tools invariably rely on the availability of high-quality purified protein. The simple fact that it is challenging to isolate and purity GPCRs from cell membranes is a bottleneck in GPCR drug discovery research that has limited the utility of these technologies in this setting. Consequently, the full potential of GPCR drugability remains to be exploited, and there exists an innovation gap in delivering purified receptor reagents for discovery programmes.

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To resolve this dichotomy, researchers have relied on detergent solubilisation of GPCRs, which has enabled some impressive advances towards this end. However, owing to the inherent instability of these membrane-bound proteins in the unnatural environment of detergent micelles, this approach requires highly specialised and expensive method development on a case-by-case basis, with no guarantee of success. As a result, progress has been too slow and limited in its ability to fully exploit the full potential of these technologies for drug discovery.

An exciting solution to these problems has been to avoid the use of detergents altogether, and to isolate and purify membrane proteins while they remain in their original phospholipid environment. This has been enabled by specialised polymers, such as SMA [3–5]. These can solubilise cellular membranes by dissecting out and enveloping small portions of the phospholipid bilayer (Fig. 1). The resulting polymer lipid particles are encapsulated by the polymer and contain membrane proteins that remain stable through interactions with native membrane. We call these materials 'PoLiPa', and, specifically, these have become known as SMALPs when they are prepared through the use of SMA. Importantly, it is becoming clear that PoLiPa offer a simple and generic approach to purify membrane proteins.

In this review, we discuss the application of the PoLiPa approach to GPCR purification and summarise current progress in exploiting this technology for GPCR drug discovery. We also highlight areas of exciting opportunity where it is anticipated that PoLiPa-GPCRs can be applied to structural, biophysical, and biologics discovery technologies. We highlight gaps in our knowledge and the limitations of the technology, with a view to aiding the development PoLiPa technology as an attractive tool for drug discovery research.

What opportunities in GPCR drug discovery can be enabled by better access to purified receptors?

Historically, screening and lead optimisation against GPCR targets has been restricted to assay readouts that use whole cells or isolated membranes. Therefore, radioligand competition binding and functional cellular readouts (such as calcium flux) have been the mainstay of GPCR drug discovery for decades. While clearly useful tools for developing new drugs, these empirical techniques have their limitations, most notably that they give insufficiently precise information on the ligand–protein interaction to drive rational drug design toward highly specific agents or allosteric modulators. As well as being largely represented by small-molecule orthosteric ligands, the GPCR drug space is focussed on a relatively small number of well-established target subtypes, which, in many cases, are each addressed by numerous different drugs [6].

Emerging trends in GPCR drug discovery suggest that there are growing opportunities for success by moving away from applying conventional strategies to well-established targets. This is evidenced by a gradual increase in the numbers of approvals and/or clinical trial progressions of allosteric modulators, biologics, and molecules targeting previously undrugged targets or even orphan receptors [6]. Modern target identification strategies, such as genome-wide association studies (GWAS) [7] and CRISPR-Cas9 [8] screening, are driving an emerging 'target first, pharmacology second' strategy. Cellular mRNA expression profiling through 'GPCRomics' is also identifying previously unimagined roles for GPCR targets [9]. Consequently, new and unexplored drug targets will increasingly be brought to the fore to expand the potential druggable GPCRome. Moreover, advances in our understanding of GPCR molecular pharmacology are beginning to enable drug discoverers to exploit the potential of optimising ligand bias [10], binding kinetics [11,12], and receptor allostery [13], and to explore alternative chemical space [14] to develop better therapeutics with improved on-target efficacy and reduced adverse effect liability. Therefore, there is much scope to develop new therapeutics against novel and existing GPCR targets.

The current global market value for GPCR medicines is US$2.6 billion, which will increase markedly if industry can successfully apply innovative solutions to exploit these novel areas of research. To capitalise on these opportunities, a comprehensive suite of orthogonal discovery technologies are required. Table 1 summarises several of these crucial drug discovery techniques. For example, to exploit unexplored pharmacological and chemical space, and to address previously intractable targets, an essential development in GPCR hit identification will be the enablement of biophysical binding assays, in the same way as these have become routine for many soluble proteins. Advances in GPCR structural biology will also improve our ability to characterise hit matter and enable the rational design of new molecules using computer-aided methodologies. Used together, structural and biophysical approaches can bolster our mechanistic understanding of receptor function and consequently strengthen efforts to rationally develop drugs with improved activity and selectivity profiles. All of this is beginning to become possible within a small number of drug discovery centres and for a limited subset of GPCR targets where purified protein is accessible. We anticipate that the development of fast and generic methods for GPCR purification, potentially enabled by the PoLiPa approach, will dramatically increase access to the protein reagents that are necessary to kick-start a revolution in GPCR research. Consequently, this may be the key to unlocking the latent potential of GPCR drug discovery, and deliver crucial new drugs in the future.

Limitations with existing detergent-based purification approaches

There has been considerable success in using detergents to purify receptor targets for the application of these technologies, and this has driven a renaissance in GPCR drug discovery over the past decade. However, a key problem is the inherent instability of GPCRs in detergent micelles, which are only poor mimics of the native phospholipid environment. Highly specialised, time-consuming and costly method development has been required to overcome this challenge. For example, thermostabilising mutagenesis [15,16], protein fusion tagging [17], detergent screening and novel detergent development [18–20], stabilising nanobodies [21], and protein-stabilised nanodiscs [22] have been applied to ameliorate the unfolding of GPCRs that occurs upon removal from their native phospholipid environment.

Importantly, however, it is still not a trivial task to generate purified GPCR protein reagents. Methods developed for one receptor might not be readily extrapolated even to closely related subtypes, meaning that the timelines, costs, and risks must be rebased for each new GPCR drug discovery programme. Even techniques that ultimately present the receptor in a detergent-free

