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Advanced applications of Nanodiscs-based platforms for antibodies discovery

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ABSTRACT

Due to their fundamental biological importance, membrane proteins (MPs) are attractive targets for drug discovery, with cell surface receptors, transporters, ion channels, and membrane-bound enzymes being of particular interest. However, due to numerous challenges, these proteins present underutilized opportunities for discovering biotherapeutics. Antibodies hold the promise of exquisite specificity and adaptability, making them the ideal candidates for targeting complex membrane proteins. They can target specific conformations of a particular membrane protein and can be engineered into various formats. Generating specific and effective antibodies targeting these proteins is no easy task due to several factors. The antigen's design, antibody-generation strategies, lead optimization technologies, and antibody modalities can be modified to tackle these challenges. The rational employment of cutting-edge lipid nanoparticle systems for retrieving the membrane antigen has been successfully implemented to simplify the mechanism-based therapeutic antibody discovery approach. Despite the highlighted MP production challenges, this review unequivocally underscores the advantages of targeting complex membrane proteins with antibodies and designing membrane protein antigens. Selected examples of lipid nanoparticle success have been illustrated, emphasizing the potential of therapeutic antibody discovery in this regard. With further research and development, we can overcome these challenges and unlock the full potential of therapeutic antibodies directed to target complex MPs.

1. Introduction

Since the approval of the first monoclonal antibody (mAb) in 1986, mAbs and antibody-related treatments such as antibody-drug conjugates (ADC) have become the major products in the biopharmaceutical market [1]. According to recent reviews, the US FDA has approved 130 mAb products for treating diseases across different target classes and therapeutic areas [2]. The apparent reason for the continued growth of mAbs is their ability to access various modes of action, depending on the molecular target, and their high specificity, tolerance, and lower risk of safety issues compared to other therapies [3–5].

While the mentioned breakthrough in biotherapeutic drug design,

Abbreviations: ADC, Antibody-drug conjugates; APLNR, apelin receptor; AQP2,10, aquaporin-2,10; β1AR, beta1-adrenergic receptor; CB1, CB2, cannabinoid receptor types 1,2; CCR1, C-C chemokine receptor type 1; CD1d, Antigen-presenting glycoprotein CD1d; CFPE, cell-free protein expression; cryo-EM, cryo-electron microscopy; CHRM2, Muscarinic acetylcholine receptor M2; CysLTR1, CysLTR2 - Cysteinyl leukotriene receptor types 1,2; ENT1,2, Equilibrative nucleoside transporter 1,2; ET(A/B), ETB-R, Endothelin receptor type A and B; GABAAR, γ-aminobutyric acid receptor; GLP-1R, Glucagon-like peptide 1 receptor; GPCRs, G protein-coupled receptors; HDL, high-density lipoprotein of apolipoprotein A-1 (ApoA-1); H(1–3)R H1R, H2R, H3R, Histamine receptor H1, H2, H3 N30αβTCR - N30β (Vβ13Dβ1Jβ1.1Cβ2) from two αβ chains of T-cell receptor; mAb, monoclonal antibody; MP, membrane protein; MSP, membrane scaffold protein; NTR1, Neurotensin receptor type; NSP, nanodisc scaffold peptide; NSPr, nanodisc scaffold peptide, reversed; OR1A1, Olfactory receptor 1A1; PTMs, post-translational modifications; SapNPs, Saposin lipid nanoparticles; SMA, styrene maleic acid; SMALPs, styrene-maleic acid lipid particles; SLC1A5, Amino acid transporter; SLC6A19, Sodium-dependent neutral amino acid transporter B(0)AT1; SorCS2, VPS10 domain-containing receptor; TRPV1, Transient receptor potential cation channel subfamily V member 1; VLPs, virus-like particles.

