Methods 180 (2020) 111–126

Contents lists available at ScienceDirect

Methods

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

Generating therapeutic monoclonal antibodies to complex multi-spanning membrane targets: Overcoming the antigen challenge and enabling discovery strategies

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ARTICLE INFO

Keywords:
G protein-coupled receptors
Ion channels
Transporters
Drug discovery
Monoclonal antibodies

ABSTRACT

Complex integral membrane proteins, which are embedded in the cell surface lipid bilayer by multiple transmembrane spanning helices, encompass families of proteins which are important target classes for drug discovery. These protein families include G protein-coupled receptors, ion channels and transporters. Although these proteins have typically been targeted by small molecule drugs and peptides, the high specificity of monoclonal antibodies offers a significant opportunity to selectively modulate these target proteins. However, it remains the case that isolation of antibodies with desired pharmacological function(s) has proven difficult due to technical challenges in preparing membrane protein antigens suitable to support antibody drug discovery. In this review recent progress in defining strategies for generation of membrane protein antigens is outlined. We also highlight antibody isolation strategies which have generated antibodies which bind the membrane protein and modulate the protein function.

1. Introduction

Monoclonal antibodies (mAbs) continue to be a fast-growing class of drugs with a recent review highlighting 76 antibody-based therapeutics approved for clinical use [1]. The growth in development of antibody therapeutics is illustrated by a near tripling in the number of antibody therapeutics on the market in the period 2010–2019 [2]. The continued growth of mAbs as effective therapeutics is built on the ability to access a variety of mechanisms of action, their high potency and the high specificity of antibodies to their molecular target leading to antibodies being well tolerated and carrying a lower risk of unanticipated safety issues than many other therapeutics [3]. The therapeutic effectiveness of antibodies is linked to the ways in which antibodies can achieve their effects. Antibodies can directly bind to and neutralise target proteins to block function (e.g. cytokines) and inhibit pathophysiological cellular signalling pathways [3]. Antibodies can bind to targets on the cell surface and engage, via their Fc domains, with components of the immune system such as immune-effector cells and complement to cause cell killing and cellular depletion [3]. Antibodies may also bind receptor targets on the cell surface and inhibit the action of pathophysiological mediators or directly activate signalling pathways [3]. In addition to the modes of action of individual mAbs, they also have a longer duration of action than small molecule drugs due to their long half-life (~11–30 days) in plasma [4]. Monoclonal antibodies have been particularly effective in the areas of Oncology (where tumour cells are eliminated through antibody effector functions, antibody-drug conjugates, or bispecific antibodies that engage killer cells) and Immunological diseases via the inhibition of inflammatory signalling pathways [1].

Typically, the antibodies developed to date have targeted largely soluble mediators (e.g. TNF, IL-17) and cell surface receptors (e.g. IL-5R, PD-L1, IL-4R) [5]. In the case of cell surface receptors, these are often receptors with a single transmembrane helix and large extracellular ligand binding domain. There is, however, significant interest and rationale for exploiting antibodies to target more complex polytopic integral membrane proteins which are associated with a variety of pathologies [6,7]. These complex integral membrane proteins are characterised by multiple transmembrane spanning domains and include G protein-coupled receptors (GPCR), ion channels and transporters which are members of large protein families. Analysis of the human genome predicts the existence of approximately 350 non-olfactory GPCRs, around 400 annotated ion channel genes and 1500 transporters [7–9]. Targeting these proteins with small molecules has proven successful as illustrated by the clinical success of targeting GPCRs and ion

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https://doi.org/10.1016/j.ymeth.2020.05.006
Received 24 February 2020; Received in revised form 21 April 2020; Accepted 13 May 2020
Available online 15 May 2020

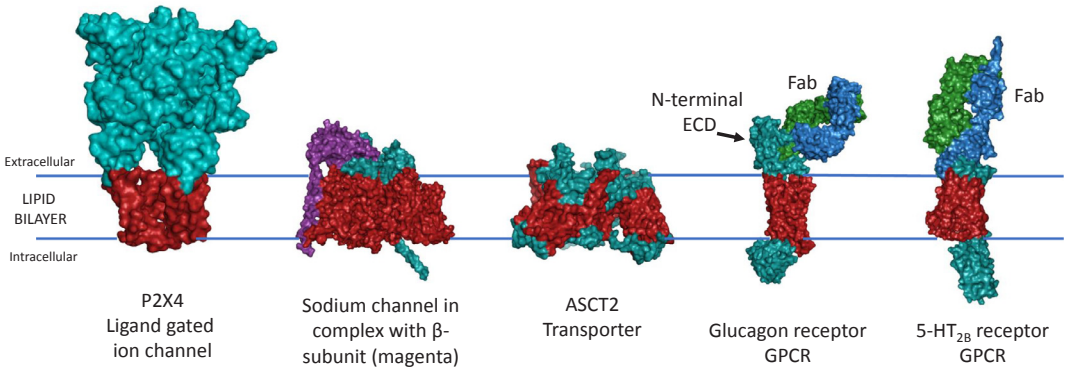

Fig. 1. Topologies of complex multi-spanning membrane proteins and interactions with antibodies. Selected structures of complex membrane proteins illustrating different topologies and variations in exposed surface extracellular regions which provide potential epitopes for antibodies. Structures showing surface representations were generated using PyMOL (Schrödinger) and information derived from the Protein Databank; P2X4 – 4DW1 [189]; $5HT_{2B}$ receptor – 5TUD [190]; Glucagon receptor – 5XF1 [191]; Eel sodium channel in complex with beta subunit – 5XSY [192]; ASCT2 transporter – 6GCT [193]. The $5HT_{2B}$ and glucagon receptor structures are solved as complexes with Fabs (heavy and light chains coloured blue and green) which target the receptors. Amino acid residues exposed on the extracellular surface of the membrane for the ion channels and GPCRs are shown (deep teal) and transmembrane regions are shown in firebrick red. These residues were coloured according to transmembrane and extracellular regions defined in their respective UniProt entries for the membrane proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

channels. A recent review showed that small molecule drugs to GPCRs and ion channels represent the first (33%) and third (18%) largest classes of marketed drugs respectively [10]. Small molecule drugs can, however, face translational challenges which hamper progress to the clinic due to issues such as toxicity and lack of target selectivity that may lead to off-target effects. For those GPCRs with peptide ligands, in addition to improved specificity and selectivity, antibodies may be better-suited to modulate the activity of these GPCRs, where the relatively larger size of the peptide ligand-receptor binding interface may limit the effectiveness of small molecules.

Given this background, these target classes present an opportunity to exploit the exquisite specificity of antibodies and other features such as their long serum half-life and ability to engage effector function. GPCRs, ion channels and transporters typically exhibit multiple conformational states and can bind a wide variety of small molecule, peptide and protein ligands and, in the case of ion channels, respond to changes in membrane potential. We can envisage antibodies which bind these targets and act through a variety of mechanisms: antibodies may act as direct antagonists or agonists by binding to GPCRs and ion channels where they interact with native ligands and toxins or modulate pharmacologically relevant conformational states by binding to allosteric sites. Monoclonal antibodies (mAbs) may also be used to exploit modes of action which involve cellular depletion via target binding and recruitment of effector functions mediated by customised antibody Fc domains [11]. This potential is reflected in a growing preclinical and clinical pipeline of mAbs and alternative scaffolds targeting GPCRs, ion channels and transporters [7,12]. To date, however, there are relatively limited numbers of marketed biologics addressing these target classes. In the case of GPCRs, mogamulizumab, an anti-CCR4 mAb has been approved for clinical use in Japan [13]. This humanised, afucosylated anti-CCR4 antibody causes depletion of CCR4 expressing cells in patients with T-cell lymphomas by enhanced natural killer cell activation. More recently, erenumab was approved in the US as a first-in-class mAb which targets the CGRP receptor and is being used clinically as a therapeutic for migraine [14]. These few approvals demonstrate the untapped potential of developing mAbs against these target classes; however, there are no approved antibody therapeutics that target ion channels or transporters, which reflects the technical challenges involved in isolating monoclonal antibodies against these target classes. Issues arise due to the difficulties in purifying and stabilising multi-spanning membrane proteins to generate pure, conformationally-relevant protein antigen for discovery and screening and in overcoming immunological tolerance arising from sequence conservation. That said, our knowledge of complex membrane proteins is growing rapidly due to the development of new technologies for the preparation and structural characterization of membrane proteins, such as cryo-EM which has been particularly effective in solving structures for membrane proteins [15,16]. In this review, we explore the technical challenges in developing mAbs against complex integral membrane proteins and focus on technologies that facilitate membrane protein antigen preparation. We also explore antibody discovery strategies, encompassing antibody display and immunization, which enable isolation of antibodies that bind these targets and modulate their function.