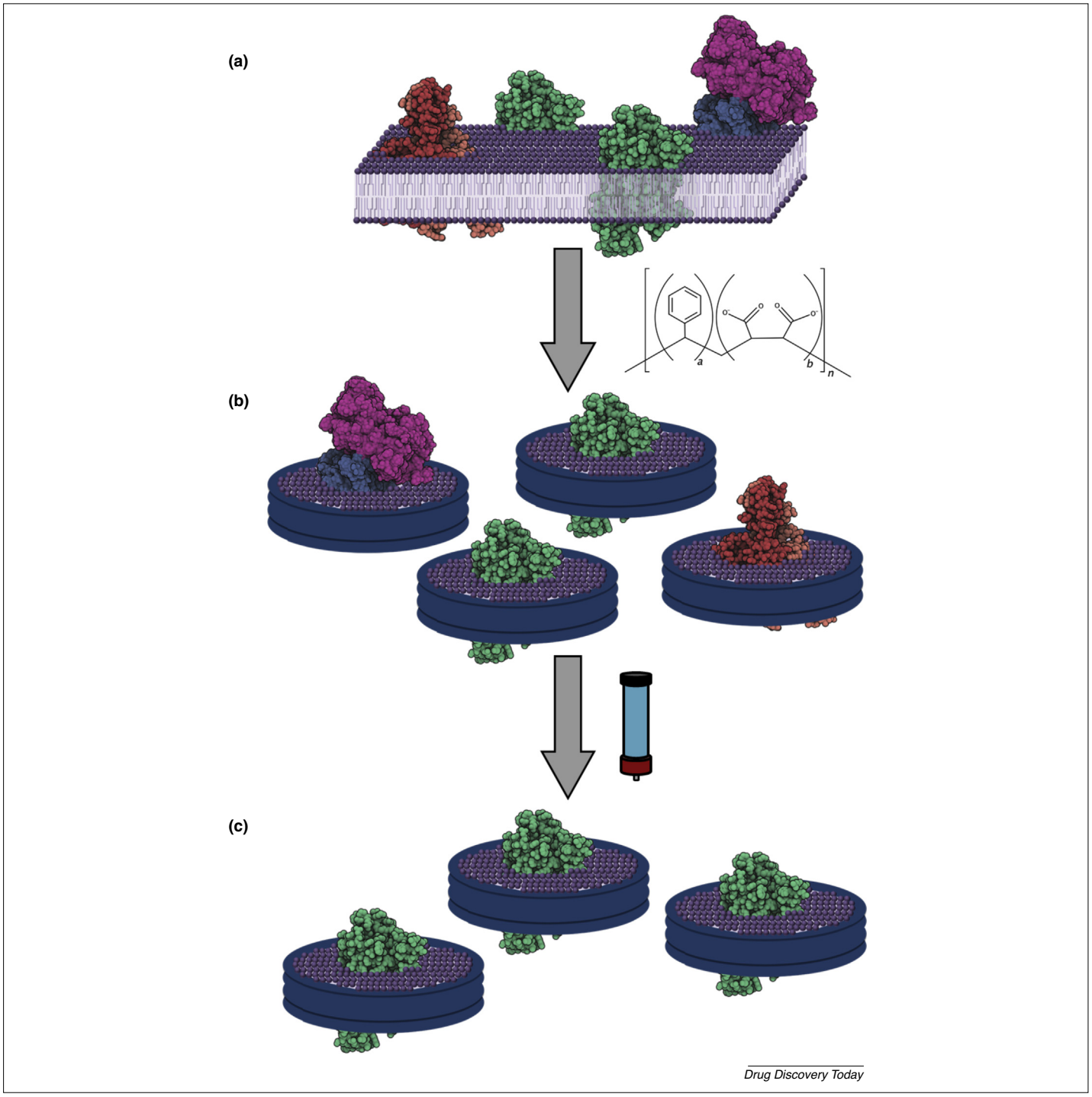

FIGURE 1

Schematic summary of polymer lipid particle (PoLiPa)-mediated membrane protein purification. **(a)** The G-protein-coupled receptor (GPCR) of interest (in green) is expressed in a cellular system and presented either as whole cells or isolated membranes (phospholipid bilayer is represented in purple). **(b)** Expressing membranes are exposed to polymer (e.g., styrene-maleic acid co-polymer; SMA). The polymer inserts into the cell membrane and solubilises it by creating particles of lipid bilayer-containing membrane proteins (PoLiPa). The polymer remains part of this complex, forming a belt surrounding the lipid/protein (in blue). PoLiPa are formed of the entire membrane proteome. **(c)** The protein of interest can be isolated from the PoLiPa cell lysate using conventional protein purification techniques. The protein remains embedded in its original native membrane throughout the purification process and downstream applications. Membrane protein structures used are as follows (Protein Data Bank codes): green, 4BVN; red and orange, 3UM7; and blue and pink, 2LEG.

environment (e.g., nanodiscs) require a preceding detergent-solubilisation step and, therefore, are prone to the same stability challenges. Consequently, the preparative techniques are only available for a handful of well-investigated, well-behaved targets. Given that GPCRs targets that are presently regarded as obscure and/or challenging assume a more prominent place in drug discovery, these shortcomings are going to be ever more keenly felt. For these reasons, there is great demand for novel approaches that offer generic tools for the rapid and straightforward generation of purified GPCRs.

TABLE 1

A brief description of important drug discovery technologies that are enabled by access to high-quality purified target protein reagents

Technique	Description	Application
SPR	Molecular interactions from mobile phase with a protein immobilised on thin gold film result changes in resonance angle. Changes are dependent on changes in molecular weight	SBDD; FBDD; biologics, DEL, and HTS confirmation; K_d; on/off rates
MST	Molecular interactions modulate changes in fluorescence of soluble labelled target protein in heat gradient. Changes are result of hydration, molecular weight, and charge	SBDD; FBDD; biologics, DEL, and HTS confirmation; K_d estimation
Native MS	Electrospray ionization MS can detect changes in quaternary state of unlabelled protein in gas phase	Label-free binding, post-translational modifications
Cryo-EM	Medium-to-high resolution structure determination of biomolecules. Electron beam passes through frozen samples and interacts with molecules, which project an image onto detector	SBDD; FBDD; atomic interactions, conformational analysis
X-ray crystallography	Diffraction of X-rays by regularly spaced atoms of crystalline material	SBDD; FBDD; atomic interactions
NMR	Protein structure and conformation dynamics interpreted from the the magnetic properties of atomic nuclei	SBDD; FBDD; molecular interactions K_d; on/off rates

Overview of PoLiPa technology

The beauty of the PoLiPa technique arises from its simplicity and generic applicability. In essence, it offers a way to cut the desired protein (in this case a GPCR) out of the plasma membrane while it is still associated with a portion of its native phospholipids and, therefore, it completely avoids any need for detergents. PoLiPa are formed through the spontaneous solubilisation of biological membranes by the polymer, and PoLiPa containing the membrane protein of interest can be isolated by conventional purification techniques. Given that the protein remains in its original membrane environment, it is highly stable, and no additives, manipulations, or mutations are required to ensure that native folding and function remain intact after isolation. Therefore, with minimal optimisation, the original protocol published for this technique [23] can theoretically be applied to any membrane protein (or even membrane protein complex) in any cellular expression background.