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discovering antibodies against membrane protein (MP) targets remains challenging [6,7]. It is noticeable that MPs are crucial elements in many pathological conditions, which makes them an indispensable type of molecular target for therapeutic interventions [8,9]. Despite the vast number of unique drug targets belonging to different MP classes, such as G protein-coupled receptors (GPCRs) [10], ion channels [11], and transporters [12]. Currently, 90 approved full-length monospecific mAbs are directed against 40 unique types of membrane proteins available [13].

The primary obstacle to discovering antibodies against MP targets is obtaining protein samples in a stable and soluble format that can be used for initial selection and secondary screening of antigen-specific binders [14,15]. Membrane proteins are challenging to recombinantly produce compared to soluble antigens [16], their highly hydrophobic transmembrane domains are anchored or immersed on a lipid bilayer to comply with the native functionality [17,18]. Multiple approaches are employed to overcome this challenge, such as genetic immunization [19,20], detergent-solubilized target membrane proteins [21,22], and whole cells [23,24]. Altogether, the mentioned methods have limitations, including low yields, purity, and conformational stability of membrane proteins in lysates or whole cells. Additionally, in vitro antibody screening against whole cells is complicated by many irrelevant protein antigens on the cell surface and the complexity of highly specific interfacing of mammalian cells with yeast or phage particles. Due to the increasing need for advancements in the development of innovative multispecific antibodies [25–27] and chimeric antigen receptor T cell therapies [28,29], advanced platforms such as virus-like particles (VLPs) [30], nanodiscs (NDs) [31], and styrene maleic acid lipid particles (SMALPs) [32] are becoming widely utilized to address the complex problems of binder identification. Notably, the demands and advances of studying membrane protein structure and function have contributed to developing a more effective methodological arsenal for the pace and success of antibody drug discovery [33–36].

This review will briefly discuss the current state-of-the-art production and stabilization of membrane proteins, focusing on the various discoidal lipid nanoparticles available. We will then explore the benefits of nanodiscs-based platforms and examine studies where these model membrane systems have been used successfully to address the challenges posed by the nature of membrane proteins, with a particular emphasis on their applications in antibody discovery. In the final section, we will discuss potential future applications of nanodiscs, particularly concerning developing a universal approach suitable for structural and functional membrane protein characterization and innovative antibody-based drug development.

1.1. Heterologous expression and lipid nanoparticles systems for MPs

The first step in studying membrane proteins is to produce the protein of interest in sufficient amounts for structural and functional studies. The main goal is to produce a high amount and functionality of target protein - despite significant progress in the last 30 years, this task is still challenging and requires a series of trial-and-error experiments [37]. The choice of the best host organism for heterological expression depends on many factors [38] (Table 1). Some researchers have developed specified expression systems to improve target protein yield by exploiting metabolic and signaling pathways, or protein folding machineries with post-translational modifications (PTMs) [39]. However, such cases serve specific problems for individual protein targets being tackled. In some cases, it is better to mutate the protein to stabilize the structure but not interfere with its functionality. Another bottleneck for membrane proteins is that the lipid composition in the host's membranes will be different, and this could also influence the folding rate and effectiveness or be crucial if some specific types of lipids are needed for protein proper functioning.

Today, most recombinant proteins are produced in the following expression hosts - *Escherichia coli*, *Saccharomyces cerevisiae*, *Pichia pastoris* - as relatively simple, fast, and cheap. For example, microbial expression is still the primary technique in NMR structural studies because protein needs isotopic labeling [65,66]. Yeasts are capable of producing several post-translational modifications - such as

Table 1
Expression platforms for human MP drug targets with recent examples.