2. Challenges in identifying functional antibodies to complex membrane proteins

The generation of monoclonal antibodies specific to target proteins commonly involves well-established platforms, including hybridoma technologies, antibody display technologies or combined methods in which immunisations are performed and immune display libraries are generated and mined for lead antibodies [17–19]. Identification of functional antibodies to GPCRs, ion channels and transporters presents challenges due to technical issues in expressing and purifying correctly-folded integral membrane proteins and the structural and functional diversity of target proteins. Membrane proteins are often expressed naturally in low abundance and heterologous expression often requires evaluation of multiple expression systems to obtain high expression levels (see for example, McCusker et al. [20]). Purification of integral membrane protein antigens requires methods to extract the protein from the lipid bilayer whilst maintaining protein conformation. Strategies to prepare antigens for antibody discovery will also vary due to the diverse structures present among GPCR, ion channel and transporter families. In some cases, large extracellular domains may be targeted by antibodies and be expressed and isolated as functional, well-folded domains. In other cases, the extracellular loops of target proteins are small and inaccessible (see Fig. 1). However, as demonstrated in Fig. 1, structures of antibody:membrane protein complexes reveal how antibodies can effectively target these proteins. In the following sections, we review methods for membrane protein antigen preparation with a focus on new developments and strategies to isolate functional antibodies.

3. Heterologous expression of complex membrane proteins

The critical step in any antibody discovery campaign is the

generation of high quality, well-validated target protein that can be used as both antigen and in screening for the desired binding and functional properties. Whilst synthetic peptides have been successfully used in antibody discovery (reviewed in Lee et al. [21]), we have focused this review on strategies to produce intact, natively-folded target proteins. For example, cells overexpressing the protein of interest can be used as a source of antigen for both *in vivo* and *in vitro* antibody discovery and can be used in binding and functional screening, including reporter assays, electrophysiology, radioactive ligand binding or radioactive ion uptake as reviewed by Colley et al. [22]. However, heterologous protein expression is commonly required to extract target proteins from host cell membranes, purify and stabilise the protein outside of its normal native lipid environment. Successful heterologous expression of multi-spanning membrane proteins remains challenging and several recent reviews highlight current strategies [23–25]. For all expression systems, it is important to note that high-level expression does not always equate to functional, well-folded protein; one must always confirm the quality of the prepared antigen. For example, despite showing the highest expression levels of serotonin transporter and hERG (K_v11.1) in *P. pastoris*, the highest functional expression was observed in HEK 293 [26,27].

3.1. Bacterial expression

Complex membrane proteins derived from eukaryotes are typically not well-suited for expression in bacterial hosts such as *E. coli* due to differences in post-translational modifications, lipids and pH [28]. Despite these limitations, there are a few examples of successful bacterial expression of GPCRs and a recent approach employing directed evolution in *E. coli* to derive higher levels of expression and stability has met with some success [29–31].

3.2. Yeast expression

Successful membrane protein expression in yeast species such as *Saccharomyces cerevisiae* and *Pichia pastoris* has been reported, where yeast is an attractive option due to the ability to scale-up production in large fermenters at high cell densities [32]. One noteworthy development is the application of directed evolution in yeast to isolate proteins with improved levels of expression and stability and, interestingly, the increased expression seen in yeast is maintained when the evolved constructs are transferred to an insect cell line for expression with up to 26-fold increased functional expression [33]. The methylotropic yeast *Pichia pastoris* has also been used as an expression host to express GPCRs, ion channels and transporters [34]. Although *Pichia* has historically required a rather cumbersome expression process, a recent publication has demonstrated a more facile auto-induction procedure that can be applied to obtain high-level membrane protein expression [35].

3.3. Insect expression

Insect cell expression of complex membrane proteins has proven valuable, particularly in the case of GPCRs [36] and insect cell expression has been used extensively to support structural studies of GPCRs [37]. The two most common insect cell lines are *Spodoptera frugiperda* (sf9 and sf21) and *Trichoplusia ni* (Hi5). Insect cell expression supports high expression levels, large scale culture and post-translational modifications such as glycosylation, albeit with simpler glycosylation patterns comprising oligo-mannose sugar chains.

3.4. Mammalian expression

Perhaps not surprisingly, when considering human membrane proteins as antigens, the expression system with the greatest success has been mammalian cells (e.g. human embryonic kidney (HEK293) or chinese hamster ovary (CHO) cells) overexpressing the target membrane protein. Membrane protein expression can be achieved by transient transfection of expression vectors using a transfection reagent (e.g. lipofectamine) or stable integration of the transgene [38,39]. Each method has its advantages and limitations and both methods typically require evaluation of several expression constructs to achieve optimal expression levels. For example, sometimes overexpression of target antigens can overwhelm the secretory and endoplasmic reticulum transport pathway [40,41] or result in toxicity. One approach to limit this phenomenon is to provide temporal control of gene expression by using an inducible expression system. In these cases, expression of target genes is regulated by an inducible promoter system; these systems include those responsive to tetracycline (T-Rex), doxycycline (Tet-On 3G) and cumate. Andréll *et al* (2016) showed that stable cell lines expressing membrane protein under the control of the tetracycline-inducible promoter system showed improved production [42].

Given the difficulties in obtaining high quality antibodies to complex membrane proteins, detection of overexpression with tool antibodies can be challenging and other strategies to detect protein expression are required. A well-established method to enable detection of membrane proteins is the inclusion of epitope tags in extracellular regions of the protein that can then be detected with anti-tag-specific antibodies, although tag incorporation may risk perturbing protein folding and function (see Maue for review [43]) or introduce an undesired immunodominant epitope. Systems such as Thermo Fisher's Tag-On-Demand bypass these risks by employing amber suppression technology to enable production of C-terminally tagged and native membrane proteins from the same expression construct. In our hands, amber suppression was rare and required isolation of a stably-transfected clone containing the tRNA synthetase and aminoacyl tRNA with the desired characteristics; however, having achieved this, expression of the tag could be controlled by exogenous addition of a non-natural amino acid [44]. The inclusion of epitope tags enables purification of recombinant proteins for use in downstream screening and binding assays.

A widely employed alternative to epitope tags is direct fusion with fluorescent proteins (FPs) such as green fluorescent protein (GFP), typically fused to the C-terminus of the membrane protein. Such FP fusions allow direct screening for membrane protein expression level using the signal from the FP as a proxy for expression of the full-length membrane protein-FP fusion. Although GFP is the most widely used FP, a recent publication which examined the use of mEGFP, mVenus, mCerulean and mCherry as fusion partners concluded that mVenus systematically gave the highest membrane protein yields [45], although the exact mechanism by which FP fusion increases yields has not been elucidated. The addition of FP fusion tags provides many benefits for facilitating studies on membrane proteins. FP fusions enable sorting of single cell clones via fluorescence-activated cell sorting which allows high expressing stable clones to be isolated. FP fusions can also be used for fluorescence microscopy or imaging enabling subcellular localisation of the protein and verification of cell surface expression as opposed to formation of intracellular aggregates or mis-trafficked protein that can occur at high-levels of expression. Finally, FP fusions can be used to monitor detergent solubilisation and the quality of protein with techniques such as fluorescence size exclusion chromatography (FSEC) [46]. It is important to note that FPs are immunogenic themselves and mitigating strategies need to be developed.

Issues that arise during the generation of high-expressing stable cell lines (e.g. toxicity, cloning and outgrowth of single cell clones) can be avoided by using of large-scale transient transfection. These methods include use of transfection reagents including cationic polymers such as polyethyleneimine and lipid-based reagents. Most promising in this regard is use of flow electroporation technology which is commercialised by MaxCyte [47] and is capable of transfecting up to 2×10^{11} cells in less than 30 min (using the MaxCyte STX or VLX instruments) with high transfection efficiency and viability.

Once a suitable overexpression system has been identified, overexpressing cells can be exploited for antibody discovery. We have had success using lentivirus transduction as a means for generating stable cell pools and clonal cell lines expressing the gene of interest (reviewed by Elegheert *et al* [48]). We have also described the use of the Jump-In cell line system (Thermo Fisher) which allows targeted integration of genes into a specific genomic locus to overexpress the fMLP receptor [49]. Recombinant cells expressing the target protein may be used directly as an immunogen for antibody discovery and screening. They may also be used to derive purified protein or to generate a wide range of complex membrane protein antigen formats which are described in the following section. It is also worth noting that recombinant expression vectors used to express membrane proteins in cells may be adapted (e.g. by introduction of strong promoters; inclusion of T-cell helper epitopes) so that plasmid DNA may be used directly as an immunogen *in vivo*. This is described in Section 5.3.

