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Production of membrane proteins in industry: The example of GPCRs

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ABSTRACT

Whereas membrane proteins make up ~23% of the human proteome, it is estimated that membrane proteins constitute more than 60% of current drug targets. With membrane proteins forming such a high percentage of drug targets relative to their abundance within the proteome, it is little wonder that drug companies need to rapidly access high quality membrane proteins for their drug discovery process. Newly devised technologies, such as rapid gene synthesis, novel detergents, and protein thermostabilisation strategies allow conventionally 'undruggable' membrane proteins to be drugged. In this review, we survey the state-of-the-art gene design, expression and purification strategies, and protein thermostabilisation methods used within a modern drug discovery programme, with a focus on G protein-coupled receptors.

1. Introduction

The ability to generate high quality protein reagents is a critical component of the drug discovery process. Within this process, proteins may be required for several applications including assay reagents, antigens, and reagents for structural biology. Approximately 700 of all currently approved drugs (~35%) target one class of membrane proteins, G protein-coupled receptors (GPCRs) [1]. This further highlights the importance of the ability to generate high quality membrane proteins. The ability to rapidly access such reagents has been revolutionised through the production of synthetic cDNAs coding for proteins of interest. Low cost, rapid gene synthesis provides the industry with a platform to make multiple constructs that may be required for the plethora of functions that may arise within a drug discovery campaign. However, the added structural complexities of membrane proteins (such as GPCRs), over soluble protein targets means that a high number of constructs need to be designed to increase the probability of a stable protein being produced. In this review, we survey the most recent gene design technologies, expression and purification strategies, and protein thermostabilisation methods used to enable and accelerate GPCR drug discovery.

2. Construct design of membrane proteins

The ability to successfully design a stable membrane protein construct is driven through in silico and experimental data fused to an understanding of its required purpose (e.g. crystallography versus surface plasmon resonance studies). A construct designed for structural studies will differ to one destined for use within assays. For proteins used for structural studies, sequences coding for flexibility and heterogeneity may be removed [2], whilst a protein used as an assay reagent will generally need to be fuller in length and may also need to contain post-translational modifications (PTMs) and a designated affinity tag [3]. Around 1,690 unique membrane protein structures are currently contained within the Protein Data Bank (PDB). When a protein of interest is contained within this database, this allows for a focused construct design paradigm to be undertaken. When direct structural information is not forthcoming, the use of secondary structure prediction programmes such as phyre 2 [4] offers a surrogate mechanism by which the designer can reduce the number of variables and deliver a focused set of constructs for experimental interrogation.

As previously mentioned, the design of protein constructs within an industrial drug discovery setting requires that the end use of the reagent needs to be carefully considered. For assay reagent generation, ensuring generation of the full length protein with the correct PTMs can be critical [5]. The role of PTMs (e.g. glycosylation, phosphorylation, and fatty acid acylation) has been shown to be essential for functional expression of a number of membrane proteins [6]. In the case of GPCRs, removal of PTMs can have a deleterious effect on both protein expression and function [7]. Glycosylation has been demonstrated to play a role within the protein folding pathway, acting as a quality control checkpoint so that only correctly folded protein is trafficked to the cell surface membrane [8]. Understanding the location and type of modification present on a protein allows for an informed decision to be

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made when designing a construct. For X-ray structural studies removal of glycans can be critical for successful crystallisation. A pre-requisite for the formation of ordered crystals is essential so that the proteins are uniform both in structure and conformation. Post-translational modifications, which vary depending on the expression system used, include glycosylation, phosphorylation, and palmitoylation [9,10]. These can be eliminated by truncation of extramembrane regions or by specific site-directed mutagenesis of the receptor. The use of glycan prediction servers like NetNGlyc provides a means to analyse the protein of interest and design a protein that is going to be fit for purpose. Whilst PTM analysis software offer a tool to predict which modifications will be 'fit for purpose' within a drug discovery programme, experimental data are also required.

In contrast to crystallography, where sample PTM heterogeneity can impede progress, cryo-electron microscopy (cryo-EM) is less constrained by these factors [11]. The cryo-EM structure of the wild-type human calcitonin receptor (CTR) for example, required minimal modifications with only the addition of N- and C-terminal tags. These tags were added to aid protein purification and expression. Replacement of the native signal peptide of the CTR with a haemagglutinin (HA) signal peptide improved protein expression levels, leading to a receptor with a more 'native-like' PTM profile. The protein structure revealed density in the extra-cellular domain (ECD) around residue 130 corresponding to predicted glycosylation in this region [12]. Only through the use of cryo-EM to this protein could this important interaction be observed, mutation of either Asn 125 or Asn 130 to Asp was detrimental to salmon calcitonin affinity and function suggesting that this glycosylation is required for normal agonist activity [12]. This highlights how PTMs can be critical in enhancing our ability to understand interactions between ligands. However, the addition of a PTM to a protein of interest is unfortunately an often-missing part of the structural enablement of a drug discovery programme.

The explosion in membrane protein structural data over the past 10 years has been phenomenal. In 2006, only one unique GPCR structure was deposited in the PDB [13]; by 2019, 67 unique GPCR structures have been released. Several technologies have driven this growth including protein thermostabilisation, protein fusions, lipidic cubic phase (LCP), and more recently cryo-EM. In the case of the first non-rhodopsin GPCR structure, the human β_2 adrenoceptor (β_2AR), a combination of LCP and the introduction of a protein fusion into intracellular loop three (ICL3) was required for successful crystallisation. In this case the T4 lysozyme (T4L) replaced most of the third intracellular loop of the β_2AR [14]. This was achieved through careful optimisation of the constructs. Residues 2–161 of T4L were inserted between residues 231–262 of the β_2AR to mimic the predicted distance of 15.9 Å between TM5 and TM6, as determined from the structure of rhodopsin. Following on from this initial success, the same approach was subsequently applied to numerous GPCRs leading to their structural determination [15–17]. Even with the abundant structures that are available to date, the introduction of a fusion into a GPCR requires a significant amount of both experimental and in silico analysis to identify the correct junction and fusion partner [18]. The ability to rapidly synthesise numerous genes with variations of the type fusion and position and then screen them has enabled successful structural determination.

The use of artificial intelligence (AI) based methods to predict the molecular structure of proteins offers a glimpse of how future protein constructs may be designed. Fast, yet accurate predictions of an unknown protein's 3D structure can yield a pre-screened set of candidate proteins to be investigated further via large-scale protein expression. Google's AI firm, DeepMind debuted an algorithm called AlphaFold, which uses three different neural networks [19]. The first level generates fragments based on the torsion backbone. From there, the fragments are combined, and the proteins are scored using a combination of two different techniques. One is based on co-evolutionary residues, and the other is a 3D approach based on scoring networks. Finally, energy models are applied to this combined protein model and improvements are made to get the final protein. The ability to rapidly look at protein constructs in silico with a high degree of accuracy and use this as one of the primary tools to design and triage constructs will become an ever-growing part of the protein design toolkit.