Expression platform	Features	Drawbacks	MPs	Sources
Prokaryotic cells	Technically well-established and simple genetic engineering; cost-effective bioprocess and high-density cell cultures; rapid growth and target-specific optimization; high production yields; suited for directed evolution for protein expression/stability optimization; capable of thriving in minimal media for NMR protein structure determination.	Common problems with target MPs are aggregation and instability; refolding of denatured MPs produced as inclusion bodies is tricky; residual endotoxin removal is obligatory for immunization; there is a lack of native-like PTMs (no glycosylation and complicated disulfide bond formation).	CB2 CB1 β1AR NTR1 SLC1A5, SLC6A19	[40] [41] [42] [43] [44]
Yeast cells	Technically well-established and susceptible to genetic engineering; cost-effective bioprocess and high-density cell cultures; rapid growth and target-specific optimization; high production yields; suited for directed evolution for protein expression/stability optimization; capable of thriving in minimal media for NMR protein structure determination.	Cell walls may impede MPs' purification; Non-native PTMs, commonly targeted MPs, are N-glycosylated (hyper-mannosylated), although strains have been glycoengineered to incorporate more human-like sugar structures.	CHRM2 H1R AQP10 AQP2 ENT1, ENT2	[45] [46] [47] [48] [49]
Insect cells	Technically well-established with relatively simple genetic engineering and transfection procedures; very high production yields.	Fast expression rates from the strong polyhedrin promoter can lead to misfolding, aggregation; extended bioprocess duration required for MP production; simplified PTMs, such as N-glycosylation (but lacking sialic acids.	APLNR CysLTR1, CysLTR2 H1R, H2R, H3R TRPV1	[50] [51] [52] [53]
Mammalian cells	Native or native-like PTMs, a lipid environment, and folding machinery (e.g., chaperones) are accessible; inducible expression by transient transfection and stable cell lines can be generated and optimized for expression via high-throughput approaches.	Potentially low expression levels and yields; some MPs may be toxic to the cell when overexpressed (e.g., transporters and ion channels); bioprocess scaling-up is costly and difficult; time-consuming optimization of target protein expression.	CD1d β1AR CCR1 OR1A1 N30αβTCR GABAAR	[54] [55] [56] [57] [58] [59]
Cell-free system	Reaction scale and formats are adjustable during target-specific optimizations; rapid reaction rates - the quickest MPs production method; toxic targets are not a concern due to the absence of living cells; a fit-for-purpose system with the ability to provide a membrane-like environment and chaperones directly to reaction.	Existing systems often lack PTM machinery, and crucial factors that assist with expression and folding necessitate additional components (e.g., endoplasmic reticulum microsomes); expensive renewable energy sources.	ETA-R, ETB-R SorCS2 β1AR GLP-1R γ-secretase	[60] [61] [62] [63] [64]

glycosylation, phosphorylation, and lipidation of target proteins, although these patterns can differ between species [67,68]. As for the main disadvantages, it is worth mentioning that the difference in host membrane lipid composition between eukaryotes and prokaryotes and the lack of post-translational modifications for *E. coli* led to improper folding of the target protein [69,70]. This obstacle could be overcome by developing different strains or vectors that allow the protein of interest to co-express other specific proteins for correct folding [71,72].

In scientific research, eukaryotic proteins often require eukaryotic hosts for proper PTMs and native folding. The lipid environment also plays a crucial role in protein function, making these hosts ideal for studying such proteins in a more native lipid environment. The two most widely used host types for MPs are insect cells infected with *Baculovirus* [73] and various mammalian cells [74].