At present, the most well-characterised polymer used for membrane protein solubilisation is SMA, which is formed from alternating hydrophobic (styrene) and hydrophilic acid (maleic acid) monomers. In the proposed model for the membrane-solubilising activity of SMA [24,25], the polymer makes strong interactions with the lipid membrane and inserts itself deep into the hydrophobic core of the bilayer. This initially destabilises the membrane, leading to its disruption. Then, the system is thermodynamically driven toward the formation of discs of phospholipid bilayer encapsulated by a belt of stabilising polymer [26]. The amphipathic nature of the polymer means that the hydrophobic phenyl moieties can interact with the alkyl tails of the phospholipids, while the hydrophilic carboxylic acid moieties surround the PoLiPa with a protective cage, interacting with the aqueous phase and rendering the whole complex water soluble.

Advantages of PoLiPa-purified GPCRs

Importantly, PoLiPa enable the solubilisation of GPCRs without the requirement for stabilising mutations or detergent optimisation, both of which would otherwise contribute significantly to the time taken to successfully generate folded protein of sufficient quality and quantity. It is estimated that, in experienced hands, a thermostabilising mutagenesis campaign will take up to a year [27], while each receptor subtype also requires time-consuming *de novo* detergent screening to select the most suitable detergent and its concentration. Given that the PoLiPa can be viewed as a more generic approach, an existing expression system could be rapidly and easily adapted for purification.

Detergents and mutations can also perturb the native pharmacology and function of the GPCR [15,28]. Therefore, by using the PoLiPa technique, a more physiologically relevant form of the receptor can be presented to drug discovery assays. Furthermore, detergent-derived reagents require the continued presence of the detergent in assay buffers to maintain the integrity of receptor-containing micelles. This can lead to issues with buffer compatibility with some bioanalytical techniques. Crucially, the PoLiPa system is completely detergent-free. Not only is the GPCR presented to the assay without detergent, but also at no stage in its preparation is any detergent required.

Another key advantage offered by PoLiPa is the permanent presence of the native membrane, which is often crucial for GPCR stability. As such, PoLiPa-GPCRs demonstrate impressive longevity in storage and resistance to freeze–thaw cycles [29], making them convenient and practical research tools. Moreover, the constitution of the cellular phospholipid environment surrounding the protein is fully represented in the PoLiPa, an observation that has been used as a diagnostic tool to investigate the preferred membrane composition of a given protein [30]. Given that lipids have been shown to allosterically modulate GPCR activity [22,31] it is likely that crucial pharmacology is maintained in the PoLiPa system, making it a more physiologically relevant approach for biological assays. Indeed, a recent publication [28] compared GPCR purification systems with and without the continued presence of phospholipids (detergent micelles and high-density lipoproteins) and found a clear impact on receptor pharmacology. Additionally, some GPCR structures have revealed ligand-binding pockets at the interface between the receptor protein and the

TABLE 2

Reported examples of important drug discovery techniques applied to PoLiPa-GPCRs and non-GPCR targets

Technique	PoLiPa-GPCRs	Other membrane protein PoLiPa preparations	Refs
Radioligand binding	Adenosine A2A; vasopressin V1a; dopamine D1	Multidrug resistance-associated protein 1; equilibrative nucleoside transporter-1	[29,34,35,94]
Ligand-observed mass spectrometry	Neurotensin NTR1[b]; β2-adrenergic receptor[b]	NP[a]	
GTPγS	Ghrelin receptor, melatonin MTR1, dopamine D2	NP	[36,46]
FRET/BRET	Ghrelin receptor, dopamine D2	SecYEG proton-conducting channel	[36,46,96]
Fluorescence spectroscopy	Adenosine A2A		[51]
SPR	Cannabinoid CB1	NP	[37]
MST	Dopamine D1	NP	[35]
Cryo-EM	NP	AcrB efflux pump; alternative complex III; P-glycoprotein; acid-sensing ion channel; KimA proton-coupled potassium pump	[54,55,92,93,97]
X-ray crystallography	NP	HwBR bacteriorhodopsin	[53]
NMR	NP	CzcD diffusion facilitator protein; cytochrome b5	[62,63]
Native MS	NP	GlpG rhomboid protease, KtrB potassium channel, AcrB efflux pump, ABCG2 ATP-binding cassette	[66,98]

[a] NP, no published examples.
[b] In-house data presented in this review.

membrane [32]; maintaining these features in the PoLiPa might be crucial for observing the same pharmacology as the cellular system. In short, the correct receptor membrane environment makes PoLiPa-GPCRs tractable yet highly physiologically relevant.

Importantly, PoLiPa integrate well with common practical techniques for protein expression, meaning that no specialist technology is required. Mammalian, insect, yeast, and bacterial expression of membrane proteins have all yielded correctly folded PoLiPa purified receptors [33]. Furthermore, standard purification techniques, such as NiNTA affinity chromatography, streptavidin-based affinity chromatography, immunoaffinity chromatography (such FLAG and HA), and size-exclusion chromatography, are compatible with PoLiPa with only minor modifications (reviewed in [33]). However, it is likely that further refinement of purification strategies will be needed, involving construct design and protocol optimisation.

PoLiPa-GPCRs have been demonstrated to be correctly folded and pharmacologically intact. As summarised in Table 2, a range of orthogonal techniques against multiple receptor subtypes have shown that PoLiPa-GPCRs can bind known ligands, activate G proteins, and exhibit correct folding. Therefore, it appears that the PoLiPa do not have a detrimental influence on receptor conformation (although this is discussed in further detail later).

The properties of PoLiPa preparations also make them, in principle, potentially highly amenable to many relevant drug discovery technologies, although a thorough examination of this compatibility remains to be fully assessed (as discussed later). High levels of purity are achievable, as demonstrated across several GPCR targets to date [29,33–37]. PoLiPa are typically of an suitable size for biophysical assays, being approximately 10 nm in diameter (equivalent to a DDM detergent micelle) [38]. Importantly, they also show a tight monodisperse size distribution [38], which can be crucial for several bioanalytical and assay techniques. The yield of protein purification for SMA is also highly comparable to DDM, where the percentage solubilised for both across three different membrane proteins (albeit non-GPCR) were similar between the two purification strategies [38].