3. Expression of recombinant receptors

The expression of membrane proteins is notoriously difficult and has been one of the major bottlenecks when targeting them for drug discovery, which typically requires milligram quantities of active protein. Several expression systems have been developed for the expression of human membrane proteins, each with their own advantages and disadvantages. To date, the majority of work has focused on four main expression systems: bacterial (*E. coli*) [20], yeasts (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris*) [21], insect cells (*Spodoptera frugiperda* Sf_9, Sf_{21}, *Trichoplusia ni* High Five, and Drosophila Schneider S2*)* [22,23], and mammalian cells (CHO, HEK, COS-1) [24]. However, other systems have been investigated; these include cell-free expression systems and the more unusual use of whole organisms (*Drosophila melanogater*, *Xenopus laevis*, and silkworm) [25–27]. Whilst this large array of systems is available, the requirements within a drug discovery programme is typically restricted to more native-like systems such as insect and mammalian cells, which have the ability to be scaled from screening levels of millilitres to production levels of hundreds of litres using fermenters or wave bioreactors.

3.1. Insect expression

Of the 353 GPCR structures deposited in the PDB, insect cells have been the most successful expression system accounting for approximately 85% of the deposited structures. The first non-rhodopsin GPCR structures to be deposited, the human β_2-adrenergic and A_{2A} adenosine receptors [14,28] were achieved using *Spodoptera frugiperda* Sf_9 cells, whilst the structural determination of the turkey β_1AR was achieved using *Trichoplusia ni* High Five cells [29].

The relative success of insect cell expression for GPCRs relates to their ability to generate multi-milligram quantities of high-quality protein. A wide range of insect cells are commercially available including: *S. frugiperda* Sf_9, Sf_{21}, High Five, and *Drosophila* Schneider S2 cells, allowing screening of different cells to improve expression levels. Insect cells have been used to express a variety of different GPCRs [30] with very respectable yields. For example, turkey β_1AR has been expressed at levels of 7 mg/L in High Five cells, whilst expression in Sf_9 cells could only yield 1.25 mg/L [31]. The eukaryotic, baculovirus-based expression system has been used for over 30 years and relies on the viral transfection of insect cells to achieve protein production. Subsequently, a number of technological advances such as MultiBac in which multiprotein complexes can be generated from the insertion of the genes into an engineered baculoviral genome [32] have resulted in systems, which are more efficient and user friendly than the original.

Insect cell expression provides a platform in which most post-translational modifications required for functional protein can be achieved. In some cases, insect cell expression results in heterogeneous protein glycosylation. For example, when the D_2 dopamine receptor was expressed in Sf_9 cells, large amounts of unglycosylated receptor were produced [33]. A major limitation regarding the production of native-like proteins in insect cell lines is the lack of complex type N-glycans, often leading to severely reduced functionality. N-glycans found on insect cell expressed proteins are mainly of a high mannose type or non-fucosylated and core-fucosylated tri-mannose structures [34]. Therefore, considerable efforts have been made in modifying insect cells such as SweetBac cells, which have a more homogeneous, human-like glycosylation machinery [35]. Whilst an insect cell expression system provides a more native-like intracellular environment compared with *E. coli* and yeast, differences exist in their lipidic

membrane environments. Insect cells are typically grown at 27 °C and the types of lipid required to maintain membrane fluidity at this temperature are different to that of a native mammalian cell membrane. Insect cell membranes are very low in cholesterol and have no phosphatidyl serine in their plasma membranes. In addition, they have a comparatively high phosphatidyl inositol content and following infection, an enrichment in phosphatidylcholine is observed [36].

Whilst insect cell expression appears to contain a number of the desirable attributes required in an expression system to produce human membrane proteins, several drawbacks exist. These drawbacks prevent insect cells from being the universal system required for large-scale structural studies of GPCRs. Even with recent improvements to the baculovirus expression system it can take up to one month to generate large quantities of recombinant high titre baculovirus. The long virus generation process can provide a barrier for its use within a drug discovery programme, where often rapid generation of multiple protein constructs is required. To circumvent the long viral generation process, virus-free, transient gene expression (TGE) systems have been developed. For example, High Five cells have been used as an efficient protein production system for haemagglutinin subunit 1 [37].

3.2. Mammalian expression

Mammalian cells have been used to express a wide range of receptors [38]. Mammalian cells have all the cellular machinery required to correctly translate, fold, modify, and insert the protein into the cell membrane. However, incomplete or heterogeneous glycosylation is often a problem when receptors are over-expressed at high levels [39].

Heterologous protein production can be achieved through transient or stable expression and a wide variety of cell lines are available (e.g. CHO, HEK, COS-1, etc). Transient expression of GPCRs has been widely used for several years although its use as a tool in drug discovery has been limited due to cost and scalability. Transiently transfected cells usually express protein through the cytomegalovirus (CMV) promoter after 24–72 h post transfection, with a steady decrease thereafter. This system provides a quick and relatively easy method of screening protein expression and allows for rapid site-directed mutagenesis. Whilst lipid-based transfection methods have proved impractical for large-scale protein expression, other methods such as the use of recombinant virus (e.g. Semliki forest virus (SFV) [38] or BacMam [40,41]) have shown promise. To date, much of the work carried out on viral transfections has centred around the use of BacMam, which has a high rate of success in the expression of membrane proteins [42]. A limitation of the BacMam system is the requirement of virus propagation, isolation, and determination of virus titre. Virus titre is an important factor in relation to expression levels.

Stably transfected mammalian cell systems offer a solution to some of the problems suffered by using transiently transfected cells. Stable cell lines provide a constant source of recombinant protein, although their generation can be particularly time consuming. The use of inducible promoters may be particularly advantageous with respect to membrane proteins as constitutive expression can lead to cell toxicity [43]. In order to obtain a stable cell line, the expression construct is stably integrated into the host's cell genome; this typically requires the use of a selective marker (e.g. antibiotic resistance) as integration events are rare. Advances in generating stable cell lines have made this process simpler, with the use of the lentivirus [44] and the Flp-In T-REx™ [45] systems for example. However, the extended timelines required for generation for the stable system limit its use when multiple constructs are rapidly required.

Several issues still exist that have prevented the widespread use of mammalian cell culture as a tool for membrane protein biology; these reasons include high cost and scalability. Complex media requiring the addition of expensive antibiotics needed to maintain the expression of selective markers for stable cell growth has been a major stumbling block. Problems also exist in scaling up cell production. Whilst mammalian cells such as HEK293 are able to grow in suspension, making them amenable to fermentation, overall yields can be poor as cells struggle to survive in suspension cultures [46]. Anchorage dependent cells can be grown in cell factories or on microcarrier beads, although issues relating to cost and practicality can make obtaining large quantities of cells difficult. A growing number of membrane proteins have been expressed and purified from mammalian cell cultures to quantities required for biochemical and structural studies such as the recent hERG channel structure where GnTi suspension cells were successfully used [47].