4. Complex membrane protein antigen formats

Due to challenges with lipophilicity, conformational flexibility and low stability, complex membrane proteins have required optimization of many different expression systems and antigen formats to enable antibody discovery efforts. The formats used range from simple soluble regions of the protein – such as soluble extracellular domains or peptides – to proteins purified in detergent or lipidic environments to complex membranous environments up to and including whole cells. The various formats available are summarised in Table 1. In this review, we will focus primarily on the more complex antigen formats alongside a brief review of detergents. Due to qualities that more accurately recapitulate the native context, these more complex systems have often succeeded in isolating functional antibodies to membrane proteins where simple antigens such as peptides and protein:detergent complexes have failed.

4.1. Detergents

In general, detergents are the least stabilising of all the different formats for solubilising and isolating complex membrane proteins, at least partially due to their stripping away of critical lipid molecules and loss of lateral membrane pressure. A further shortcoming is that those detergents that are most stabilising tend to have longer acyl chains and these chains can mask extracellular epitopes on target proteins and block antibody binding. Despite these limitations, several advances have been made in the development of novel detergents or methods for target stabilisation. An approach now widely applied to overcome low stability is the introduction of multiple point mutations within a target's transmembrane helices that increase thermostability, as with Sosei-Heptares StaR technology. The resulting stabilised molecules retain function when extracted in shorter chain detergents like decylmaltoside (DM), unmasking potential antibody binding epitopes. The other major advance has been the development of novel detergent series such as the Calixarenes [50], the neopentyl glycols [51] (for example the widely used lauryl maltose-neopentyl glycol, LMNG) and derivatives thereof [52,53], for which there are numerous reports demonstrating increased stability over more traditional detergents [54–56]. LMNG appears to possess a particularly stable interaction with membrane proteins with a very low off rate (four orders of magnitude lower than dodecylmaltoside) [57], as demonstrated by the reported ability to deplete a sample of excess protein-free LMNG micelles and obtain a homogeneous, correctly folded target [58].

4.2. Discoidal particles

4.2.1. Nanodiscs and peptidiscs

Possibly the most widely used systems for investigating the structure and function of membrane proteins after detergent systems are those that can be broadly grouped into a family of disc-shaped nanoparticles, or nanodiscs. Following their successful application in the membrane protein structural biology community, these approaches have also been applied to industrial antibody discovery programs. The original nanodisc format was developed by Sligar and colleagues in the early 2000s [59], and borrows the principle concept from naturally occurring high density lipoprotein particles observed in humans. The major protein component of the particles is an amphipathic, largely α-helical protein called apoliprotein A1. Truncated forms of apolipoprotein A1 termed membrane scaffold proteins (MSPs) were developed that were combined with lipids to form nanodiscs, lipid bilayer discs with the hydrophobic edge encircled by a belt composed of two copies of MSP. Since the development of the original nanodisc format, truncated and extended variants of MSPs have been developed, which permit a greater range of disc diameters capable of accommodating small and large membrane proteins (extensively reviewed by Denisov and colleagues [60]. A recent modification to the classical nanodisc format called 'peptidisc' uses a short amphipathic bi-helical peptide derived from two repeats of a sequence in apolipoprotein A1. Peptidiscs offer some advantages over the nanodisc format as no exogenous addition of lipids is required and peptidiscs can accommodate a range of proteins without optimisation. Following reconstitution with the peptide, excess detergent can be removed by simple dilution [61]. More recently, a single step on-column purification and peptidisc reconstitution has been described. By addition of a biotinylated variant of the peptide, the resulting peptidiscs can be immobilised via streptavidin for affinity determination (and potentially for phage selections) [62].

Encapsulation of a membrane embedded protein in an MSP nanodisc requires the prior solubilisation of the protein with detergents with the risk to stability and activity that entails. The MSPs themselves can be readily expressed in bacterial hosts and purified with a series of detergent wash steps [63]. Of note, while MSPs are potentially immunogenic themselves, species-specific MSPs can be expressed matched to the intended immunised animal. For example, when a mouse is to be immunised, a nanodisc can be reconstituted using a mouse MSP. Nanodiscs are reconstituted my mixing the detergent-solubilised membrane protein, the MSP and detergent-solubilised phospholipids, followed by removal of the detergent on adsorbent polystyrene beads. Following reconstitution, nanodiscs can be purified from excess reagents using affinity chromatography directed to an epitope tag on the target protein. A YidC homolog (a prokaryotic membrane protein insertase) and an ion channel embedded in nanodiscs have been successfully used as antigen for phage display to isolate conformation specific Fabs [64]. A recent report describes the use of nanodiscs containing a human/bacterial ion channel chimaera to isolate channel-binding antibodies. The nanodiscs were biotinylated, fluorescently labelled with Alexa Fluor 647-strepavidin and used to FACS sort hybridoma cells expressing surface B cell receptor specific for the ion channel [65]. A further recent publication describes the use of nanodiscs loaded with human apelin receptor as an immunogen [66]. Apelin receptor nanodiscs were used to immunise camels and a library of single domain antibodies was prepared and selected against the receptor in proteoliposomes.

4.2.2. Salipro

An alternative nanodisc format first described in 2016 is the Salipro (Saposin-lipid-protein) particle system [67]. Here, the amphipathic lipid-binding protein Saposin A serves an analogous purpose as apolipoprotein A1, where Saposin A wraps around the exterior of the lipid disc. The process for loading Salipro particles with a membrane protein target is similar to that required for classic nanodiscs: the target must be initially solubilised in detergent and then mixed with Saposin A and lipids prior to removing detergent. Saposin A has a fold containing 3 disulphide bonds that make it highly stable and may contribute to the stability observed for membrane proteins incorporated in Salipro particles. One potential advantage Salipro particles have is that particle

Table 1
Antigen formats used in antibody isolation strategies.

Format	Detergent extraction	Stability	Purity/complexity	Orientation	Comments/Applications
Peptide	No	+ + +	High purity, chemical synthesis.	N/A	Particularly suited to cases of N-terminal peptide interaction with ligand. Tendency to isolate simple binders without functional effect on target. Challenging to recapitulate correct fold in simple peptide.
Globular extracellular domain (ECD)	No	+ + + +	High purity via standard protein purification methods.	N/A	Only applicable to a subset of integral membrane proteins with a large, functional extracellular domain.
Protein:detergent complexes	Extracted and maintained in detergent	+	When protein is epitope tagged, high purity achievable via affinity and orthogonal chromatographic methods.	Both intracellular and extracellular epitopes exposed	Generally low stability[1]. Continued presence of detergent required; potential interference in assays. Protein:detergent micelle liable to dissociate *in vivo*.
Calixarenes	Yes – calixarenes are a novel detergent class	+ +	Epitope tags enable high purity preparations.	Both intracellular and extracellular epitopes exposed	Compared to conventional detergents, calixarenes have been shown to provide significant thermal stabilisation
Lipid nanodiscs	Initial detergent extraction	+ +	Epitope tags enable high purity preparations.	Both intracellular and extracellular epitopes exposed	Large number of membrane scaffold proteins of different length to accommodate different size target proteins.
Peptidiscs	Initial detergent extraction	+ +	Epitope tags enable high purity preparations.	Both intracellular and extracellular epitopes exposed	Format derived from nanodiscs with MSP replaced with apolipoprotein A1-dervied peptide. Reported to be readily adaptable to different protein sizes.
Salipro particles	Initial detergent extraction[2]	+ +	Epitope tags enable high purity preparations.	Both intracellular and extracellular epitopes exposed	Conceptually similar format to nanodiscs, with apolipoprotein replaced with saposin A. Reported one size fits all approach.
SMALPs	No – direct extraction from membranes/cells	+ +	Epitope tags enable high purity preparations.	Both intracellular and extracellular epitopes exposed	Direct extraction enables isolation of target with native lipids, maintaining stability and function.
Proteoliposomes	Initial detergent extraction	+ + +	Purity dependent on material to be reconstituted.	Both intracellular and extracellular epitopes exposed	Reconstituted format allowing precise control of lipid content tailored to application.
Magnetic proteoliposomes	Initial detergent extraction	+ + +	Purity dependent on material to be reconstituted.	Only extracellular epitopes exposed	As for proteoliposomes, but magnetic core offers two advantages: enforced correct orientation of target and possibility of immobilisation on magnetic beads.
Virus-like particles	No	+ + +	Complex enveloped particle containing a subset of the plasma membrane proteome and intralumenal Gag protein.	Only extracellular epitopes exposed	Productions from stable cell lines or transient expression of target via expression of viral Gag gene. Immunogenic Gag protein can aid in breakage of tolerance.
Membranes	No	+ + + +	Complex samples containing entire plasma membrane contents.	Dependent on precise membrane format, both intracellular and extracellular epitopes exposed	Tetragenetics isolate membranes from *Tetrahymena* enriched in membrane protein targets.
Whole cells	No	+ + + +	Highly complex sample containing membrane and soluble proteins; screening against parental cells required.	Only extracellular epitopes exposed	Provide the highest level of stability and allow the same cells to be used for immunisation and functional screens. Possible use of syngeneic cells to reduce off target binders
DNA	No	(Non-protein antigen)	Can be used to drive production of a single target *in vivo*.	Only extracellular epitopes exposed (upon expression *in vivo*)	Provides production of the target protein *in vivo* following immunisation, theoretically giving no background of other non-host proteins. Possibility to also produce immune stimulatory molecules with multi-cistronic vectors.