Insect cells, such as Sf9, Sf21, and Hi5, are commonly utilized as expression systems to produce numerous human GPCRs [51,75], ion channels [76], and ABC transporters [77]. This system offers a natural advantage as baculoviruses can easily insert and replicate inside the host cells. The system can provide posttranslational modifications by incorporating the gene of interest protein into the viral genome and inducing the insect cell to produce the target protein. However, insect cells produce different glycosylation patterns than mammalian cells, making it preferable to avoid glycosylations that are not functionally critical. Additionally, the system has the capacity and flexibility to accommodate multiple gene expressions. The system is safe to manipulate since baculovirus does not infect humans. Nevertheless, the system's downsides include higher prices than *E. coli* for growing media and other compounds, strict cell block and laboratory equipment regulations, a lower yield per liter cell culture, and a careful transfection procedure. Mammalian cells are widely used for antibody generation and protein production for functional assays, with one of the most commonly used cell lines being from human embryonic kidney cells (HEK293) [78,79]. These cells have been used to produce 25.3% of eukaryotic membrane proteins of known structure in the last decade, mainly owing to their ease of transfection, high protein yields, and the increasing use of electron microscopy for structure elucidation. Low recombinant expression levels are a significant challenge in membrane protein studies, but new approaches are being developed, and mammalian systems are showing increasing success [80]. Many proteins expressed in mammalian cells form large complexes such as tetramers or hexamers, which is an advantage for studying ion channels and multiprotein complexes [39]. Other commonly used cell types include baby hamster kidney cells (BHK-21), monkey kidney fibroblast cells (COS-7), and Chinese hamster ovary cells (CHO) [81,82], which are especially useful for therapeutic protein production. Mammalian cells typically require special incubators as they grow at an optimal temperature of about 37 °C and require a supply of 5% CO_2. Although they usually grow in adherent cultures, HEK293 and CHO have been adapted to grow in suspension, making their scale-up much easier [83]. Mammalian cells provide endogenous posttranslational modifications required to express MPs alongside authentic translocation and trafficking machinery. Thus, mammalian proteins are expressed entirely folded, with post-translational modifications, and in a delicate lipid environment [84]. Novel cell line creation allows scientists to increase target protein yield, which is helpful for structural studies such as X-ray crystallography or cryo-electron microscopy (cryo-EM). However, practical approaches for producing directly in mammalian host cells isotopically labeled proteins required for NMR are under further development [85,86]. The ExpiCHO cell line was preferred for its high transfection efficiency, resulting in a high protein production yield and better quality for MPs [84] and antibody production [87]. Despite being an advantageous alternative to simpler expression systems for expressing proteins that require complex PTMs that cannot be performed in other expression systems, mammalian cells have some disadvantages. These include high cost, demanding culture conditions, susceptibility to contamination, and low protein yields, which may discourage their use, especially for structural studies. In some cases, due to its toxicity, the membrane protein may undergo auto-proteolysis, making it preferable to maximize the yield of the target protein by expressing it in a denatured state into inclusion bodies. After purification, refolding is necessary to transfer the protein into membrane-mimicking media, with thermodynamics guiding the establishment of the correct fold. Specific charged detergents or lipids could also be helpful if there is data about specific protein-lipid interactions.

The cell-free protein expression (CFPE) systems efficiently produce target membrane proteins without the need for living cells [88]. They are developed using prokaryotic or eukaryotic cell extracts, removing all cellular components not required for target protein production [89]. The process is initiated by adding suitable DNA or RNA templates along with components like amino acids, NTPs, or specific cofactors to initiate the in vitro protein expression process [90]. The CFPE systems used for MP production are mainly based on T7, or SP6 promoter systems synergized with extracts derived from different sources [91], for instance, *E. coli* [92], Sf21 cells [93], *Leishmania tarentolae* [94], CHO cells [95], BY-2 tobacco cells [96], and wheat germ [97]. The well-known limitations of CESF application for producing complex protein targets (such as MPs, VLPs, antibodies, and enzymes), particularly the lack of PTMs, represented a significant challenge actively being addressed. Several approaches at the cell-free extracts level can facilitate protein folding and disulfide bond formation [98]. N-linked glycosylation can be achieved by directly incorporating oligosaccharide transferases and the microsomal fraction in the cell-free extracts. The CFPE systems have been successfully employed widely for efficient MP production due to their high potential for mimicking native biological environments to save native functional and structural properties [99]. The mentioned systems allow for mimicking these environments by directly supplementing the expression reaction with stabilizing or solubilizing agents, for example, by adding detergent micelles, lipid vesicles (liposomes), or nanodiscs [100,101]. Nowadays, we have vast opportunities for membrane protein expression, all of which have advantages and drawbacks. Still, there is no universal system for every protein - this field of science is still state-of-the-art.