4. Expression analysis

The need for large numbers of constructs to be taken through in parallel to fuel an expansive drug discovery programme requires an efficient screening strategy. To successfully identify and triage constructs several different assays are required to assess aggregation, active protein, and proteolysis profiles. The ability to measure the aggregation state of a protein during expression is a critical parameter. A high throughput assay to assess membrane protein aggregation can be achieved through fluorescent tagging of the target protein either covalently (fusing green fluorescent protein (GFP) [48]) or non-covalently (tagged with a fluorescent probe) [49]. The resulting unpurified protein can then be analysed by fluorescence-detection size-exclusion chromatography (fSEC) [48].

The ability to identify high expressing constructs or clones with the correct pharmacology is another critical part of the construct triage strategy. Radioligand binding (RLB) assays are seen as the gold standard for measuring both the affinity of ligand binding and total protein expression levels [50]. Whilst radioligands are not always available, the use of fluorescently tagged ligands [51] or conformational sensitive antibodies [52] offers another mechanism to measure protein expression levels. Whilst fSEC is primarily used to measure protein aggregation, it also offers a semi-quantitative tool to measure expression levels by peak integration of GFP. With an understanding of proteolysis using automated CE (capillary electrophoresis) Western blot systems, a rapid and informed assessment of many constructs is possible.

5. Thermostabilisation

GPCR crystallisation is challenging because GPCRs are usually unstable in detergent. To address this problem, different strategies reviewed in Milić and Veprintsev [53] have been developed to increase their stability. Briefly, academic research groups started to screen for more stable orthologs when human receptors were not stable enough [54–56]. The first thermostabilising mutation was introduced as a rationally engineered disulphide bridge in bovine rhodopsin [57]. This pioneering work led to the resolution of several rhodopsin mutants [58–61] and provided information for the design of other stabilising mutations such as the E122$^{3.41}$W mutation (Ballesteros-Weinstein numbering [62]) in the human β_2 adrenoceptor [63]. The first antibody fragments have also been developed to stabilise GPCRs [14,64] and GPCR-G protein complexes [65].

In 2007, the Laboratory of Molecular Biology developed a stabilising technology [66], which was later commercialised and further developed by Heptares Therapeutics, known as StaR™ (Stabilised Receptor) technology to purify and crystallise GPCRs. A StaR protein is a GPCR with a small number of point mutations which greatly improves the protein's thermostability, without disrupting its pharmacology. This technology is based on making a series of mutants where in turn every amino acid residue is changed to alanine (or to leucine, if it had already been an alanine) and testing the thermostability of each mutant. The most stabilising mutations are then combined [67]. Specifically, the increase in stability that each individual mutation gives to the receptor is determined by measuring the apparent melting temperature (Tm), which corresponds to a 50% decrease in functional binding after

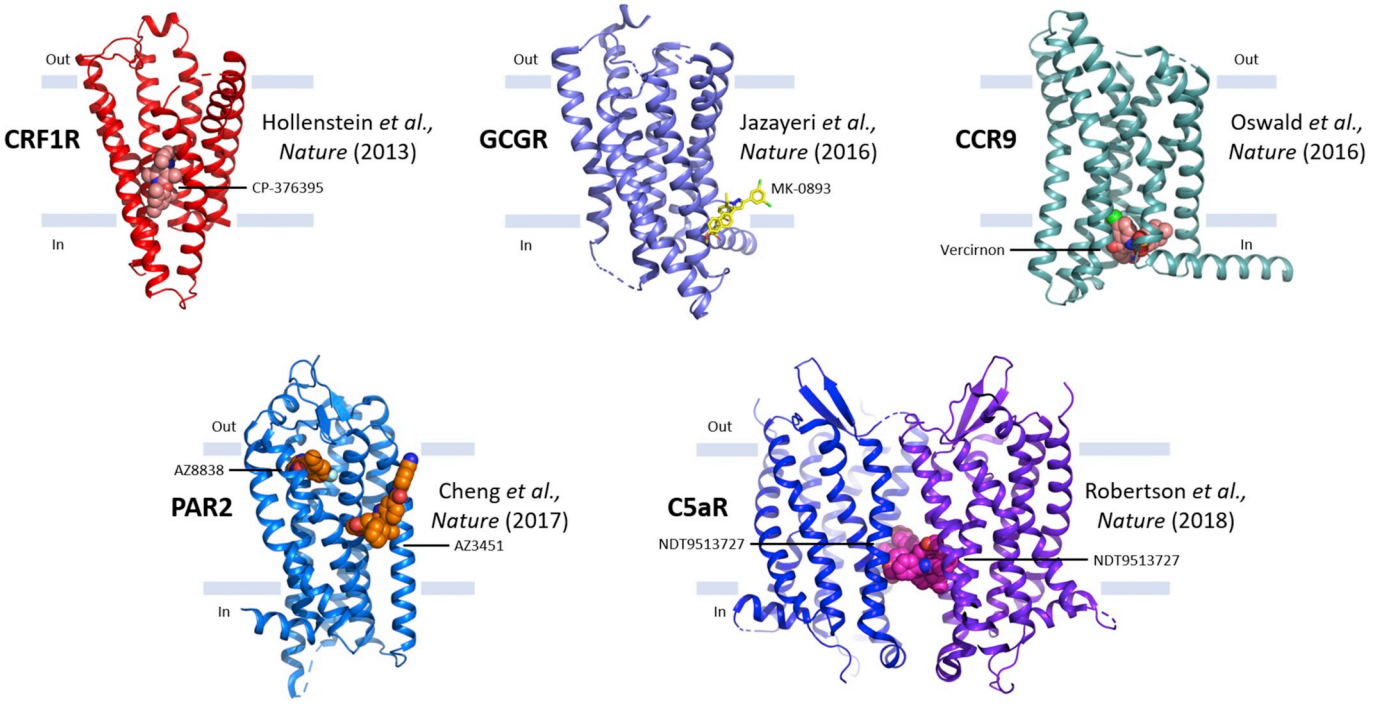

Fig. 1. Recent StaR X-ray structures revealed unexpected allosteric binding sites. This opens new opportunities and challenges for modulation in GPCR drug discovery. PDB accession codes for CRF1R, GCGR, CCR9, PAR2, and C5aR are 4K5Y, 5EE7, 5LWE, 5NDD/5NDZ, 5NDZ, respectively.

heating the receptor for 30 min. The StaR approach has led to the publication of important crystal structures [54,56,68–77] (Fig. 1) and to a transformation in structural data on GPCRs.

Now, Heptares Therapeutics, which has become Sosei Heptares, incorporates the SaBRE and CHESS platforms, which employ molecular evolution techniques to obtain a wide variety of GPCR variants with enhanced stability and versatility [78–80]. These GPCRs are evolved from populations containing hundreds of millions of variants of an ancestor GPCR and can be easily expressed in bacteria [81], yeast, or insect cells [82,83]. This unique technology has already enabled the crystal structures of class B parathyroid hormone 1 receptor bound to a peptide agonist [82] and class A human neurokinin 1 receptor in complex with several antagonists to be solved [83].