[1]Although stabilised receptors (StaRs®) and enabled membrane proteins (EMPs™) offer more stable variants of the format.
2Salipro AB report near-simultaneous extraction with detergent and transfer to Salipro particles.

size is adaptable simply by tuning the Saposin A to lipid ratio, rather than requiring switching to different MSP variants as for nanodiscs. There are several reports of the use of Salipro particles used to determine Cryo-EM structures of complex membrane proteins [68–71]. A recent article details a more concerted method for reconstitution of Salipro particles – termed DirectMX – where the proteins and lipids from a membrane are solubilised in the mild detergent digitonin and then the membrane protein and native lipids are reconstituted into particles by simple dilution [72]. An advantage of such concerted solubilisation methods (which also applies to SMA – see below) is that they are more likely to preserve interactions with weakly interacting associated proteins. To date, there is a dearth of published information on the use of Salipro particles in antibody discovery, although the company commercialising the system (Salipro Biotech) have an application note describing collaborating with a pharmaceutical company to immunise mice with ion channel-loaded Salipro particles to derive monoclonal antibodies [73].

4.2.3. SMALPs

A third discoidal lipid nanoparticle has been developed over the past decade termed the SMALP (styrene–maleic acid lipid particle) in which the role of the amphipathic proteins in nanodiscs is replaced with a polymer. Styrene–maleic acid (SMA) is an amphipathic copolymer of styrene and maleic acid available commercially from several sources. SMA is able to insert directly into lipid bilayers and form pores [74,75], which leads to the extraction of membrane proteins in lipid bilayer discs bounded by the SMA polymer. In these nanoparticles the styrene groups of SMA interact with the hydrophobic acyl chains of the lipids and the maleic acid moieties interact with solvent. One major advantage of SMALPs over the related nanodisc formats is that SMA allows direct extraction from lipid bilayers (either as purified membranes or whole cells), both overcoming the destabilising effects of detergents and co-purifying the membrane protein with native lipid. The resulting particles are highly stable with membrane proteins retaining function at 4°C for weeks and amenable to freeze-thaw. A potential advantage of SMALPs for antibody discovery is that use of a non-protein scaffold avoids generation of anti-MSP/Saposin antibodies that would require counter screens to deplete antibodies that were specific for nanodiscs or Salipro particles.

Although SMA can extract a wide range of different membrane protein classes and the resulting particles are stable, there are several drawbacks with the original SMA polymers. These include their interference with UV spectroscopy due to absorbance in the UV range by the styrene moieties. SMA (and SMALPs) also precipitate in the presence of divalent cations and at low pH (pH $<$ 7), presenting an issue for the study of proteins requiring divalent cations or low pH for function. Several alternative maleic acid copolymers have been developed as reagents that can substitute for SMA and alter particle properties. The most widely used of these is diisobutylene maleic acid (DIBMA, sold by BASF as Sokalan CP9), where the aromatic styrene groups are replaced with acyl chains. DIBMA offers the advantages of being transparent in the UV range for UV spectroscopy and can tolerate higher concentrations of divalent cations [76–78]. DIBMA also leads to a lower level of ordering of lipid acyl chains in the particles than the styrene groups of SMA, which may support a wider range of membrane protein functions. A range of SMA derivatives with modifications to the maleic acid component have been reported which alter the negative charge to a neutral or positive charge and allow use of the polymers at pH and salt concentrations inaccessible to SMA [79,80]. Of interest for antibody discovery applications, where the ability to label or immobilise antigens is critical, SMA can be modified with cysteamine, yielding a polymer with sulphydryl groups (SMA-SH) that can be further derivatised with biotin or fluorophores [81].

SMALPs and related polymer-bound lipid discs have acted as a critical enabling technology in the structural and functional studies of membrane proteins. The particles are compatible with a wide range of biophysical assays used to interrogate membrane protein function. There is a single example where SMALPs were applied to crystallisation: bacteriorhodopsin purified in SMALPs was transferred to a lipidic cubic phase and successfully crystallised and had its structure solved at 2.0 Å resolution [82]. Following the “resolution revolution” in cryo-EM, the technique has been applied to determine the structures of two membrane proteins embedded in SMALPs to sub-nanometre resolution in 2018. The multidrug efflux transporter AcrB was solved to a resolution of 8.8 Å and exhibited an equivalent structure to those determined using other methods [83]. The second structure was of a bacterial electron transport chain component, alternative complex III–cyt aa3 supercomplex with a mass of 464 kDa and a total of 48 transmembrane α-helices [84]. This structure demonstrated the potential for SMALPs to purify large, native membrane protein complexes and allowed the resolution of multiple natively co-purifying lipid molecules.

The use of SMALPs in antibody discovery in published works is limited. A team at Amgen have used SMALPs to isolate the class A GPCR cannabinoid receptor 1 (CB1 receptor) in a highly stabilised form [85]. The authors demonstrated the use of GFP-fused CB1 SMALPs as tools to label cells for sorting via FACS, applying the SMALPs to both yeast and mammalian display systems and obtaining specific labelling. The CB1 SMALPs were additionally used as analytes in SPR to detect binding to a CB1 specific Fab, although a high level of non-specific binding of a control SMALP was detected. Another context where SMALPs have been employed was to verify that an scFv raised to the extracellular domain of Parathyroid hormone receptor 1 (PTH1R) also bound to the full-length receptor. Here, His-tagged PTH1R was expressed in mammalian cells and isolated in SMALP format and SMALPs were immobilised for SPR via interaction with an anti-His antibody on the chip surface – the authors were able to confirm the scFv interacted with the full-length receptor in SMALPs with the same affinity as to the isolated ECD [86].

4.3. Complex membranous environments

Although the discoidal lipid formats offer the advantages of having no detergent present, increased thermal stability cf. detergent micelles and co-purification with native lipids, they are not truly generally applicable formats. Some target membrane proteins are not amenable to the discoidal formats for several reasons, including large size, poor extraction efficiency from the membrane, loss of binding partners required for correct conformation/function. In these cases, more complex membranous systems can be applied that more closely resemble the native cellular environment. Such systems include spheroidal particles such as virus-like particles, extracellular vesicles and proteoliposomes and cruder membrane preps.

4.3.1. Virus-like particles

Virus-like particles (VLPs) are non-infectious broadly spheroidal structures whose formation is driven by a viral capsid protein, and which originally came from basic research into the structure and function of viruses. Of interest for use in antibody discovery are the enveloped VLPs whose formation is driven by the overexpression of a structural Gag protein from a lentivirus or other retrovirus family member. The VLPs are generated simply by overexpressing a Gag protein in a cell line of interest – the Gag protein localises to the intracellular surface of the plasma membrane and induces curving of the membrane that leads to budding of VLPs. The VLP surface is composed of a layer of lipid bilayer extracted from the plasma membrane and contains a subset of the membrane proteome embedded within it. The most commonly used viral Gag proteins are those of the lentivirus HIV-1 and the retrovirus murine leukemia virus (MLV), both driving production of VLPs with diameter 100–200 nm. The resulting VLPs can be derived from cells overexpressing a membrane protein target and are often enriched 10–100-fold for the target as compared to the levels in the cell. To convert VLPs into more useful tools, they can be readily modified, e.g. by genetic fusion of a component to a fluorescent protein

to allow their use in flow cytometry. Traditional methods for purification have involved sucrose density gradient ultracentrifugation or incubation with PEG to precipitate the VLPs followed by a low speed centrifugation step. More recently, chromatographic methods have been developed to achieve higher levels of purity, including the use of ion exchange [87,88] and bind-elute size exclusion chromatography [89,90]. In the latter method, a crude VLP prep is applied to a resin containing beads with a porous shell and an interior with hydrophobic and charged ligands – VLPs are larger than the pores in the shell and pass through the column while impurities enter the bead interior and are removed from the VLPs.

VLPs isolate membrane proteins in their native phospholipid bilayer environment and without the constraints imposed on bilayer fluidity and curvature seen in the discoidal formats. As a result, membrane proteins are often obtained in a highly stabilised, functional form and with interacting proteins present, making them ideal tools for antibody discovery and screening. VLPs can be used in multiple applications during the therapeutic antibody development process. They can be applied both for *in vitro* selections in the context of phage display and for immunisation (or both in combination), where the highly immunogenic viral Gag protein can act as an adjuvant to boost the immune response. VLPs can also be used in screening antibodies, being highly amenable to ELISA and other screening formats, and can be immobilised on SPR chips for affinity determination.