The NDs platform was created by Stephen Sligar's research group [102] to facilitate the study of membrane proteins. Nanodiscs are self-assembled disc-shaped particles containing lipids surrounded by a membrane scaffold protein (MSP) belt. The idea for this platform was inspired by natural high-density lipoprotein (HDL) particles, which are responsible for transporting cholesterol in the blood and are formed of apolipoprotein-A1. The MSP is a genetically engineered α-helical protein that mimics the function of apolipoprotein A-1 (ApoA-1). Its non-interacting N-terminal domain is truncated to reduce size heterogeneity, similar to HDLPs. Initially, the first type of membrane scaffold protein, MSP1, was developed with its sequence based on the native ApoA-1 but without the globular region [103]. The MSP1E1D1 variant of MSP1 introduces a novel approach by removing the first 11 amino acids in the Helix 1 region of the original MSP1 sequence 3 (MSP1D1) [104]. This variant is an N-terminal histidine-tagged protein with a TEV protease cleavage site, offering a unique feature. Additionally, it presents a novel duplication of the Helix 4 sequence of the original MSP1 sequence, positioned between the parent Helix 4 and Helix 5 segments of MSP1D1 [105]. Two MSP molecules and lipids form a 10 nm disc that can combine with a membrane protein. Later, different variants of MSP were developed to vary the NDs' diameter by adding or cutting different helixes in the MSP sequence [106]. The large multidomain membrane protein complexes needed bigger disc sizes. However, for structural methods such as protein NMR spectroscopy, the dimensions of the particles of interest are crucial for obtaining useful structural data [107–109]. The main advantage of the NDs approach compared with detergents in membrane protein studies is the ability to mimic the bilayer properties of native membranes. Different kinds of lipids could assemble into nanodiscs with different individual properties, such as headgroup charge, hydrocarbon chain length, and saturation - all of these parameter's bilayers give rise to different lateral pressure profiles

and phase transition behavior and influence membrane protein insertion, folding rate, and stability [110–112]. Also, specific lipids could establish protein-lipid contacts and be crucial for membrane protein functioning [113]. However, unlike liposomes, nanodiscs cannot provide asymmetry in inner and outer phospholipid layer lipid composition [114]. Later, additional variants of nanodisc technology were developed based on intein [115] or sortase molecules [116] to produce circularized nanodiscs where N- and C-terms covalently linked showed enhanced stability and defined sizes (large up to 50 nm) and shapes used for membrane proteins structural studies [117,118].

Another way to stabilize lipid bilayer fragments is to use MSP and other scaffolds, such as saposin proteins [119]; these proteins natively modulate lipid membranes in an acidic environment found in lysosomes [120]. Saposin-lipoprotein nanoparticles (SapNPs) stabilize membrane protein complexes in detergent-free buffer systems for functional and structural studies, such as high-resolution structure determination by single-particle cryo-EM [121]. In some cases, saposin scaffold proteins allow extraction and reconstitution directly in bilayer nanodisc, skipping the detergent purification step [122]. Due to the SapNP's unique and flexible nature, they adapt to the size of the MP) they incorporate by adjusting the number of saposin A scaffold protein molecules, forming a belt around the MP-lipid complex. Unlike the size dependency of MSP-based NDs, the size of SapNPs is not restricted by the scaffold protein's length [123]. Out of the wide range of similar proteins, saposin A is a preferential choice because it can bind the largest range of lipids [124]. A newly developed alternative approach, DirectMX, has emerged in the literature [125]. It focuses on incorporating MPs into SapNPs without using detergents. The method uses crude solubilized mammalian membrane extract in the presence of a low amount of digitonin, a mild detergent that increases the fluidity in the membrane and can enhance saposin A «open-state» activity.