Drug discovery techniques with demonstrated application to PoLiPa-GPCRs

Radioligand binding

When known ligands are labelled with a radioisotope, their binding to the target protein can be easily monitored by counting radioactivity after physical separation of bound from unbound ligand. Traditionally, isolated membranes from overexpressing cell lines have been used for GPCR radioligand binding assays, and separation of bound from unbound ligand has been achieved by filtration through glass fibre. For soluble PoLiPa-GPCRs, this method has been adapted by use of desalting filtration columns, from which ligand–protein complexes are specifically eluted [29]

Given that this technique relies on the existence of labelled ligands, it can be readily applied to PoLiPa receptors, although these might not offer any significant advantage over membrane preparations in this setting. However, this technique can be useful to demonstrate that folded and intact GPCRs are produced by PoLiPa technology.

Ligand-observed LC-MS binding

An adaptation of the radioligand-binding approach is to use tandem liquid chromatography mass spectrometry (LC-MS) to quantify the concentration of unlabelled ligands bound to the GPCR, typically after chemical denaturation of the GPCR–ligand complex. This has been previously demonstrated with GPCRs presented in isolated membranes [39,40], as well as detergent-purified receptors [41]. Furthermore, as shown in Fig. 2, in-house data demonstrated that PoLiPa-GPCRs are also highly amenable to this technique in a 96-well format. Saturation binding analyses of small-molecule antagonists to the β2-adrenergic receptor and the neurotensin 1 receptor demonstrated high-affinity binding with low nonspecific binding levels.

Therefore, ligand-observed LC-MS can take the place of radioligand binding for standard pharmacological analysis and competition-mode screening. However, this technique offers significant advantages. Most obviously, being label-free, it allows a wider choice of tracer ligands (limited only by their amenability to

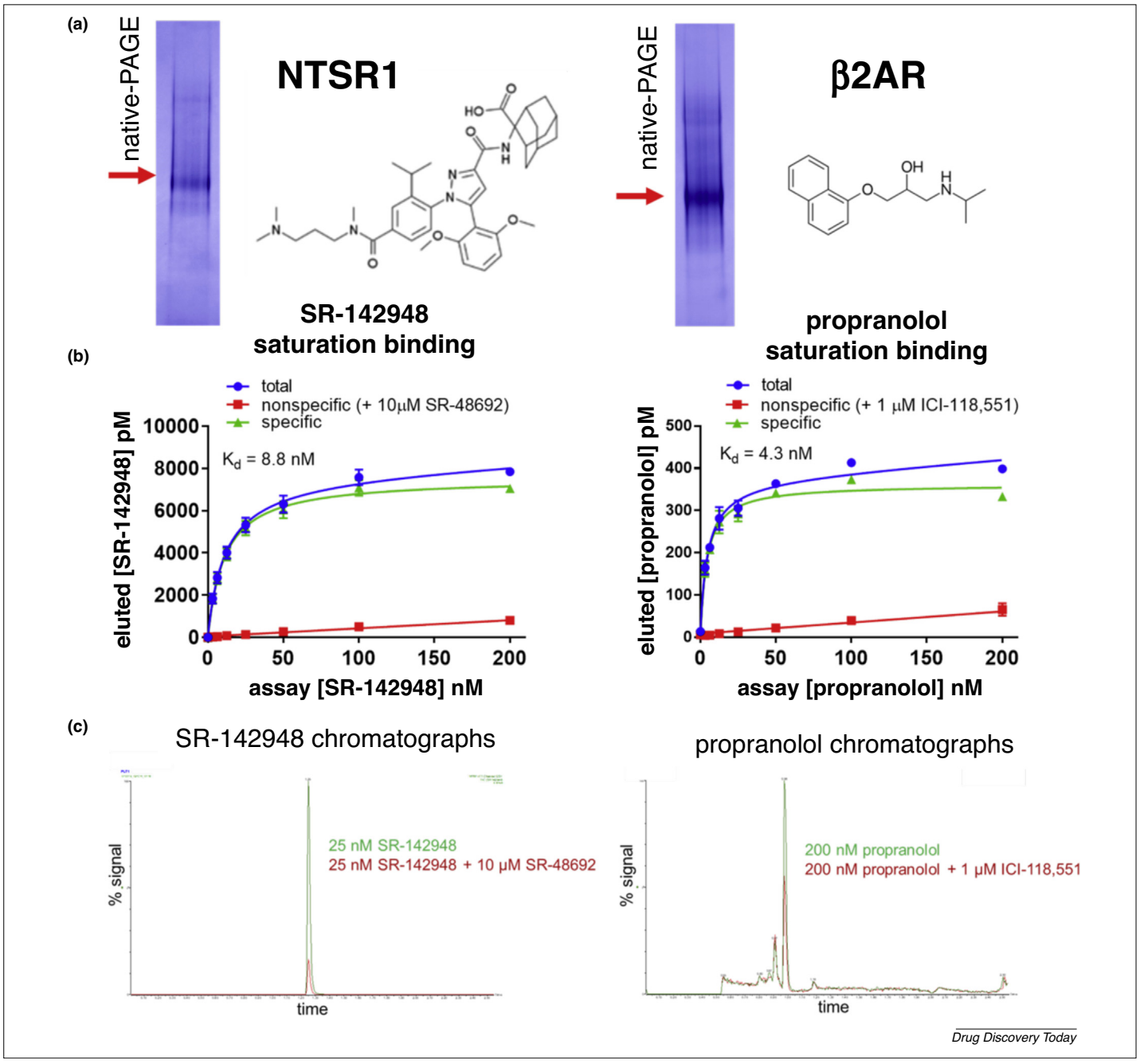

FIGURE 2

Ligand-observed liquid chromatography–mass spectrometry (LC-MS) binding assay for polymer lipid particle (PoLiPa)-NTRS1 (left) and PoLiPa-β2AR (right). **(a)** Native-PAGE gels showing the respective PoLiPa-G-protein-coupled receptor (GPCR) (red arrows), and the small-molecule antagonists used as tracer molecules by LC-MS detection. **(b)** Saturation binding analysis of the tracer antagonists against the respective PoLiPa-GPCR. Protein and a concentration range of the ligand (SR-142948 for NTSR1 or propranolol for β2AR) were incubated for 60 min at room temperature before separation of bound/unbound ligand. The protein was then denatured to release bound ligand, and the concentration of bound ligand was measured by LC-MS (interpolation from a standard curve). No-specific binding was estimated by displacement with a structurally distinct small-molecule compound (SR-48692 for NTSR1 or ICI-118,551 for β2AR). Affinity Kd values were calculated from specific binding curves and showed expected pharmacological profiles. **(c)** LC-MS traces for the respective tracer molecules in the total or nonspecific binding experiments.