The leading company in the development of VLPs for antibody discovery is Integral Molecular (Philadelphia, USA), who have developed an MLV-based VLP platform. They have reported use of VLPs in immunisation, phage display and screening, isolating antibodies against a range of membrane protein types, including GPCRs (e.g. CB1R and C5aR), ion channels (P2X family members) and transporters (GLUT4). In the case of GLUT4, VLPs were used to immunise chickens, which are more evolutionarily divergent and were used to ensure a good immune response against the highly conserved transporter [91]. Plant derived VLPs (produced in *Nicotiana benthamiana*) displaying on their surface a GPI-anchored malarial protein have been used to immunise human immunoglobulin transgenic mice to isolate antibodies that can block parasite development [92].

4.3.2. Proteoliposomes and magnetic proteoliposomes

Proteoliposomes are another near-native membranous format consisting of a lipid bilayer vesicle (a liposome) into which a membrane protein is reconstituted. Proteoliposomes have a key difference to other cell-derived vesicular formats in that they are a reconstituted format more akin to nanodiscs, and hence no native lipids are present in the final particles. The absence of native lipids has the disadvantage that lipids critical for correct folding or function can be lost. However, there is also the advantage that the lipids present in the liposome can be carefully controlled. Lipids can be screened for those that provide long-term stability and retention of function. Proteoliposomes can also be doped with lipids that provide additional functions: phosphoryl lipid A can be incorporated to act as an adjuvant [93,94]; fluorescent lipids can be added to allow use in various binding assays; and, biotinylated lipids can be incorporated, allowing immobilisation on streptavidin coated plates or beads [95]. Biotinylated proteoliposomes also enable an antibody:target interaction assay termed the biotinylated liposome-based interaction assay (BiLIA) [96].

Proteoliposomes are generally derived in one of two processes. In both cases, the first step is the generation of protein-free liposomes. Liposomes are formed by mixing the desired lipids at a defined ratio in chloroform and then drying the lipids on the surface of a glass container to remove the solvent. The lipid film is then hydrated in buffer and liposomes prepared either via sonication or through extrusion of the lipid suspension through a polycarbonate filter of defined pore size. One method for transfer of a membrane protein to the liposomes is to detergent solubilise the membrane protein and prepare protein:detergent micelles. These are then mixed with liposomes saturated with detergent to enable incorporation. Following an incubation step, the detergent is depleted from the preparation to yield the final proteoliposomes [97]. An alternative method to form proteoliposomes avoids the use of destabilising detergent. Instead, the membrane protein target is translated from an mRNA in a cell-free system in the presence of liposomes and is directly incorporated [94,96]. A further modification to the reconstitution process allows generation of paramagnetic proteoliposomes [98,99]. In one example, paramagnetic beads were coated with the Rho 1D4 antibody which was used to immobilise detergent-solubilised CCR5 chemokine receptor on the bead surface via a C-terminal Rho 1D4 tag. Detergent-solubilised lipids were then coated onto the beads and detergents were removed via dialysis. The use of paramagnetic beads confers two advantages. Firstly, by using an epitope tag for immobilisation, it ensures correct orientation of the membrane protein in the proteoliposomes membrane, in comparison to a non-magnetic proteoliposome where orientation is random. Secondly, the magnetic properties of the bead can be used to pull down the beads for wash steps during phage selections.

A recent example of the use of proteoliposomes as an antigen format for discovery of antibodies targeting ion channels employed the protozoan *Tetrahymena thermophila* as an expression host. The company Tetragenetics have developed tools to enable the genetic manipulation of the organism to drive stable overexpression of target proteins, particularly ion channels and transporters. Following overexpression of the $K_v1.3$ potassium channel, protein was extracted, purified and reconstituted into proteoliposomes and these preparations were used in immunisation strategies in chickens and llamas to isolate potent and specific channel blocking anti-$K_v1.3$ antibodies [100].

5. Antibody generation strategies

Having established methods for generating validated preparations of purified antigen a variety of different approaches can be considered to generate antibodies. There are broadly two approaches to generate antibodies targeting complex membrane proteins (CMPs) such as ion channels, GPCRs and transporters. These typically rely on immunisation of laboratory animals, harnessing the natural immune response, or *in vitro* display methods, such as phage, yeast or mammalian display [18,101,102]. Most functional antibodies to date against CMP targets have been generated via immunisation either in isolation or via generation of immune display libraries. In this review we will consider both immunisation strategies and phage display strategies and how these can be tailored according to the antigen generation strategies adopted to drive antibody discovery campaigns.

Table 2 provides a summary of some recent published literature covering antibody generation to CMPs. The main hosts used for antibody generation are rodents, with the species of choice being mice (whether native or transgenic humanised). This likely reflects their ready availability within Academia and Pharma, ease of handling, and well-established methodologies for antibody generation via the mouse hybridoma approach [103]. However, other hosts are increasingly being used with camelids providing an established platform for nanobody generation and some examples of chickens being used very effectively [104].

Immunisation strategies used to generate antibodies which functionally modulate CMPs fall into three broad categories in terms of approaches to introduce target proteins of interest as immunogens: (1) engineered cell lines overexpressing the target protein, (2) purified or enriched protein antigen derived from a heterologous expression system, and (3) genetic immunisation using a cDNA antigen expression plasmid. These immunisation methods are not mutually exclusive, and often multiple antigen formats are used in orthogonal approaches and/or parallel immunisation strategies to ensure isolation of a broad panel of antibodies [105].

Table 2
Monoclonal antibody isolation strategies for G protein-coupled receptors, ion channels and transporters.

TARGET	TARGET CLASS	ANTIGEN FORMAT	ANTIBODY ISOLATION METHOD	ANTIBODY FUNCTION/ REFERENCE	NUMBER OF HITS	FUNCTIONAL HITS
Endothelin A receptor	GPCR	Overexpressing Cells; N-terminal ECD	Immunisation/Mouse	Inhibitor [194]	613	Multiple; 1 mAb selected
Apelin Receptor	GPCR	Thermostabilised receptor in nanodiscs	Immunisation/Camel; single domain antibody phage library	Inhibitor [66]	186	106; 1 antagonist converted to agonist by structure-based design
ASIC1a	Acid sensing ion channel	ASIC1a protein in nanodisc	Phage library selections	Inhibitor[195]	6 described	1
P2X4	Ligand gated ion channel	P2X4 purified protein	Phage library selections	Inhibitor/Potentiator [196]	33	6 inhibitors; 8 potentiators
$K_v1.3$	Voltage-gated ion channel	Plasmid DNA; Purified $K_v1.3$ reconstituted in liposomes	Immunisation/Chicken Immunisation/Llama	Inhibitor [133]	69	10
GLUT4	Glucose transporter	GLUT4 virus-like particles	Immunisation/Chicken; Immune phage display library	Inhibitor [91]	29	2 state-specific mAbs
ASCT2	Glutamine transporter	Rat hepatoma cells expressing human ASCT2	Immunisation/Rat	Inhibitor [197]	Not disclosed	1 antagonist

Selected recent examples of antibody isolation strategies generating antibodies which bound to GPCR, ion channel and transporter targets and modulated target protein function.

5.1. Immunisation with engineered recombinant cell lines

Engineered recombinant cell lines designed to over-express the target protein may be used directly as a source of membrane protein in its native conformation for use as an immunogen. These cell lines may also be used as a source of target protein for purification and reconstitution methods or provide a source for target protein enrichment via generation of VLPs. The cells can also be used a tool to screen for antibodies that bind to the target CMP, using the parental host cell to eliminate any non-CMP target binders. Typically, HEK or CHO cells are used as the host for transgene expression. These cell lines are readily available and have been used extensively for transgene over-expression either transiently or by generation of stable cell lines. The primary advantage of using over-expressing cell lines for immunisation is that they present the target protein to the immune system in its native conformation. This approach allows for the generation of binders to the target in its native, folded conformation, maximising the chances of generating functional binders to the target. Since neither HEK or CHO cells are derived from a commonly used hybridoma host, the whole host cell proteome, in addition to the target CMP antigen, will potentially be recognised as non-self by the host immune system. Whilst this may have some benefit in that the host cell proteins could produce an adjuvant effect to boost immune response to the target immunogen, the host cell proteins will also give rise to an animal immune response. This will have an impact on the breadth and depth of antibody generation to the target CMP. It is therefore important to screen recombinant cell lines to ensure high level expression of the target CMP. This can be very challenging for GPCR, ion channel and transporters and in our experience is very target dependent. We typically aim for expression levels of 10^6 target proteins per cell. However, even with this level of over-expression (and immunisation protocols using 10^7 cells per animal) the actual amount of target protein introduced as immunogen has been calculated to be less than a tenth of what would be administered for a purified target protein. Given this challenge, strategies to prime the immune response (e.g. by using DNA encoding the target as immunogen) followed by a boost with recombinant cell lines could be explored on a case by case basis.