To date, almost one third of all published GPCR structures stem from thermostabilised mutants and directed evolution variants.

6. Solubilisation

6.1. Cell lysis and membrane preparation

The first step in isolating GPCRs is the preparation of cell membranes. Insect and mammalian cells are easy to break in comparison to yeast cells and a freeze-thaw cycle complemented with a single pass through a microfluidizer is enough to complete the lysis. It is important to work on ice and to add protease inhibitor cocktail tablets to the lysis buffer to prevent proteolysis. Cell membranes are isolated by ultracentrifugation and often washed once or twice with high-salt buffers (1 M NaCl) to remove peripheral membrane proteins. Subsequently, the concentration in total membrane protein is estimated by using copper-based protein assays, including the BCA and Lowry methods. Assessing the quality of membrane preparations by Western blotting and radioligand binding assays is essential as this will determine the yield of functional purified receptors. This provides a stop/go check point for any purification attempt. If a GPCR has suffered significant proteolysis and/or the membranes contain low amounts of receptor (less than 5 pmol/mg), then the expression conditions must be revisited.

6.2. Ligands as stabilising tools

One of the most significant contributors to obtaining a high resolution structure is the choice of ligand [72,73,76,77,84]. The presence of a ligand plays an important role in stabilisation. When a ligand interacts with a GPCR binding site (either allosteric or orthosteric), it reduces the entropy of the receptor by locking it into a single confirmation, which can in turn promote and maintain the correct folding of the protein. When considering a panel of potential ligands to enable structural studies several factors need to be considered including ligand physical chemical properties, affinity, kinetics, and thermal stability.

The ligand property that is most closely correlated with crystallisation is ligand induced thermostabilisation. There are different approaches to measure ligand induced thermostabilisation including microscale fluorescent thermal-stability assay (CPM assay) [85], fSEC [86], and radioligand binding [66]. A ligand inducing a high melting temperature (Tm) value implies that the receptor has formed a stable complex with the bound ligand and in turn generated a stable conformational state. Analysis by Zhang et al. suggested that a Tm value higher than 55 °C identified through a CPM assay system is usually required, although not sufficient, for successful crystallisation and subsequent crystal diffraction [84]. Physical chemical properties of ligands also need to be considered; identification of ligands that are soluble and remain in solution at the high concentrations >1000 fold over the K_D to provide complete occupancy of the ligand-binding site is property to consider. Whilst solubility is a significant parameter, the ligand also needs to be stable over the course of the purification, crystallisation, and structural determination whilst not degrading.

The receptor binding affinity (K_D) is another important consideration. A receptor ligand complex is dynamic, with the K_D composed of an association rate k_a (M^{-1} s^{-1}) and dissociation rate k_d (s^{-1}). The ability for the ligand to bind, occupy, and subsequently stabilise the receptor complex is critical for receptor stabilisation. An ideal ligand should

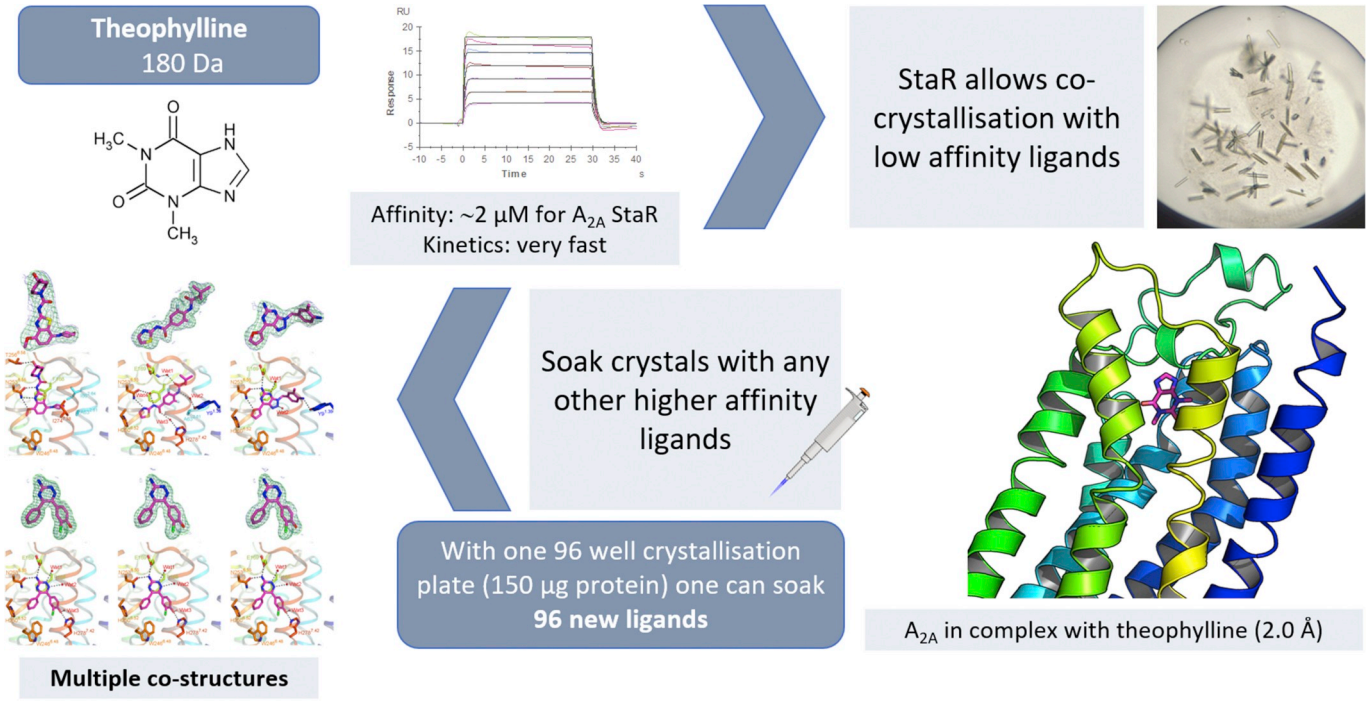

Fig. 2. Towards high-throughput GPCR crystallography. Theophylline binds to the thermostabilised A_{2A} receptor with relatively low affinity (pK_D = 5.7) and with fast kinetics. This ligand is used as a low-affinity carrier for the purification and the crystallisation of the stabilised receptor using the lipidic cubic phase technique. Crystals are then soaked in solutions containing different selective A_{2A} antagonists such as 1,2,4-triazine derivatives which bind with higher affinities (pK_D > 8) and exhibit slow off-rates. This *in meso* soaking method can yield multiple high-resolution co-structures and accelerate structural throughput for a given stabilised GPCR (adapted from Rucktooa et al. [87]).

have a fast association rate and a slow dissociation rate, however in some cases ligands have a slow association rate of >1 h and the ligand can be added either during the expression or the membrane preparation step [70,74]. Whilst high-affinity ligands often result in high-resolution structures, low-affinity ligands can offer a certain degree of stabilisation and present the advantage to be exchanged at a later point during the purification process for further applications such as fragment screening or *in meso* soaking [75,87] (Fig. 2).