LC-MS detection). Not only does this enable study of more obscure targets for which radiolabelled orthosteric ligands are not available, but also allows screening for allosteric binding sites on GPCRs, where there is often a similar lack of assay reagents. Moreover, the increased flexibility in choice of ligand can help overcome issues with nonspecific binding of the available labelled ligands, which can limit assay development. Finally, avoiding the cost and safety implications associated with radioisotope usage is another a major benefit of the LC-MS approach.

Another key advantage of this technique is the ability to perform direct-binding experiments, where the test ligand is detected rather than observing its competition with a fixed tracer ligand. This method has shown success in small-molecule [42], fragment [43], and natural product [44] screening, wherein the

association with the target protein of individual components of screening libraries is measured by LC-MS, and compared with a negative control. The throughput of these methods can be vastly increased by using the power of MS to detect individual compounds from mixtures in a single experiment; for example, validated hit identification in a cocktail of 20 000 compounds was successful against the adenosine A2A receptor [45]. The direct-binding approach is also attractive in that it does not discriminate in favour of any particular binding site (as would otherwise be dictated by the tracer). This can offer exciting opportunities to identify novel allosteric drugs, and gain deeper understanding of novel allosteric sites. In the field of orphan GPCR discovery, this approach could also be instrumental in identifying novel binders and accelerating the development of new drugs against these targets, because there is no pr-requisite for characterised known binders to be available.

PoLiPa-GPCRs can enhance the power of technology by allowing easy access to high-purity receptor reagents presented in physiologically -relevant states. Therefore, nonspecific and/or nonrelevant binding events that would otherwise be difficult to eliminate can be greatly reduced, thereby increasing the precision of direct binding screening.

G-protein activation assays

GTPγS assays are a key tool in GPCR drug discovery, providing a readout for G-protein coupling and, hence, receptor activation. A functional readout is important because it facilitates the classification of agonist and antagonist compounds. Typically, accumulation of the nonhydrolysable GTP analogue GTPγS labelled with [^{35}S] in isolated membranes is used to quantify GDP/GTP exchange on Gα subunits as a result of agonist-mediated activation of the receptor. More recently the fluorescent GTPγS assay [36] offers an attractive alternative to the [^{35}S]GTPγS radioligand approach because it is a homogenous no-wash procedure and, therefore, simpler and more amenable to automation. This also enables kinetic analyses of receptor activation, which could be key in compound profiling cascades. Finally, the cost and safety benefits of avoiding radioactivity are also obvious. Easier access to large amounts of purified GPCR by the PoLiPa route has the potential to greatly increase the impact of this assay.

Logez *et al*. [36] demonstrated using a fluorescent GTPγS assay that PoLiPa purified ghrelin and melatonin-1 receptors showed functional activity. This work is important because it validates the intact G-protein coupling and agonist pharmacology of PoLiPa-GPCRs. Similarly, PoLiPa-dopamine D2 receptor and D2/ghrelin receptor heteromers have been studied using this system, allowing discrimination between the kinetics of G-protein activation between the homomeric and heteromeric GPCR [46]. These authors also used Förster resonance energy transfer (FRET) biosensors for G-protein heterotrimeric complex formation to uncover activation and distinct conformational states of the Gα subunit to be studied.

SPR

Surface plasmon resonance (SPR) is a direct-binding biophysical approach that detects the change in mass upon ligand binding to the target protein, which is immobilised on specialised chips. It has begun to have utility in fragment screening against detergent purified GPCRs [47,48], although the technique is currently limited by access to purified proteins. SPR is extremely valuable in characterising compound binding kinetics (k_{on}/k_{off}), and can also detect allosteric ligands.

Although there are no published examples of SPR screening against PoLiPa-GPCRs, it has been demonstrated that, in principle, the techniques are compatible. By flowing PoLiPa-CB1 receptors over an immobilised antibody to the GPCR, a specific binding signal was detected [37]. However, further work is required to optimise PoLiPa for SPR, because the same authors found that attempting the reversed format by immobilisation of the PoLiPa-GPCR did not generate a specific binding signal [37], and this is the format that would be more useful for compound screening. It is likely that the high negative charge of the SMA polymer used in this case prevented adequate immobilisation of the PoLiPa to the SPR chip. We anticipate that future polymer development will identify polymers with better assay compatibility (as discussed later).

MST

Nanotemper AG manufactures 'MST' instruments that measure the thermofluorescent changes that occur when fluorescently labelled target proteins are placed in a thermal gradient. These changes occur through two phenomena called thermophoresis (the movement of molecules in thermal gradients) and temperature-related intensity change (TRIC; where compound binding alters the local environment of the fluorophore and hence its response to heat). Compound binding will change these properties and, therefore, alter the fluorescence in a quantifiable way. MST is a homogenous direct-binding approach highly amenable to high-concentration fragment screening and affinity determination, but, unlike SPR, it is solution based and does not require immobilisation to a chip. MST has recently been applied to fragment hit identification against a kinase target [49], and is routinely used by Domainex for fragment-based drug discovery (FBDD) programmes against soluble proteins.

Although there are no examples of MST GPCR screening in the public domain, we envisage that this technique will become a key tool in GPCR hit identification, because PoLiPa increase the availability of the appropriate protein reagents. It has already proven utility in characterising ligand binding [20,50], as well applications in mechanistically characterising G-protein coupling [22], against detergent purified GPCRs. PoLiPa-GPCRs could enable this technique to be more readily applied for GPCR drug discovery, because a combination of the two offers a rapid and straightforward path for FBDD. There is early promise because a recent study demonstrated the compatibility of PoLiPa-GPCRs with MST to determine ligand binding [35]. However, these investigators tracked a fluorescently labelled ligand rather than the target protein, suggesting that some further method development is required to establish direct-binding screening assays with labelled GPCRs. Perhaps a key challenge will be optimising the assay conditions to enable successful screening and, in particular, understanding the effect of polymer electrostatic charge on the MST readout.

FRET and BRET

FRET exploits the transfer of energy that occurs when two fluorophores are in close proximity, whereas bioluminescence resonance

energy transfer (BRET) capitalises on the energy transfer between a luminophore and a fluorophore. This has numerous applications in drug discovery depending on the configuration of the labelled molecules, such as sensors for intracellular signalling molecules (e.g., cAMP), ligand-binding assays, protein–protein interactions, and protein conformational dynamics.