Given the potential to generate antibodies to the host cell proteins, alternative approaches can be used in which cell lines are generated in a host cell which is more closely matched genetically to the host animal (i.e. a syngeneic approach). If a mouse is the desired host animal, this approach can employ either a general mouse cell line or a specific isogenic mouse cell line [106]. In the case of isogenic host cell lines, the host expression cell is theoretically silent to the immune system except for the human CMP transgene it is carrying [106,107]. A further option to optimise the immunogenic response to the CMP target is to immunise mice in which the mouse homologue of the target CMP is deleted from its genome (i.e. a transgenic knockout mouse), although not all genetic knock-outs are viable. Alternatively, one can immunise a non-transgenic mouse with cells from the same genetic background which have the human target CMP inserted into its genome – a transgenic knock-in mouse [108]. In a further development aimed at improving generation of antibodies to CMP targets, a recent publication described the use of cells expressing the target antigen and cell membrane bound cytokines as adjuvants. Huang et al. described establishing 'cell adjuvants' by expressing membrane bound mouse IL-2, IL-18 or GM-CSF on BALB/3T3 cells which were shown to stimulate splenocyte proliferation *in vitro*. These cells were initially tested with transient overexpression of a simple, single-pass transmembrane protein (ecotropic viral integration site 2B (EVI2B)), with GM-CSF found to elicit the highest anti-IVEI2B response. Of more relevance to antibody discovery on CMP targets, the authors went on to co-express membrane bound GM-CSF and the chemokine receptor GPCR CXCR2, eliciting an improved anti-CXCR2 response, with the isolated mAbs demonstrating specific binding to CXCR2 overexpressing HEK cells [109].

5.2. Immunisation with purified or enriched recombinant proteins

A plethora of approaches can be considered for immunizing with purified or enriched samples of protein antigens. At the simplest level, a synthetic peptide will represent the sequence of an extracellular loop or N-terminus and can be used as antigen (reviewed in Lee et al. [21]). Membrane protein-derived peptides can be successfully used in antibody discovery particularly where binding is sufficient for the desired antibody function such as those for antibody-dependent cellular cytotoxicity (ADCC, e.g. mogamulizumab) or antibody-drug conjugates. However, peptides alone may not recapitulate the structural context and folding required to use as an antigen to generate the intended therapeutic and for the purpose of this review we will focus on recombinant protein generation strategies. The choice of strategy employed to express and purify a recombinant CMP target can be directed by what is known about the topology and structure of the protein. In some cases, the CMP may have a well-defined extracellular domain (ECD) which folds into a conformationally relevant structure. In these cases, it is possible to express a recombinant version of the ECD and purify as a soluble protein which can be used as an antigen. This approach is exemplified by Class B GPCRs which have well defined N-terminal domains that bind peptide hormone ligands. An example of this is the isolation of antibodies by phage display to the N-terminal ECD of glucose-dependent insulinotropic polypeptide receptor (GIPR) [110]. In another example, the complex of the ECD of the CGRP receptor and the ECD of the accessory protein, receptor activity-modifying protein-1 (RAMP1), was used to generate anti-CGRP receptor antibodies following immunisation of the Xenomouse [111].

Where the CMP targets have less well-defined extracellular domains the proteins may be expressed in a heterologous expression system and purified. This requires empirical approaches to define the best expression system and the optimal method for detergent extraction and subsequent purification. Proteins in detergents are often unstable and several methods are available to improve stability. This can include mutagenesis of the protein to improve thermostability and maintain ligand binding in detergent micelles (an approach successfully used by Sosei Heptares [112]), use of specialised detergents or polymers which improve stability (e.g. calixarenes, SMA [56,113]) or reconstitution into lipids [95]. These methods have been described in detail in Section 4. Only after a protein preparation has been validated by biophysical and pharmacological characterization may it be used to support antibody discovery efforts.

5.3. Genetic immunisation

Genetic immunisation discovery strategies utilise DNA expression constructs encoding the target gene of interest which is typically delivered to a host animal by the intradermal route using a variety of techniques and technologies. These include simple needle injections (including Dermajet) [114,115], biolistic particle delivery systems such as Gene Gun (Bio-Rad) [104,116,117], where DNA coated inert gold particles are fired into cells in the dermis, or via electroporation [118–122]. Electroporation requires the injection of a DNA vector containing the transgene into skin or muscle in solution before applying microneedle electrodes around the injection site. A high-voltage electrical pulse is then applied which causes opening of nanopores in cell membranes and uptake of the DNA expression construct. With each of these protocols, introduction of the DNA expression vector into the cell drives protein synthesis and expression of the target CMP antigen to stimulate an immune response. This method of immunisation circumvents some of the issues relating to use of cell lines expressing the target protein as the antigen is expressed in a host cell that is regarded as self by the immune system. Furthermore, *in vivo* expression should ensure the protein adopts the correct conformation in the host cell membrane and is post-translationally modified. Expression constructs containing the gene of interest are facile to generate and relatively inexpensive to produce and can be manipulated to change promoter strength or use promoters tailored to specific host tissues. However, compared to cell, protein and peptide-based immunisation strategies, the immune response to DNA immunisation is markedly lower giving lower serum titers and is slow to develop, which means a prolonged antibody generation campaign may be required and often significantly fewer lead antibodies are derived from these methods (reviewed in Saade and Petrovsky, 2012 [123]. However, it is interesting to note that in one study comparing multiple protocols, a combined DNA/cell method, although giving the lowest antibody titer produced the highest number of antigen specific clones [105]. Whilst this study did not use complex multi-spanning membrane proteins as antigens, the observation may be translatable to these types of antigen. Examples of priming the immune response with DNA immunization in combination with other sources of antigen (e.g. cells, membranes or protein) are described later in this section.

Several other approaches have been described for delivery of expression plasmid DNA to initiate immunisation. Hydrodynamic tail vein (HTV) injection of expression plasmid DNA (see for example Takatsuka *et al.* [122]; Hazen *et al.* [124]; CRTH2 patent application (Genentech) [125]) is an efficient procedure to deliver DNA to the liver [126]. This procedure relies on force generated by the rapid injection of a large volume of a physiological solution to increase the permeability of cell membranes of perfused organs, and thus deliver DNA into these cells. Three key aspects of the procedure are critical for success: correct insertion of the needle into the vein, the volume of injection, and the speed of delivery [127].

Hazen *et al.* [124] described the generation of antibodies to the 12-transmembrane spanning transporter, multi-drug resistant protein 4 (MRP4). In this example, MRP4 expression plasmid was co-administered with plasmids expressing the 'molecular adjuvants' mouse Flt3 and GM-CSF. The plasmids were administered by HTV injection and antibodies binding to extracellular surface epitopes were obtained. Conventional immunisations of Balb/c and MRP4 C57BL/6 knock out mice were unsuccessful using detergent-solubilised membrane protein or MRP4 expressed on HEK293 and LnCAP cells, as was the immunisation of MRP4 KO mice with insect cell-derived MRP4 protein. Only DNA immunisations resulted in antibodies that bound specifically to cell-surface expressed MRP4. Furthermore, co-administration of molecular adjuvants improved the reproducibility of the serum response to MRP4. However, there was only a marginal increase in the number of hybridomas expressing extracellular MRP4 binding antibodies. In this example it is noteworthy that different promoters were studied (CAGGS and CMV promoters) and the CAGGS promoter increased the overall efficiency of DNA immunisation compared to the CMV promoter allowing a reduction in the frequency of immunisations whilst maintaining a high immune response to the target protein. This group used the same technique to generate antibodies to CRTH2 (described in a patent application [125]). Takatsuka *et al.* [122] also employed an immunisation regime delivering CCX-CKR and GroEL expression plasmids (employing GroEL as an adjuvant) using *in vivo* electroporation immunisations, followed by a final boost by HTV injection to obtain anti-CCX-CKR antibodies.

The use of viruses as a method to deliver antigen encoding genes to cells is also an interesting strategy that has been explored. Genetic immunisation using adenovirus as the delivery vehicle has been described by Amgen for two CMP targets: the iron transporter Ferroportin [119,128] and transient receptor potential ankyrin 1 (TRPA1) ion channel [129]. In these studies, mice received a priming injection of Adenovirus containing a CMV-driven expression construct encoding the target gene of interest and a T helper epitope (PADRE [130]). Subsequently three plasmid-based immunisations were given by intradermal injection followed by *in vivo* electroporation and a final intraperitoneal boost with membranes from cells expressing the same CMP-PADRE constructs. In the case of Ferroportin, two BALB/c mice were used to generate 4000 hybridomas and 37 Ferroportin binding antibodies were

identified, of which one was functionally active. The authors also generated Ferroportin antibodies from two strains of transgenic mice (the Xenomouse™). In this case mice were immunised with either HEK293 cells over-expressing Ferroportin or membrane preparations from cells over-expressing Ferroportin via sub-cutaneous or intraperitoneal injection. This more conventional hybridoma approach in which 7600 hybridoma supernatants were screened led to identification of 200 Ferroportin binding antibodies of which 11 were functionally active (Ferroportin patent application, Amgen [128]).