When using low-affinity ligands, typically only 50–100-fold over K_D can be achieved due to solubility problems. The screening process to identify stabilising ligands is an iterative one as described in Fig. 3 below.

6.3. Optimisation of solubilisation conditions

As a pre-requisite to any purification, GPCRs must be solubilised in detergents. In many cases, thiol groups of free cysteine residues are blocked with iodoacetamide 15–30 min prior to solubilisation to prevent protein aggregation by intermolecular disulphide-bond formation. As a rule of thumb, membranes are solubilised at 10–15 mg total membrane protein per mL of solubilisation buffer. Above this threshold, the extraction efficiency usually drops rapidly, especially when using the newly developed maltose neopentyl detergents such as lauryl-maltose-neopentyl glycol (LMNG). Below 5–10 mg/mL, the risk of stripping away from the detergent micelles endogenous lipids important for GPCR function and/or stability increases. It is difficult to predict how a detergent solubilises a GPCR, therefore the optimal detergent or detergent/lipid combination for solubilisation must be tested empirically; this used to be found by running Western/dot blots [88] or ultracentrifugation dispersity sedimentation assays [89]. However, these methods are cumbersome and do not provide enough qualitative information.

A much faster and versatile strategy, initially developed by Eric Gouaux [48], is now applied to GPCRs to find the best detergent and solubilisation conditions, including salt concentration, pH, and additives such as ligands. In this approach, the target GPCR is covalently fused to GFP at its C-terminus and solubilised with different detergents at distinct membrane concentrations. After ultracentrifugation, the resulting unpurified receptor is analysed by fSEC. This requires only nanogram quantities of unpurified GPCR and gives information about the solubilisation efficiency as well as the expression level, the degree of monodispersity, and the approximate molecular mass of the solubilised receptor. As an alternative to GFP tagging, fluorescent ligands, where available, or peptide-based fSEC probes if the receptor is histidine tagged [49], are added during solubilisation and the fSEC detector set-up on the ad hoc excitation/emission wavelengths. The advantage of using fluorescent ligands is to inform on the fraction of receptors which is still active after solubilisation. When such fluorescent ligands are not available or too difficult to synthesise, surface plasmon resonance (SPR) can be used instead to identify detergents that produce active solubilised receptors [90].

6.4. Solubilisation of GPCRs

Whilst detergents and GPCRs can interact in an unpredictable manner, stabilised receptors have been effectively solubilised in *n*-dodecyl-β-D-maltoside (DM) (1.5–2%, w/v) [54,68–70,72,73,75,76], *n*-dodecyl-β-D-maltopyranoside (DDM) (0.5–1.5%) [71,77], and LMNG (1%) [56,74]. DDM and LMNG are mild detergents and thus, depending on the Tm of the receptor, they may offer a good starting point. However, because of their low critical micelle concentration (CMC) (0.0087% and 0.001%, respectively) and large micelle size (72 kDa, >90 kDa), it is very difficult to exchange DDM or LMNG for another detergent. Furthermore, due to their large micelle size, even using a 100 kDa concentrator, detergent will concentrate during the purification process, which can lead to protein losses (due to the high concentration of detergent) and problems with setting up trays for both lipidic cubic phase and vapour diffusion. DM is a harsher detergent than

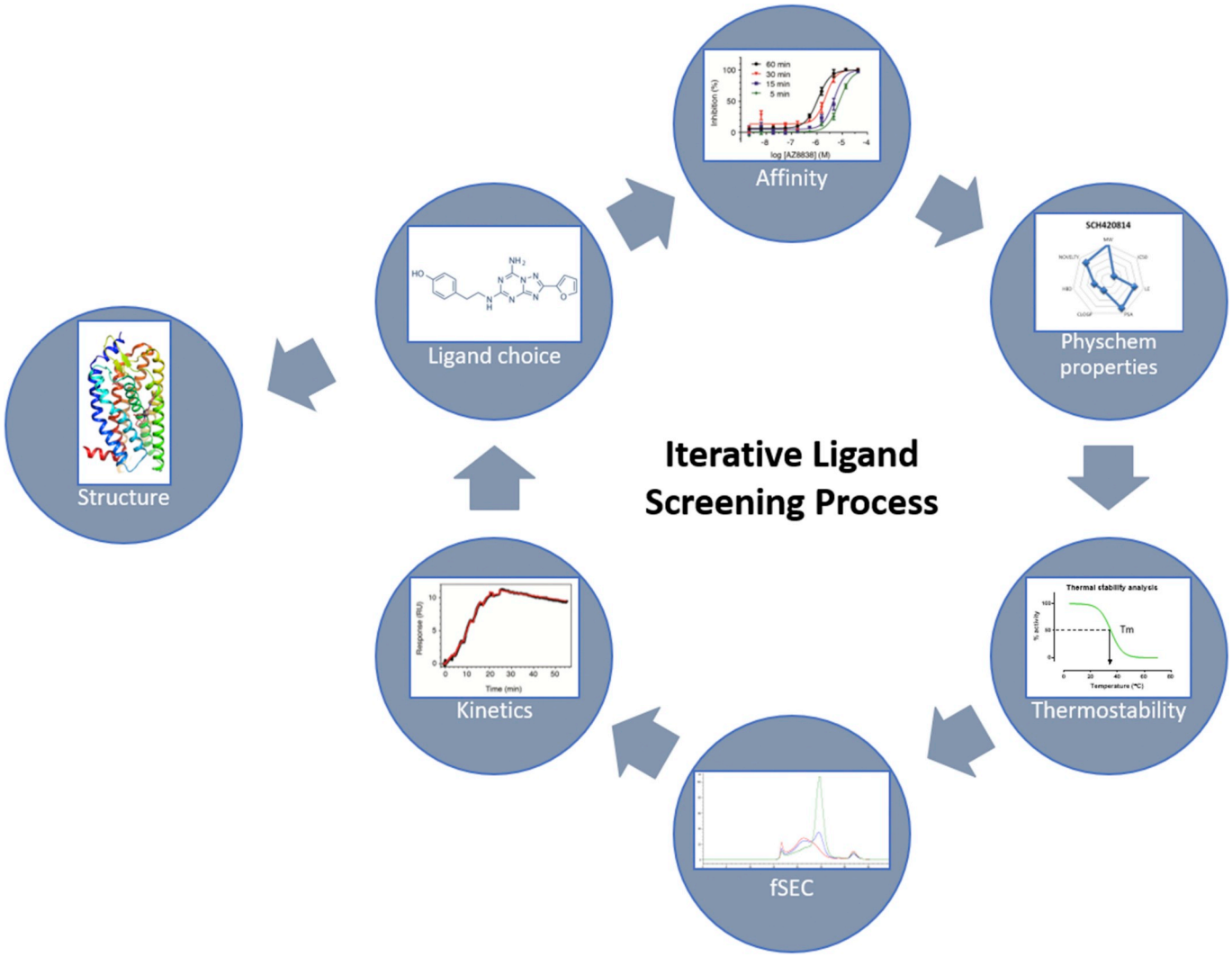

Fig. 3. Receptor ligand screening cascade.