PoLiPa were used to purify heteromers of dopamine D2-ghrelin receptors, and FRET was investigated using fluorescent tags on each receptor subtype to suggest a tetrameric complex comprising two of each of the receptors [46]. Intramolecular FRET (two fluorophores strategically placed within the same protein molecule) enables measurement of protein conformational dynamics. This has also been demonstrated with PoLiPa-purified ghrelin receptor, where ligand-mediated conformational changes corresponded to their pharmacology [36]. In these cases, the PoLiPa approach offered an easily manipulated system to study the complex subtleties of receptor function and pharmacology using FRET, which might otherwise have been far more challenging. It is likely that PoLiPa will accelerate these discoveries by offering a quick and straightforward source of reagents.

Fluorescence spectroscopy

Protein conformational dynamics can also be reported by measuring changes in fluorescence emission spectra of fluorescent residues because these properties change with the altered environment that accompanies conformational movement. Not only can this demonstrate ligand binding, but it can also distinguish between compound pharmacological profiles (because these are consequences of different ligand-mediated conformational changes). The PoLiPa-adenosine A2A receptor was site-specifically labelled with a fluorophore and produced an increased emission in the presence of an inverse agonist, whereas an agonist caused smaller increases [51]. The same authors also used label-free intrinsic tryptophan fluorescence spectroscopy to demonstrate ligand-specific effects on conformational change. There are several reports using this approach that have broadened our understanding of molecular pharmacology (e.g., efficacy and ligand bias) via ligand-specific conformational dynamics with detergent-purified GPCRs (as reviewed in ref [52]).

Drug discovery techniques with potential future applications to PoLiPa-GPCRs

X-ray crystallography

The PoLiPa method has been used for the X-ray crystal structure determination of bacterial rhodopsin (to 2 Å resolution) [53]. This study demonstrated the successful application of lipid cubic phase technology to the PoLiPa-purified protein and, therefore, indicates that it is possible to solve membrane protein crystal structures without having to remove the protein from its lipid environment. There are, at present, no examples of crystal structures for GPCR targets in the public domain. This might reflect a lag in the adoption of this technology and also the potential requirement for method developments to adapt the PoLiPa system to crystallography protocols.

Should further success be forthcoming in this field, then there is great potential to use PoLiPa for expanding GPCR crystallography. A key advantage of the PoLiPa system is that the protein can be presented in its endogenous conformation ensemble(s), whereas stabilisation, mutation, and fusion approaches tend to induce selective conformations that might be preferable for purification, but are not necessarily physiologically relevant. It is also probable that there are differences in receptor conformational dynamics in a detergent micelle compared with the natural membrane, and this could adversely impact the derived structure and consequent drug discovery efforts. We anticipate that GPCR crystal structures will begin to emerge via the PoLiPa route, and that this technology can be transformative in terms of opening rapid and straightforward access to new targets. There appears to be a correlation between access to a solved structure of a target GPCR and the subsequent success of progressing new therapeutics against this target into clinical trials [6], which highlights the importance of this technology in driving the development of new medicines. In short, this is expected to invigorate an already exciting GPCR structural revolution.

Cryo-EM

Using PoLiPa purified samples, cryo-electron microscopy (EM) structural determination (<4 Å resolution) has been demonstrated for a bacterial efflux pump [54], as well as an entire electron transfer chain supercomplex [55]. Cryo-EM has undergone an exciting revolution in recent years, where developments in technology have increased protein structure resolution to levels comparable with crystallography, without the need to form protein crystals.

However, cryo-EM is most effective against high-molecular-weight species (>100 kDa), and has proven challenging for GPCR targets because of their relatively small size and the detection limits for high-resolution structure determination. One way around this has been to image receptors in complexes with G protein or antibodies, which has helped to elucidate crucial information regarding receptor activation mechanisms [56]. However, PoLiPa offer a more straightforward approach to study monomeric receptors because of the size advantage in applying the technique to the whole particle (protein, lipid, and polymer). Interestingly, it has been demonstrated that intact PoLiPa protein can be extracted from electrophoresis gels and visualised using EM [57], which has exciting implications for studying proteins in their native state. There is great potential for PoLiPa to enable an explosion in GPCR structural biology, affording access to structure-based drug discovery (SBDD) throughout the GPCR drug discovery community.

NMR

Whereas the structural details elucidated using either X-ray crystallography or cryo-EM continue to prove highly valuable for GPCR drug discovery, these techniques only offer static insights into this highly dynamic family of proteins. Nuclear magnetic resonance (NMR) offers structural information on the dynamics of receptors as they adopt a variety of conformations depending on specific binding partners. Indeed, studies using NMR have greatly contributed to the understanding of the GPCR signalling in recent years, providing insights into the structural basis of partial agonism [58], biased agonism [59], and allosteric coupling [60]. The application of these insights is key to elucidating the drug mechanism of action and could assist with rational drug design.

NMR can also monitor weak binding interactions, which makes it a valuable tool in FBDD. Indeed saturation-transfer difference

NMR has been successfully used for fragment screening against a detergent-purified adenosine A2A receptor [61].

The use of PoLiPa in conjunction with solution-state NMR is unlikely because their relatively large particle size, which leads to slow protein tumbling and consequently broad linewidths. However solid-state NMR does not suffer from any drop in data quality as the particle size increases, and there is a growing body of publications using solid-state NMR to study membrane proteins purified with the PoLiPa system [62,63].

Native MS

Native MS describes the electrospray into the gas phase of target proteins in a near-native state, which enables the characterisation of the protein species present in the original solution. Compound binding can be identified as the introduction of additional species into this population, which has proven application in high-concentration fragment screening [64]. Presently, there are no examples in the public domain of using native MS for screening against GPCR targets. However, native MS does have proven utility for detecting ligand binding to a detergent-purified purinogenic GPCR [65]. Interestingly, the PoLiPa method has been successfully used to present three diverse non-GPCR targets for native MS studies [66]. Therefore, there is potential to exploit the future availability of purified GPCRs obtained by the PoLiPa technique for developing native MS screening assays.

Additionally, native MS has been applied to investigate the oligomerisation status of membrane proteins purified with PoLiPa [66]. This could be advantageous for the functional characterisation of GPCR dimerisation, which can be a key aspect of drug action.