The use of viral vectors as tools for delivery of expression cassettes is intriguing. Indeed, there is a great deal of work in the vaccine field already with Adenovirus immunisations, which show this method induces potent transgene product–specific T- and B-cell responses (reviewed in Lasaro & Ertl [131]). Furthermore, the Adenovirus itself may potentiate the immune response to the target gene by being highly immunogenic in its own right. Viral transduction may be more efficient than naked DNA delivery methods, and the transgene expression is longer lived than plasmid-based methods of delivery. However, therein potentially also lies the drawback for this route of genetic immunisation. The persistence of antigen expression and the high anti-virus titres generated by repeat dosing may hinder the development of a hyper-immune response required for hybridoma generation.

The three immunisation approaches described above (over-expressing cell lines, recombinant proteins and genetic immunisation) have been used in isolation to successfully generate functional binders to CMPs. However, as a general observation it is more common to see different and complementary combinations of antigen sources being employed in parallel to maximise the diversity of antibodies isolated and increase opportunities to isolate antibodies with desired properties [114,115,117]. A large panel of binders provides drug developers a choice of antibodies, to enable the most appropriate epitope to be targeted for function, as well as finding an antibody with the best characteristics in terms of 'developability' (e.g. manufacturability, formulation) or species cross reactivity to allow for its successful development as a candidate drug.

Whilst hybridoma strategies have proved very effective to isolate functional antibodies, whether using mice or transgenic humanised mouse hosts, classical hybridoma technology does have limitations. These limitations include poor efficiency of the cell fusion process (resulting in a significant loss of antibody repertoire), poor immunogenicity of targets that have high sequence conservation in mammals and difficulties in isolating antibodies that are rodent cross reactive which is a pre-requisite for testing antibodies in animal disease models. To address these limitations alternative antibody discovery platforms have been developed including phage, yeast and mammalian display. In addition to these methods, a number of alternative strategies are emerging which allow immunisation in animal hosts but which do not rely on generation of hybridomas. These methods rely on directly evaluating the B-cells from immunised animals to directly identify antibody-secreting B cells. These methods employ microencapsulation of B cells and reporter cells and an increasing array of microfluidic techniques. A recent example of this type of approach was described by Könitzer *et al* who used immunisation of chickens with human GIPR (as chicken and human orthologues have only 37% sequence identity) followed by screening using gel encapsulated microenvironment (GEM) screens [104]. The GEM assay comprises capture of single antibody secreting B cells in a droplet which also contains particulate reporters which can be beads, cells or other particles (e.g. VLP). Secreted antibody diffuses within the GEM and binds to a target protein displayed on the reporter followed by detection with a labelled secondary antibody [132]. Using this approach, a large and diverse antibody panel was generated containing 172 unique sequences with three-quarters of the antibodies being functional antagonists [104]. This GEM based approach has also been used to identify chicken antibodies to the ion channel $K_v1.3$ [133]. Alternative single-cell droplet techniques coupled to microfluidic screening have been described by Shembekar *et al* which employed capture of antibody secreting cells and tumor cells in the same microdroplet followed by microfluidic screening and detection [134]. Commercial platforms are also emerging such as the Berkeley Lights Beacon™ system which employ nanofluidic B lymphocyte screening techniques coupled with the ability to manipulate living cells using OptoElectro positioning to move cells into so called 'nanopens' where cells secreting antibody can be detected [135]. The development and application of these technologies coupled to the use of new target protein formats for complex membrane proteins holds great promise.

5.4. Phage display

Phage display is an *in vitro* selection method for isolating affinity reagents. It is based on George Smith's discovery that foreign DNA encoding a peptide could be incorporated into the gene encoding the filamentous phage coat protein III (pIII), the peptide could be expressed on phage as a pIII-fusion, and phage could be selectively enriched by an antibody that recognised and captured the displayed peptide [136]. Later Parmley and Smith showed that a biotinylated antibody could enrich phage displaying a reactive peptide from 10^8-fold excess virions [137]. This method is now commonly known as 'biopanning' likely due to the similarity of the process for gold panning – undesirable materials are removed from desirable ones. Biopanning has shown that peptide or protein libraries displayed on phage can be selected agnostically via affinity to a desired target, where selected phage can be identified by DNA sequencing due to the link between phenotype and genotype. Of interest to antibody discovery, John McCafferty and Gregory Winter showed that phage could display the variable domains of the anti-chicken lysozyme (D1.3) expressed as single-chain Fv (scFv) and selected *in vitro* for binding to chicken lysozyme [138]. This seminal work forms the basis of antibody discovery by phage display as we know it today, where antibody fragment combinatorial phage libraries, including scFv, fragment antigen-binding (Fab), and camelid VHH (nanobody) libraries, are constructed from naïve/non-naïve immune cells [139] or synthetic sources [140] and enriched through affinity to a desired target. This *in vitro* technique can accelerate the identification of relevant antibodies for libraries derived from immune cells and can access epitopes unavailable through animal immunisation for libraries derived from synthetic sources. The composition of phage display libraries differ in their source (naïve, non-naïve, synthetic), binding format (peptide, antibody fragment [141–144], anticalin [145], affibody [146], designed ankyrin repeat protein (DARPin) [147], fibronectin type III domain [148], i-body[149], and fusion site on phage (protein III, protein VIII, protein VI, protein VII, protein IX). For the purpose of this review, we will focus on phage display libraries composed of antibody fragments (scFv, Fab and camelid VHH domains) and the role antigen presentation format contributes to the successful isolation of antibodies to integral membrane proteins.

General protocols for phage display are described elsewhere [101,150–153]. Briefly, phage display selections consist of immobilization of target antigen on a suitable support, exposure of combinatorial phage libraries to immobilised target antigens, removal of unbound phage through wash steps, and amplification of specific phage in TG1 *E. coli.* Generally, multiple rounds of affinity selection are required, which may incorporate reduced levels of target antigen, stringent wash steps, or different antigen presentation formats to drive selection of target-specific phage. Analysis of input and output phage titers is used to monitor phage enrichment during each round of selection [151], DNA sequencing is used to monitor phage enrichment and clone diversity [154] and ELISA or flow cytometry measurement in either phage or antibody fragment formats [151] is used to assess target-specific binding.

It is important to note that the expression level of antibody fragments displayed on phage may strongly influence selection efficiency. For example, it has been reported that competition between unmodified and antibody fragment-modified phage coat proteins, proteolytic

degradation, or unintended stop codons can lead to as little as 6–10% of phage displaying the antibody fragment [155,156]. Of course, expression on phage is limited by the intrinsic size, stability, and bacterial expression level of the antibody fragment to be incorporated, where small, stable and well-expressed antibody fragments are preferred. For combinatorial libraries derived from naïve and non-naïve animals, internal biases of the host immune system, including framework and CDR sequences, dictate the properties observed. However, for combinatorial libraries from synthetic sources, fragment antibody libraries based on single, optimised scaffold frameworks that exhibit desirable properties, such as those libraries in scFv [157] and Fab [158,159] formats, provide a shortcut to therapeutic discovery by phage display. In these cases, library diversity is achieved synthetically through randomization of the scaffold framework's CDR loops (sequence and length) with a bias for naturally occurring positional and chemical diversity [160].

Phage selections on membrane protein extracellular domains in part or in whole circumvent the challenges of other membrane protein display formats as protocols for soluble proteins can be used without optimization. For example, peptides of CXCR2 [161] and CCR2 [162] and soluble extracellular domains of GIPR [110], GLP1-R [163], and PTH1R [164] have been used in successful phage selections. The challenge for peptide and extracellular domain formats is identifying relevant peptides and extracellular domains that recapitulate the full-length antigen presented on cells. This generally requires additional steps to ensure antigens of appropriate relevance and quality. In work described by Boshuizen et al., peptides of CXCR2 were pre-screened to identify the IL-8 (CXCL8) binding site prior to being used in phage selections aimed at identifying CXCR2 antibodies that antagonised IL-8 binding [161]. In the absence of methods to ensure appropriate antigen quality, phage selections performed on antigens in multiple formats can promote isolation of relevant antibodies. For example, phage selections aimed at identifying scFvs that antagonise GLP1-R used both the GLP1-R extracellular domain and GLP1-R-expressing cells [163]. Sometimes, however, peptides and extracellular domains derived from target antigens are insufficient [165,166]; in this case, antigen formats that incorporate the full-length protein may better lead to the desired outcomes.