Instead of scaffold protein, it is also possible to use amphipathic molecules such as styrene-maleic acid copolymers (SMAs) to solubilize membrane patches in discoidal nanoparticles known as SMALPs. They consist of linear sequences of styrene (S) and maleic acid (MA) residues in different ratios [126]. This ratio defines hydrophobicity and acidic charge density at the critical polymer concentrations that fragment membranes into nanodiscs. Unlike "ordinary" nanodiscs based on MSP belts, these particles are highly thermostable, and detergent is unnecessary for extraction and assembling protocol [127–129]. Later, different analogs of such polymers were suggested to improve further the extraction ability in various conditions (such as low pH) or polycation tolerance [130–132]. For example, aliphatic diisobutylene/maleic acid (DIBMA) is a copolymer of maleic acid or its sugar derivative [125]. This polymer's good feature is directly extracting membrane proteins and lipids from cellular membranes without using harsh detergents for cell lysis or solubilization. Varying the polymer composition allows for attenuating the physical-chemical parameters of nanodisc particles, such as the extraction ability or stability of the protein-nanodisc complex. These could be very useful to characterize and develop libraries and panels of membrane proteins and their complexes for structural and functional studies [133–135]. The maleic acid can chelate divalent cations, destabilizing SMALPs and precipitation in buffer solutions. The styrene ring absorbs UV light and could interfere with some spectroscopic properties of the encapsulated target MP. Next-generation of SMA-like polymers overcome such obstacles. For instance, the poly(styrene-*co*-(N-(3-N′, *N*′-dimethylaminopropyl)maleimide)) (SMI), which lacks the maleic acid of SMA and has improved tolerance to divalent cations and altered pH sensitivity characteristics [136]. Recently, the SMI has been successfully applied to the A2AR, V1aR, and bovine rhodopsin studies [137]. An alternative promising approach for the lipid bilayer nanodiscs preparation is based on hydrophobically functionalized fructo-oligosaccharides/inulin polymers developed recently [138]. These novel non-ionic amphiphilic polymer-based nanodiscs can efficiently extract membrane proteins while they are stable versus pH and divalent metal ions with impressive magnetic alignment properties. As reference [139] demonstrated, electrostatic interactions can also affect the solubility, stability, and size uniformity of inulin-based polymer nanodiscs.

Nanodiscs technology provides a significant advantage in a synergetic combination with cell-free protein expression systems [140,141]. Typically, when expressing membrane proteins in cell membranes or inclusion bodies, we must use detergent to extract or solubilize the target protein. However, detergents can disrupt the spatial structure of the protein, as micelles do not provide a fully native amphiphilic environment like the lipid bilayer. Therefore, using detergents to extract membrane proteins can be problematic. Another way to overcome this issue is to use CFPS based on a cell lysate containing the cell transcription/translation machinery and pre-made nanodiscs [142,143]. In this approach, the membrane protein is expressed and inserted into the nanodiscs and undergoes co-translational folding without harsh purification by detergents. This method is a better alternative to using detergents to extract membrane proteins [144]. Also, varying the lipid composition of nanodiscs could provide more successful insertion and folding as better native membrane-mimicking properties. The outstanding prospects of lipid nanoparticles for MP studies have increased manifold over the last ten years, as evidenced by the growing number of publications [145–150].

1.2. Lipid nanoparticles-based platforms for antibody discovery applications

Traditionally, antibody discovery relied on complex techniques often fraught with limitations such as low throughput, high cost, and low success rates during the selection and expression of target membrane protein. Despite the diversity of display platforms (yeast, ribosomal, bacterial, phage) [151], the latter has been most widely used in antibody generation [152,153]. A key to auspicious antibody discovery through phage display is a mostly high-quality source of diversity for binder selection. That can be provided by different types of libraries, which are globally divided into natural, synthetic, and semi-synthetic [154], and, depending on specific needs, more highly specialized libraries can be used [155–157]. Speaking of membrane proteins in the antibody development circuit, there are significant issues with antigen presentation, which boil down to hydrophobicity of the transmembrane domains, often misfolding, aggregation, and instability. Moreover, classic antibody display approaches have different capacity limitations, and without treatments and optimizations, they can become impossible to use [158,159].