DDM/LMNG, however, due its higher CMC (0.1%) and smaller micelle size (33 kDa), this detergent circumvents the inherent problems of using DDM/LMNG. However, care needs to be taken with respect to stability in DM and solubilisation efficiency.

Cholesteryl hemisuccinate (CHS), a more soluble version of cholesterol, is occasionally added to DDM/LMNG to solubilise StaRs in concentrations ranging from 0.1 to 0.2% (w/v) [56,74,77]. This sterol induces a bicelle-like architecture when added to DDM micelles and provides a more native stabilising environment for GPCRs [91]. However, the addition of CHS provides a large hydrophobic surface for lipid retention and subsequently increases the size of the detergent-lipid micelle. This can hinder the binding of affinity tags to purification resins, and therefore the concentration of CHS is decreased by 10–100 times in purification buffers.

It is worth noting that, apart from bovine and squid rhodopsins, non-stabilised GPCRs cannot be solubilised in DM alone and are almost exclusively solubilised in DDM/LMNG, sometimes mixed with CHS, or with detergent mixtures such as digitonin (1%) and sodium cholate (0.35%) [92], DDM (1%) and sodium cholate (0.2%) [93,94], LMNG (1%) and sodium cholate (0.3%) [95], DM (1.5%) and zwitter-ionic CHAPS (0.8%) [81], and finally DDM (0.5%) and CHAPS (0.3%) [96].

6.5. New amphiphiles and calixarene-based agents

Interestingly, new detergents are being tested for GPCR stabilisation, such as trehalose-cored amphiphiles [97], asymmetric maltose neopentyl glycol amphiphiles [98], dendronic trimaltoside amphiphiles (DTMs) [99], new penta-saccharide-bearing tripod amphiphiles [100], tandem malonate-based glucosides (TMGs) [101], mesitylene-cored glucoside amphiphiles (MGAs) [102], and tandem neopentyl glycol maltosides (TNMs) [103]. One DTM for instance (i.e. DTM-A6) outperformed DDM in stabilising the human β_2 adrenoceptor and its complex with Gs protein [99]. These amphipathic agents might have potential for extracting and stabilising GPCRs, especially for cryo-EM studies.

In the same vein, Calixar developed a range of innovative calixarene-based agents to solubilise and stabilise native, full-length, and functional membranes proteins, including the human adenosine A_{2A} receptor [3].

6.6. Styrene-maleic acid lipid particles (SMALPs)

As an alternative to detergents, a styrene-maleic acid copolymer (SMA) was developed, which directly solubilises cell membranes and generates discoidal particles comparable to nanodiscs [104]. The human A_{2A} receptor was successfully solubilised and purified in such nanoscale SMA lipid particles (SMALPs) for biophysical and functional studies [105]. More recently, such SMALPs were used to solubilise, purify, and crystallise *in meso* an α-helical seven-transmembrane microbial rhodopsin. The structure was solved to 2.0 Å and is virtually

identical to the 2.2 Å structure obtained from traditional detergent-based purification and subsequent LCP crystallisation [106]. Therefore, SMALPs, which preserve the original lipid environment, might represent a valuable substitute to detergents for the purification and crystallisation of GPCRs and other membrane proteins.

7. Purification

7.1. Immobilised metal-ion affinity chromatography

Immobilised metal-ion affinity chromatography (IMAC) is commonly used as the first chromatographic step in GPCR purification. Receptors are in general tagged at the C-terminus with a deca-histidine tag (e.g. Refs. [56,68–77]), sometimes with an octa [95,96,107] or hexa (e.g. Refs. [31,54,55,108–110]) histidine-tag. However, in some instances deca-histine tags are inserted at the N-terminus between a Flag tag (DYKDDDDK) and a human rhinovirus 3C protease cleavage site (e.g. Refs. [82,83,111]). A variety of matrices is available which can tolerate the detergent concentrations used during solubilisation (1–2%). The most popular are Ni^{2+}-NTA and Co^{2+}-CMA (TALON resin). Cobalt has a lower affinity for histidine than nickel and binds fewer non-specific proteins, therefore TALON metal affinity resin may help to remove non-specific contaminating proteins, especially when receptors are expressed at low levels. In some cases, batch mode increases GPCR binding to IMAC resins and help to retain solubilised membrane lipids in detergent micelles [74].

7.2. Strep-tag purification

Several GPCR ligands (e.g. histamine receptor antagonists) contain an imidazole ring which may prevent binding of His-tagged receptors to IMAC resins. When such ligands are used or when receptor purity after IMAC is not satisfactory, GPCR constructs can be engineered to include a Strep-tag at the N- or C-terminus. The Strep-tag purification system is based on the highly selective and easily controllable interaction between the Strep-tag II peptide (NH_2-WSHPQFEK-COOH) and the biotin binding site of an engineered streptavidin called Strep-Tactin. Strep-tag II binds Strep-Tactin nearly 100 times tighter than streptavidin, but can be eluted under gentle, physiological conditions. Rapid, one-step Strep-tag purification can result in active protein with a very high degree of purity [76].

7.3. General considerations about affinity tags

The yield of membrane proteins in affinity tag purification steps can be affected by the partial burial of the tag in the detergent micelle. This is usually more noticeable with large micelles such as DDM or LMNG. Improvements in recovery can be achieved when histidine tags are lengthened from His_6 to His_{10} (or from Strep-tag to Twin-Strep-tag) with the insertion of a flexible alanine linker and protease cleavage site.

The addition of small affinity tags does not normally have any effect on ligand binding properties. However, the addition of tags will often affect expression levels. For example, increasing the length of poly-histidine tags from His_6 to His_{10} may in some cases reduce expression and gains in recovery and purity that accompany increased tag length must therefore be offset against any reduction in expression. It is possible that the addition of other tags at either terminus will reduce receptor expression, and this effect will vary with tag, position, and expression system. All these factors need to be optimised to minimise any deleterious effect on expression levels.

7.4. Ligand-affinity chromatography

If there is an available resin with an immobilised ligand for the GPCR of interest, ligand affinity columns can be applied as a first or second purification step. Resins with covalently bound ligands such as XAC (xanthine amine congener) [112], neurotensin [113], alprenolol [14,31,54,65], and ABT (aminobenztropine) [92] have all been used to purify GPCRs. The major advantage of ligand affinity chromatography is that only functional receptors will bind to the resin. Therefore, the process is particularly useful where a mixed population of active and inactive GPCRs are present. Although affinity chromatography has several benefits with respect to only purifying active material, it can suffer from low binding and poor elution. In the case of the aminobenztropine resin, which has been used as a first chromatographic step for the purification of the muscarinic cholinergic receptor M2 [92], binding and elution can be extremely difficult due to the slow on and off rates coupled with a sub-nanomolar K_D for the ligand affinity column.