The potential application of PoLiPa-GPCRs in hit identification strategies

The lack of functional diversity in GPCR drugs is ultimately a consequence of the lack of chemical diversity of the hits that have been generated through traditional screening techniques. To address this, it is essential to refine the full gamut of modern hit identification strategies to be compatible with GPCRs. The earlier sections discussed specific technologies that are enabled by access to high-quality purified target protein using techniques such as PoLiPa. It is equally important to consider how these technologies can be fully integrated into the drug discovery process, and specifically how improving access to GPCR targets using PoLiPa has the potential to greatly facilitate crucial hit identification strategies.

Structure-based drug discovery

Atomic-level structural determination of target–compound complexes by X-ray crystallography or cryo-EM allows researchers to visualise ligand-binding sites and, therefore, enables the rational design of new compounds. SBDD is extremely powerful in enabling lead compound optimisation for on-target pharmacology, while simultaneously balancing selectivity and ADME considerations.

SBDD can also be coupled to FBDD and virtual screening, which have proven to be fruitful and cost-effective approaches to hit identification. These can also help to identify a multitude of allosteric binding sites or 'hot-spots' across the entire receptor and have greatly expanded our repertoire of conceivably druggable GPCR binding sites. Additionally, better understanding the link between receptor conformational dynamics and functionality could become an incredibly valuable tool for drug discovery by further specialising SBDD. For example, comparing the crystal structures of GPCRs bound to biased and nonbiased ligands has gained insight into the structural mechanisms that govern ligand bias [67,68], while cryo-EM structures of GPCR-G protein complexes have been instrumental in increasing our understanding of receptor activation mechanisms [56]. The potential to exploit these avenues will only be fully realised by quick and easy access to GPCR–ligand structures.

By exploiting the expanding repertoire of crystal structures, virtual screening against GPCR targets has shown significant progress in recent years. The PoLiPa method has the potential to enable the determination of structures for more obscure and challenging GPCR targets, and would broaden access to such structures to a larger portion of the drug discovery community: this can only make structural biology more central to GPCR drug discovery. The first nonrhodopsin GPCR (β2AR) crystal structures published in 2007 were rapidly utilised for virtual screening. New high-affinity β-blockers with diverse chemotypes (some with nM affinity) were promptly discovered [69], whereas decades of previous research had largely limited discovery of β-AR ligands to the variations on the aryloxypropanolamine scaffold. Further advances have facilitated the discovery of biased ligands against the μ-opioid receptor through exploring novel chemotypes with putative novel biological activity [70]. More recently, exploration of chemical space using virtual screening has seen a dramatic advancement, with screens against 170 million compounds with >10 million new scaffolds, as has been successfully applied to the discovery of a pM binder for the dopamine D4 receptor [71].

Fragment-based drug discovery

Fragment screening is now a well-established technique and the success of the approach was validated by the US Food and Drug Administration (FDA) approval of vemurafenib in 2011, the first drug arising from FBDD to gain regulatory approval. Subsequently, venetoclax has also been approved and many other drugs designed by FBDD have reached clinical trials.

FBDD essentially involves screening libraries of low-molecular-weight compounds (<250 Da) with the expectation of identifying hits that, although of low affinity, are highly ligand efficient (binding affinity normalised to the heavy atom count of the compound). The advantages of fragment screening include: (i) fragments are smaller than standard screening compounds and, therefore, are more likely to bind to the target, which leads to significantly higher hit rates (up to 5–7%) compared with high-throughput screening (HTS); (ii) fragment libraries offer more comprehensive coverage of chemical space, meaning that much smaller libraries (typically ~1000) can be screened with obvious cost and time implications; (iii) fragments offer greater scope for enhancing potency within the desired physiochemical property range, which is often not possible for HTS hits.

Fragment screening against GPCR targets has proven challenging because of the limitations of high-concentration screening (typically mM) using existing techniques. Some success has been demonstrated using traditional cellular calcium flux or radioligand

competition binding assays for GPCR fragment screening [72–74], but these are not routine and likely are not sensitive to the identification of lower affinity molecules, which may still be highly relevant leads. Furthermore, they are biased towards the identification of orthosteric binders. Biophysical binding techniques (e.g., SPR, MST, and NMR) are more suitable as high-concentration screening assays. These can monitor the direct effects of test compound on the target protein, avoiding the necessity for competing tracer ligands and, consequently, enable detection and quantitation of allosteric interactions. In-roads have been made into applying SPR and NMR to GPCR FBDD using detergent purified receptors [48,61,75,76]. However, these are obviously limited to receptors with established methods for reagent generation, and there is a barrier to applying these techniques to more challenging and obscure targets.

SBDD is an underpinning technology for FBDD. Even if suitable fragment screening assays can be established without purified protein, these reagents are still required for structural biology. This is another important reason for the slow progress in GPCR FBDD, because structural information is only available for a small subset of important GPCR drug targets. The identification of fragment binding sites on the target protein through structural analysis is central to development of high-affinity lead-like compounds from the fragment hit set. Expanding or linking fragments to grow into binding pockets is a key strategy here and requires visualisation of the fragment-binding mode [77]. Therefore, GPCR FBDD can only develop to its full potential with an increase in accessibility and availability of GPCR structural biology, as discussed earlier.

Antibody panning

Although the number of FDA-approved biologics is increasing, antibodies against GPCRs are under-represented compared with the market for small molecules addressing GPCR. This is largely because of the lack of available suitable GPCR epitopes, and is despite the enormous potential for targeting GPCRs with therapeutic antibodies [78].

Technical innovations in the field of GPCR purification are beginning to bear fruit, with discovery pipelines represented by several GPCR antibody targets [78]. PoLiPa might be ideally suited to capitalise on, and invigorate, this wave of antibody discovery because GPCR epitopes are presented in a structurally relevant form when in a pure isolated preparation that enables the specificity required for panning technologies. GPCRs are highly dynamic and allosterically regulated by the membrane environment and by post-translational modifications. Relevant forms of the receptor in a native human cell membrane environment can be presented using PoLiPa, which offers great promise for the identification of more physiologically relevant biologics with improved clinical efficacy. A recent publication demonstrated a proof of principle that PoLiPa-GPCR can be used as an epitope for flow-cytometry based display techniques [37].

DNA-encoded library screening

DNA-encoded library (DEL) screening involves generating large and diverse libraries of compounds, each of which is labelled with a unique DNA tag, and screening these by panning pools of up to 10^7 compounds against the target protein. Two key requirements for successful DEL screening are high concentration and high purity of the target protein. Therefore, traditional methods for presenting receptor targets (i.e., whole cells or in isolated membranes) make this technique challenging. However, DEL screening against the neurokinin-3 receptor in a whole-cell assay did have some success in identifying novel antagonists [79], although it is clear that this approach is not generic across all test systems.