Challenges in developing antibodies to membrane proteins arise from multiple factors and emerge at early stages. Key binding sites of potential antibodies are often immersed within the membrane and inaccessible for binding, necessitating alternative approaches for generation and purification. Another issue lies in the intrinsically low expression levels of many membrane proteins, which require selecting an expression system to enhance yield. Conformational features of membrane proteins, such as size and accessibility of extracellular domains, demand various optimizations during generation and purification to maintain their native structure upon extraction from cellular membranes and preserve protein functionality for subsequent antibody development [6].

Among the multitude of approaches proposed by the scientific community to overcome the challenges mentioned above, particular attention is drawn to systems based on the utilization of lipid membrane mimetics. In existing reviews, the main focus is placed on how discoidal particles have made a breakthrough in structural analyses of membrane proteins, with relatively few examples describing lipid discs' use in antibody development. [160]. One of the main applications of nanodiscs is their use for high-resolution characterization of complex protein structures and complexes [161,162]. However, over the past decade, they have emerged as a promising platform for antibody development against membrane proteins.

1.3. Nanodiscs

The ND platform enables the generation of antigens accessible for antibody binding while preserving their native conformation, which is crucial for antibody development and has gained significant traction in recent years (Fig. 1). One of the earliest documented applications of nanodiscs in antibody development against membrane proteins describes a comprehensive set of protocols for selection using phage display [163]. Among the crucial stages outlined in this process is the reconstitution of the target protein into nanodiscs for subsequent selection [31]. Incorporating nanodiscs into the phage display pipeline from the immunization stage to the actual panning has improved the selection process in general and enhanced the efficiency of antibody development against challenging membrane proteins [164,165]. For example – the human apelin receptor (APLNR) – one of the targets in treating chronic heart failure – is a representative of the GPCR family known for being quite complex for antibody discovery. However, using APLNR nanodiscs enabled the preservation of the natural conformation and stability of the protein for the immunization step in the study pipeline. [50]. A noteworthy approach is using nanodiscs to generate an immune response to viral or tumor-specific antigens, with episodes showcasing the successful implementation of this method. An influenza matrix-2 protein was reconstituted in nanodiscs to ensure the resemblance of its natural conformation to overcome the problem of the poor efficiency of generated antibodies [166]. Another target of the same virus was hemagglutinin, which is crucial for viral transmission in cells. A recombinant version of this protein was enclosed in nanodiscs, and results showed a stronger immune response and protection in mice compared to just hemagglutinin. Incorporation of the viral protein into nanodisc allowed the stabilization of the molecule and enabled proper epitope presentation [167]. A study was conducted with several SARS-CoV-2 spike proteins assembled in one nanodisc and used as a vaccine adjuvant system. Obtained results showed the possibility of developing broadly neutralizing antibodies to ensure future healthcare alertness [168]. The Nanodisc platform also made an appearance in an immunotherapy study against glioma models. A delivery vehicle based on synthetic high-density lipoprotein filled with antigens mix was developed and used for vaccination. The results showed the therapeutic efficacy of the aforementioned approach in combined immunotherapy against several murine glioma models, laying the foundation for future personalized vaccine pipeline development against not only glioma but other types of cancer [169].

Nanodiscs present numerous benefits, such as their adaptable size and lipid makeup, capacity to stabilize membrane proteins, and the ease of affixing affinity tags to membrane scaffold proteins (MSPs) rather than directly to the proteins. This method targets both sides of a membrane protein and streamlines the selection procedure by permitting the protein's release from the nanodisc using detergent. Additionally, nanodiscs can serve as depleting agents instead of detergents, potentially reducing the number of selection rounds. The incorporation of nanodiscs has also proven beneficial not only for selecting high-quality binders but also for capturing specific conformational states or target epitopes [170].