7.5. Size-exclusion chromatography

Size-exclusion chromatography (SEC) is often used as a final polishing step for the purification of GPCRs. It is always the case when stabilised receptors are prepared for vapour diffusion crystallisation, as this method cannot tolerate high amounts of impurities and aggregates. When GPCRs are expected to crystallise in LCP, which is a more robust crystallisation method, SEC can be skipped to avoid delipidation. Purified receptors are then passed over a desalting column (e.g. PD MiniTrap G-25) to remove imidazole/d-desthiobiotin and adjust the detergent concentration, and cleaved tags and protease are removed by reverse affinity chromatography (e.g Refs. [111,114–118]). Likewise, when the presence of endogenous lipids is critical for crystallisation, SEC can be avoided. Alternatively, lipids such as CHS or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) are added directly to the SEC buffer or immediately to the SEC pooled fractions [76] to further stabilise the receptor before concentration. Purification of GPCR-G protein complexes for crystallography, however, always include a final SEC step to remove the excess of unbound G protein [65] or mini-G, a minimal G protein [119,120]. Technically, Superdex 200 10/300 is the column of choice in GPCR purification but matrices such as Sephadex, Superose or Sephacryl are also utilised.

7.6. Detergent exchange

Detergents which are the most efficient for solubilisation are not necessarily the best ones for crystallisation. Most GPCRs are crystallised in LCP (reviewed in Ref. [121]) in DDM or LMNG, with or without CHS. These detergents have low CMCs and can be used both for solubilisation and purification-crystallisation. However, there are several examples of stabilised receptors which have crystallised using the vapour diffusion method. In this case, a key factor in obtaining strong lattice interactions is the size of the detergent micelle in relation to the size of the receptor. GPCRs often have only small hydrophilic domains outside the membrane and these can be occluded by detergents with large micelles (e.g. DDM, LMNG). Ideally, vapour diffusion crystallisation should be performed using detergents with smaller micelles (e.g. *n*-octyl or nonyl-β-D-glucopyranoside, OG/NG), but these detergents are often more denaturing than DDM/LMNG, resulting in denaturation of the receptor upon solubilisation. Therefore, a balance must be struck between shielding the hydrophobic domains of the GPCR from the aqueous environment, while at the same time, fostering interactions between the hydrophilic domains of the protein, thus driving lattice formation. Typically, this means that a stabilised receptor is purified into a mild detergent with a higher CMC than DDM/LMNG, such as DM, and is then exchanged into a detergent with a smaller micelle size, such as *n*-octyl-β-D-thioglucopyranoside (OTG) [54,76,122,123], NG [68] or HEGA-10 [124,125]. Depending on the stability of the receptor, detergents are essentially exchanged by immobilisation during affinity purification and/or size-exclusion chromatography. A lot of information is generated through the thermostabilisation process and Tm assays can act as a guide to help choose which is the most appropriate exchange detergent. Interestingly,

exchange of DM into short chain detergents did not dissociate StaR-mini-G complexes but instead enabled their crystallisation in vapour diffusion, as for A_{2A}-mini-Gs in OTG [119] and Rhodopsin-mini-Go in octyl glucose neopentyl glycol (OGNG) [120].

7.7. Small-scale purification

For the successful purification of a GPCR several factors must be optimised in order to generate sufficiently pure and active material. In a Biotech or Pharma environment where multiple GPCR targets and constructs are progressed simultaneously, scouting purification and small-scale magnetic pulldown are the two main techniques used to rapidly find the best detergent, buffer (pH), salt, ligand, and purification resin.

Scouting provides a fast and automated method to screen several different variables within the same experiment. The automated ÄKTA pure platform can be adapted to screen different constructs as well as different conditions (buffers, ligands). Typically, GPCR constructs are expressed in Thompson flasks (1–2 L of cell culture per sample, scale will depend on expression levels), membranes prepared and solubilised with the detergent(s) of choice, and lysates loaded to 1-mL affinity columns. Samples are eluted in up flow mode and peak cuts of affinity elution are injected sequentially on a Superdex 200 10/300 column. The SEC peak fractions are concentrated, and protein stability is determined by nanoDSF (e.g. Prometheus), a modified differential scanning fluorimetry method employing intrinsic tryptophan or tyrosine fluorescence. This method generates precise unfolding temperatures (Tm) which are instrumental in selecting the optimal purification conditions, and especially the best ligands for further crystallisation.

The use of Ni-NTA or Strep-Tactin magnetic beads makes it possible to perform small-scale purifications of receptors in a 96-well format, allowing for screening of many constructs and conditions in a single day experiment (e.g. different fusion proteins, ligands, detergents, buffers). The method also requires very little material as a starting point (typically membranes prepared from 1 to 10 mL cell culture per sample are enough to produce a few micrograms of purified receptor). This is important particularly for ligand screening, as it removes the requirement for purifying the receptor apo or with a low-affinity ligand and then doing ligand exchange. Instead, the ligand can be added from the beginning (pre-solubilisation), as in a large-scale purification. Additionally, the 96-well format is compatible with microscale thermostability screening using a qPCR system and thiol-specific fluorochromes such as N-[4-(7-diethylamino-4-methyl-3-coumarinyl) phenyl]maleimide (CPM) [85] or BODIPY FL-cystine [126]. This allows to generate thermal melting curves for up to 96 different purification conditions simultaneously. In parallel, purified samples (500 ng–1 μg is enough) are analysed by tryptophan fSEC to ascertain receptor monodispersity.

7.8. Proteolytic cleavage and deglycosylation

Affinity tags can prevent crystallisation and are therefore often removed through the action of proteases. Tobacco etch virus (TEV) protease and the human rhinovirus 3C protease are the most used proteases for removal of affinity tags (His-/Flag-His- or Strep-tag in almost all stabilised GPCR constructs used for crystallographic studies). These proteases are highly site-specific, easy to produce in-house, and show reasonable activities in detergents such as DM, DDM, and LMNG. GPCRs are mostly glycosylated on asparagine residues (*N*-linked glycosylation) present at the N-terminus, including the ECD of class B GPCRs, and the extra-cellular loops. More rarely, glycosylation occurs on serine/threonine residues (*O*-linked glycosylation) [127]. Glycan heterogeneity can prevent the formation of ordered crystals and therefore glycosylation sites must be removed by point mutation (sometimes in the post-translational consensus site) or truncation. When the introduction of mutations or truncations result in a loss of expression or activity, proteins can be deglycosylated by peptide-*N*-glucosidase F (PNGase F), which cleaves the core GlcNAc (*N*-acetylglucosamine) on the asparagine converting it to an aspartic acid. When expressing membrane proteins containing core α1-3 fucosylations that are resistant to PNGase F treatment [128], *Endo*-H is preferred as this glycosidase can cleave hybrid and core-fucosylated *N*-glycans, leaving a GlcNAc attached to the asparagine residue. In general, glycans which are structurally important are not fully digested by PNGase F or Endo H, and occasionally they can promote essential crystal contacts as exemplified by the resolved oligomannose *N*-glycans of the ECD in the PTH1R structure [82].