DEL screening against detergent-purified β2-adrenergic receptor [80] and protease-activated receptor 2 [81] was reported to identify novel small-molecule hit matter with novel allosteric pharmacologies. Should the PoLiPa system prove amenable to this screening technology, then it is likely that this will enable a great acceleration in DEL screening against GPCR targets

mRNA display

mRNA display is a panning technique used for peptide evolution to create molecules that can bind epitopes of the targeted protein [82]. mRNA technology creates a library of peptides with up to 10^{13} independent sequences, which can be screened using panning approaches. This approach has demonstrated the ability to select and evolve peptides with affinities in the nanomolar range that can modulate activities such as protein–protein interactions (PPI) and post-translational modifications. The technology works by building mRNA molecules that contain a 3′ puromycin, which can be translated in vitro to generate covalent mRNA–peptide fusions. To perform directed protein evolution experiments, synthesis of the mRNA–protein fusions is incorporated into an in vitro genetic cycle. High-affinity peptides are identified using immobilised panning approaches, whereby purified soluble protein is preferred to reduce nonspecific binding. The high-affinity mRNA–protein fusions are reverse transcribed to cDNA sequences amplified via PCR, resulting in a sequence that encodes a high-affinity peptide for the target of interest.

This approach has been successfully applied to the identification of novel peptide binders against a detergent purified *Drosophila* GPCR [83]. It is envisioned that the PoLiPa platform might be highly amenable to extend the application of the technique to GPCR drug discovery.

What are current limitations to PoLiPa technology?

Here, we summarise some of the most significant limitations in use of PoLiPa for GPCR drug discovery, and how these might be addressed: (i) Buffer compatibility. Low pH (<7) and high concentrations of divalent cations cause SMA to precipitate [23], making buffer composition an important consideration for downstream applications. Alternative polymers (e.g., DIBMA and SMI) can have preferable properties and could be used in these instances [34,84]; (ii) polymer charge. SMA has a large negative charge, which could interfere with some assays (e.g., chip immobilisation for SPR). Alternative polymers with altered charge (see later) might prove to be better solutions; (iii) Nonspecific binding to the polymer. The polymer is ever-present in the assay as part of the PoLiPa complex. Comparing assay readouts with PoLiPa made from different polymers could help to identify nonspecific interactions; (iv) nonspecific binding to the phospholipids. This is an inevitable consequence of presenting the GPCR in its native folded state, and must be balanced with the benefits of the highly relevant receptor conformations that are applied in the assay; (v) conformational restriction. There is evidence that protein conformation

dynamics might be restricted by the rigidity introduced by the polymer 'belt' in the PoLiPa. Transmembrane helical movement upon light activation in an archaeal rhodopsin was found by electron paramagnetic resonance spectroscopy to be limited in a PoLiPa environment [85]. This might have implications for GPCR screening because ligand pharmacology is dependent upon the conformational dynamics of the receptor and, if the full landscape of conformational ensembles are not represented, then the ability to distinguish antagonism, partial agonism, full agonism, and ligand bias might be limited. This remains to be empirically tested across a broad set of receptor subtypes and ligand classes, and requires further investigation to better understand the implications for GPCR screening. However, it is encouraging that differences in antagonist and agonist conformational dynamics in a PoLiPa-GPCR have been reported using fluorescence spectroscopy [51], and functional receptor agonism towards G protein and β-arrestin coupling has been demonstrated in the PoLiPa system [36,46]; (vi) lipid exchange. There is evidence to suggest that lipids incorporated into the PoLiPa can exchange in and out of the particle [86]. This might have consequences for the lipid environment represented within the particles because only lipids strongly associated with the protein might remain. Given that the representation of the original native membrane constituent in the PoLiPa system is a key advantage in maintaining a physiological-like environment for the target protein, the impact of this phenomenon on GPCR drug discovery assays requires further investigation and, if necessary, optimisation of purification protocols.

Optimisation of polymer chemistry for specific drug discovery applications

Clearly, for the reasons listed earlier, the choice of polymer can be crucial for achieving the optimal performance of the PoLiPa reagent in a given drug discovery technique. It is likely that there will be different polymers best suited to different downstream applications. Adapting the present standard SMA-based approach by investigating alternative polymer chemistry is an active area of current research, and is summarised in recent reviews [3,87]. The ultimate success of the PoLiPa platform in GPCR drug discovery will likely be dependent on optimisation of the polymer chemistry for different purposes.

The high negative charge of SMA might be a limitation in several settings and, to this end, positively charged (SMI) or zwitterionic (zSMA), and derivatives of SMA have been developed that could demonstrate better performance [34,88]. Acid and cation compatibilities have also been improved with SMI and DIBMA [26,84]. Polymer functionalisation to include, for example, biotin or fluorescent tags, has also been developed [89], which might have utility for immobilisation-based techniques, such as SPR and antibody panning.

Other membrane proteins

The application of PoLiPa to drug discovery is clearly not going to be limited to GPCRs, but will include other important membrane protein drug targets. For example, ion channels have been successfully purified by PoLiPa and shown to be folded and functionally intact [90–92]. Similar success has been reported in the field of transporters [93–95].

Concluding remarks

Advances in detergent-based purification of GPCRs has given us a taste of the potential for applying isolated and soluble GPCR protein reagents to structural and biophysical drug discovery methods, and, consequently, developing improved drug molecules. However, full realisation of the potential of these techniques is not being achieved because it is still too technically challenging to quickly and reliably purify new GPCR targets. PoLiPa is, in principle, a straightforward way to rapidly generate purified GPCR reagents for drug discovery. Therefore, there is great scope to use this technology to access crucial drug discovery tools for GPCR targets and, hence, to accelerate along an exciting path that realises this potential. We predict that new approaches and new targets, which have been held back by the large investment of time and money that is required to develop purified receptor by legacy methods, will be enabled by using PoLiPa. The ultimate consequence will be the development of better drugs for patients.

Although it is early days in using PoLiPa for GPCR drug discovery, with no examples of screening or structural determination yet disclosed in the public domain, we have highlighted the potential for this technique to be applied and readily adopted for these purposes. Although PoLiPa-GPCRs are, in principle, highly compatible with existing structural and biophysical technologies, further work is necessary to fully understand the limits of the system and how we can optimise specific assay approaches. In particular, specific polymer design will likely be essential to successfully deliver PoLiPa that are suitable for a variety of drug discovery applications.

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