Phage selections on full-length, purified membrane protein antigens displayed in either detergents, proteoliposomes or nanodiscs offer an alternative to peptide- and extracellular domain-derived antigens for phage selections. Unlike phage selections on peptides and extracellular domains, these formats expose both extracellular and intracellular protein domains for phage binding; isolated antibody fragments targeting the extracellular domains of membrane proteins are relevant for therapeutic applications, whereas isolated antibody fragments, particularly Fabs and nanobodies, may be used in applications aimed at locking membrane proteins in specific conformations for structural characterization or *de novo* antibody discovery efforts.

In brief, full-length membrane proteins are expressed in cells, solubilised from cellular membranes with detergents [167], and reconstituted in protein-detergent complexes, proteoliposomes or nanodiscs before or after purification. Generally, extensive detergent optimization is required and conversion to proteoliposome or nanodisc formats is preferred [98,168] as detergents have been shown to compromise the stability and homogeneity of target antigens [168]. However, successful phage selections have been reported for membrane proteins in detergents. For example, CitS [169,170], KcsA [171], transporters and a cation channel [172], and CorA [168] were isolated from Fab libraries and Bpe [173], TM287/288, GlyT1, and ENT1 [174] were isolated from nanobody libraries. Surprisingly, selections from scFv libraries have not been reported. Though detergent chain length [175] and binding moiety size can affect the availability of epitopes on membrane proteins, detergents may also influence characteristics of the binding moiety displayed on phage or the phage itself [176]. Our own experience serves as reference. When we performed phage selections on a detergent-solubilised GPCR using either DARPin or scFv phage libraries, DARPins but not scFvs could be isolated that bound and antagonised the receptor (unpublished results). Our experience suggests that scFvs may not be a suitable format for phage selections on membrane protein antigens-solubilised in detergents, perhaps, due to some instability of the binding moiety caused by detergent. Therefore, considerable thought is required for not only the platform for presenting the membrane protein but also how that choice may influence the selection of suitable phage libraries. At present, next generation detergent alternatives, such as styrene maleic-acid [113,177] and calixarene [178], have received increased attention due to their application in the stabilization, purification and structural characterization of many membrane proteins. It will be interesting to see how these detergent alternatives behave in phage selections.

Membrane proteins displayed in liposomes and nanodiscs offer a stable, native-like lipid environment for phage selections of target antigens without limitations to the antibody fragment library format. For example, successful phage selections have been reported for CCR5 [98], CXCR2 [99], VGLUT1 [179], CHRM2 [95], and CorA [168] using liposomes or nanodiscs, where some of the isolated antibody fragments possess non-linear epitopes. To further support preference for phage selections on membrane proteins in liposomes or nanodiscs over detergents, Dominick and colleagues performed side-by-side phage selections on membrane proteins displayed in either nanodiscs or the detergent dodecyl maltoside (DDM). This work showed that isolated Fabs showed bias in affinity, display format, detergent sensitivity, and non-specific binding for the platform from which they were derived, where Fabs from the nanodisc protocol consistently showed stronger affinity for target, less dependence on the display format for antigen recognition, less sensitivity to different detergents, and less non-specific binding [168]. These observations suggest that differences in epitope exposure, protein conformation, and the chemical composition of detergents and lipids in each of the formats impact the characteristics of leads isolated by phage selections; therefore, formats that closely mimic the native environment of target antigens is preferred.

Phage selections on whole cells ensures proper presentation of membrane proteins in their native context. For this format, cells, either in monolayers or in suspension, are exposed to phage libraries, washed to remove non-specific binding phage, and immobilised phage are recovered. Due to the high background of non-target-to-target antigens and complications arising from non-target-mediated phage adsorption on cells, considerable effort must be invested in the optimization of membrane protein expression constructs, generation of high-expressing cell lines, and depletion of off-target binding phage. Although cell-based selections face these challenges, selections have been successful for the isolation of antibodies/nanobodies to CCR4 [180]. In the work by Hagemann and colleagues, the authors described a competitive elution approach using the CCR4-specific ligands CCL17 or CCL22 to isolate antagonistic antibodies to CCR4 [180]. Although not GPCRs, ion channels or transporters, the successful cell-based phage selections on integrin α11β1 [166] and CD44 [181] highlight advantages that cell-based phage selections offer over other antigen display formats. For example, Gallo and co-workers reported that cell-based phage selections yielded more diverse, potent, and selective antibodies for the integrin α11β1 than those obtained through conventional phage selections on the recombinant extracellular domain [166] and Kavousipour and colleagues showed that cell-based phage selections resulted in nanobodies with improved specificity than those derived from protein- and peptide-based campaigns [181]. Therefore, cell-based phage selections can be successful but may require efforts to optimise target antigen expression and deplete non-target binding phage.

Recently, methods to accelerate generation of high-expressing, stable cell lines, such as that described in the Tag-on-Demand approach [44], and methods to improve enrichment of target-specific phage in cell-based selections have been developed [182]. In this work, Jones and colleagues described an optimised cell-based phage selection protocol that utilised transient expression of the target membrane protein

antigens fused to green fluorescent protein, which enabled enrichment of high expressing cells along with immobilised phage by fluorescence-activated cell sorting (FACS) [182]. This approach creates a natural phage deselection and selection step; phage immobilised on cells that do not express the target antigen well are deselected, whereas phage immobilised on cells that express the target antigen well are selected for phage amplification. The result is phage enriched for target-specific reactivity. The protocol also demonstrated the importance of alternating cell lines and incorporating stringent washing steps to remove cell-specific and non-relevant phage binders [156]. Furthermore, this work highlights that the structure of the target antigen influenced cell-based phage selections; elongated proteins like CD117 with five immunoglobulin domains yielded more unique binders than CD83 with only one immunoglobulin domain, where this observation could be due to several factors, including the number of antigen-specific epitopes presented, accessibility of antigens to binding phage, and, in our hands, conformational flexibility of target antigens.

Recently, work by Integral Molecular has highlighted the utility of membrane protein display in virus-like particles (VLP) for phage selections from non-naïve B cell libraries. VLPs are highly stable, immunogenic, lipid-enveloped retroviral particles capable of concentrating membrane proteins on their surface to levels greater than host cells [183–185]. They can be used directly in phage selections or biotinylated [186,187] and used in phage selection schemes incorporating competitive deselection. Therefore, VLPs represent an attractive membrane protein format for phage selections, where antibodies to GLUT4 [91], P2X7 (Integral Molecular, unpublished), GCGR [120], CB1, C5AR, CXCR5, CGRPR [188], P2X3 (Chambers R. Integral molecular presentation: new approaches for mAb discovery against GPCRs, ion channels, and transporters. CHI 14th Annual Discovery on Target; 2016 Sept 19–22; Boston, USA.), and CXCR4 [149] have been reported.

6. Conclusions

The considerable growth of approved mAb therapeutics to treat a whole spectrum of diseases illustrates the advances that have been made in isolation, development and clinical approval of functional antibodies. GPCRs, ion channels and transporters are attractive targets to modulate with mAbs and significant efforts are being employed discover antibodies which both bind to and functionally modulate the target proteins. These complex, multi-spanning integral membrane protein targets remain technically challenging for functional antibody discovery for several reasons. High-level expression of these proteins in heterologous systems and subsequent extraction of the proteins from the membranes as purified high quality and conformationally stable protein antigens requires a significant investment of resources and diverse strategies. Approaches to antigen generation for antibody isolation is influenced by the structural diversity of these proteins. In some cases, large extracellular domains which fold into stable structures are present which may offer a ‘simple’ route to generation of a suitable antigen. For such cases this may offer a clear strategy for antibody isolation. It is more often the case, however, that investigation of multiple antigen formats is required to ensure a stable, correctly folded protein is generated for use as an antigen. These antigen formats may then be used to support antibody discovery by application of various strategies which employ hybridoma, B cell platform, display technologies or a combination of these techniques to ensure generation of a diverse panel of antibodies. The search for functional antibodies that target complex integral membrane proteins is increasingly employing new platforms for antibody discovery. A number of emerging strategies are employing new antigen formats, alternative hosts (e.g. chickens and camelids), conformationally constrained antigens and a variety of microfluidic discovery platforms. In this review we have outlined developments in the options that are available for antibody discovery and which are shaping antibody generation strategies. The steady growth in publications describing functional antibodies identified in pre-clinical research programs along with increased numbers of antibodies entering clinical studies suggest we are overcoming the challenges inherent in targeting complex integral membrane proteins with antibodies. This provides a reason to believe that we will see further approvals for antibodies against these targets and potential benefits to patients.

7. Compliance with Ethical Standards

Conflict of Interest

Roger Dodd, Trevor Wilkinson, Zachary Britton and Darren Schofield are full-time employees of AstraZeneca, a company generating therapeutic antibodies to complex membrane protein targets for the treatment of a variety of diseases.

Funding

No sources of funding were used to support the writing of this review.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2020.05.006.

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