7.9. Quality controls

Purity of the final GPCR sample should always be examined in polyacrylamide gel electrophoresis and the protein molecular weight, the number of disulphide bridges, and post-translational modifications (especially glycosylation and palmitoylation) checked by top-down/bottom-up mass spectrometry. The determination of the exact protein concentration by quantitative amino acid analysis (QAA) and the final concentration in detergents (e.g. Molisch's test, mid-infrared spectroscopy) are also crucial for consistency in the context of crystallography. Besides, high-throughput FRAP (Fluorescence Recovery After Photobleaching) can be used to predict crystallisability of GPCR constructs in LCP [129,130]. In particular, the rate of molecular diffusion of fluorescently labelled GPCRs in LCP is positively correlated to their crystallogenesis, therefore promoting LCP-FRAP as an efficient pre-crystallisation screening method.

8. Sample preparation for cryo-EM

The wave of resolution revolution in cryo-EM has now touched GPCRs. Due to their relatively small size (most class A GPCRs are approximately 50 kDa), the initial focus has been on complexes with signalling partners such as heterotrimeric G proteins (Gs [12,131–134], Gi [135–140], and mini-Go [141]). These complexes have the advantage of being a more suitable size (approximately 150 kDa) for single particle cryo-EM studies.

As for crystallography, GPCRs are preferentially solubilised and purified in mild detergents (DDM/LMNG), using the similar purification tags and techniques as described above. Complex formation with heterotrimeric G proteins/mini-G is usually initiated with the agonist-bound and purified receptor (concentrated to at least 0.1 mM), by adding a 1.2-fold molar excess of Gα and Gβγ subunits in the presence of apyrase to hydrolyse residual GDP released from the Gα subunit upon binding to the receptor and therefore maintain the high-affinity nucleotide-free state of the complex. Many of the structures of GPCR-Gs complexes use the nanobody Nb35 to stabilise the complex and new antibodies were developed to stabilise complexes with Gi, such as the antibody fragments Fab16 [137,138,140] and Fab_G50 [136,139]. Nb35 bridges the Gs protein α- and βγ-subunits [65] whereas Fab16 and the single-chain variable fragment scFv16 bind to the heterotrimeric Gi/o and prevent GTPγS-mediated dissociation of nucleotide-free complexes (it is derived from a monoclonal antibody that was raised against a rhodopsin:Gi_1 complex [142]). However, stabilisation of the interface between the α- and βγ-subunits is not always necessary, as exemplified by the structures of the serotonin 5-HT_{1B} receptor [141] and the adenosine A_1 receptor [135]. In both structures, the α-subunit was mutated to facilitate structure determination. The 5-HT_{1B} receptor was mixed with thermostabilised mini-Go, which contains only the GTPase domain of the α-subunit (molecular weight of less than 30 kDa), and the adenosine A_1 receptor was coupled to a dominant-negative Gi_2 ($DNGi_2$), which contains four mutations in the α-subunit that alter nucleotide binding and affinity for Gβγ [143]. Alternatively, to form an active, G protein-coupled complex, receptors are co-express with their cognate G protein subunits in insect cells and stimulated with an excess

of agonist in the presence of apyrase and if necessary, a stabilising nanobody/antibody fragment. Membranes are then collected and solubilised with a mixture of LMNG (0.5%) and CHS (0.03%). Further, solubilised Flag-tagged receptor and receptor complex is purified by antibody affinity and loaded on a Superdex 200 10/300 column to separate the excess free receptor from the complex. This latter approach was used to obtain the first near-atomic-resolution cryo-EM structure of an activated GPCR, the class B calcitonin receptor coupled to heterotrimeric Gs [12]. It is worth noting that GPCR-G protein complexes in LMNG maintain their structural integrity even when diluted below the CMC of the detergent [65]. This is a major advantage as one of the main challenges in cryo-EM is to avoid high detergent concentrations, which complicate the controlled formation of thin vitreous ice in grid preparation [144]. Typically, GPCR-G protein complexes are concentrated no further than 5–10 mg/mL, far from the concentrations reached for LCP crystallisation (30–60 mg/mL). To minimise the final detergent concentration, only the central fractions from the SEC peak containing the monomeric complex are collected and concentrated. Glyco-diosgenin (GDN), a synthetic substitute for digitonin, which has a much smaller CMC than digitonin, is often added as an additive (0.01%) to the SEC buffer to help stabilise GPCR complexes [131]. If the protein complex avoids the areas of thin vitreous ice, then the specimen must be imaged in relatively thick ice at the expense of increased background noise in the micrographs. When this happens, the final sample can be spiked with near CMC concentrations of fluorinated surfactants such as octyl maltoside (FOM) or Fos-choline 8, to help decrease non-specific aggregation and improve the distribution of the protein complex on carbon grids during the vitrification process. It is important to keep in mind that the detergent micelle bound to a membrane protein affects image processing and can also disrupt GPCR dimers. Another issue is that free detergent micelles can easily be mistaken for protein particles in electron micrographs [144]. Therefore, several approaches have been explored to remove detergent in cryo-EM grid preparation. This includes the use of amphipols, which have enabled the subnanometer structure elucidation of TRPV channels and the γ-secretase complex [145,146], and nanodiscs, which have been widely used as a native-like lipid bilayer for membrane protein characterisation [147]. Recently, metabotropic glutamate mGlu5 receptor obligate dimers and native rhodopsin dimers were stabilised by self-assembly with phospholipids and the membrane-scaffold proteins 1D1/1E3D1, and their cryo-EM structures resolved in nanodiscs [148,149]. Furthermore, a newly developed scaffolding protein, saposin A (SapA), adapts its oligomerisation to maintain membrane proteins in an artificial lipid bilayer that is proportional to their transmembrane domains [150,151]. This might represent an alternative tool to prepare GPCR dimers and complexes for cryo-EM studies.

9. Conclusion

The production of recombinant GPCRs in the milligram range for biochemical and structural studies is now well established. Expression is predominantly performed in insect cells, while other systems such as BacMam are becoming increasingly important. Mild detergents such as DDM/LMNG, mixed with or without CHS, and high-affinity ligands are used in combination for solubilisation and purification, whether GPCRs are prepared for LCP crystallisation or cryo-EM studies. Thermostabilisation of GPCRs by Ala-scan mutagenesis and/or directed evolution allows Pharma and Biotech to initiate drug discovery progammes on targets that are originally expressed at very low levels. Furthermore, these thermostabilised receptors can be crystallised in vapour diffusion using harsh detergents (e.g. NG/OTG), which adds up to their versatility and increases the chance of solving their X-ray structures. Finally, single particle cryo-EM provides a prevailing direction to obtain high-resolution structure information on GPCR-G protein complexes without crystallisation. This will help to decipher biased agonism and develop safer drugs such as better analgesics to treat acute pain.

CRediT authorship contribution statement

James C. Errey: Writing - original draft. **Cédric Fiez-Vandal:** Writing - original draft.

Declaration of competing interest

The authors declare no competing financial interests.

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