Thus, antigen presentation through nanodiscs has enabled the exploration of several potential conformations of the binding region of the HIV gp41 viral envelope protein. This protein serves as an established target for the development of neutralizing antibodies, so investigating the spatial structure and binding properties of its derivatives has allowed for a deeper examination of the role and characteristics of the transmembrane domain of this protein within the framework of antibody development optimization [171].

To enhance the efficiency of nanodiscs in the phage display selection process, Dominik et al. introduced a new approach based on improving the accessibility of the target protein epitope regions. To avoid the distortion of antigen binding caused by the biotinylation of their epitopes, the authors utilized a biotinylated scaffold protein instead of

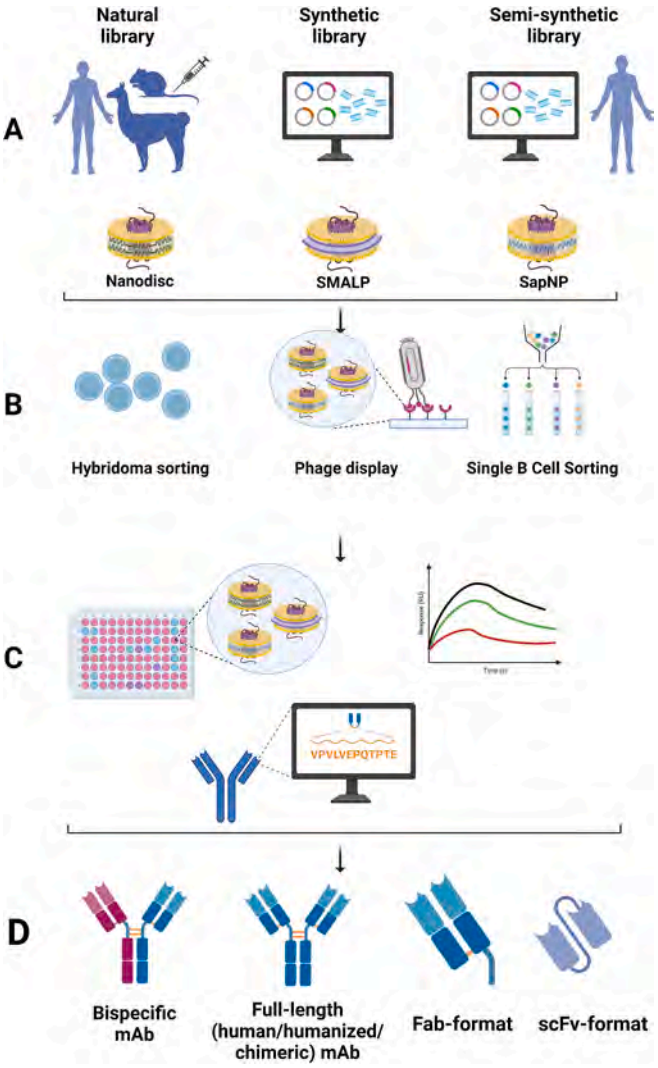

Fig. 1. Schematic illustration of antibody discovery against MPs pipeline utilizing lipid membrane mimetics. The target protein can be obtained through a variety of sources, i.e. constructed libraries for direct subsequent selection, or reconstituted in Nanodiscs, SMALPs, or SapNPs and used for immunization of selected hosts (mice, camelids, chicken, etc.). The following selection exploiting the nanodiscs platform can be performed via the chosen approach: phage (also yeast and whole cell) display, single cell or hybridoma sorting (B). Further screening and antibody characterization can be performed with the help of nanodiscs incorporated in standard workflows of established methods (i.e., ELISA, SPR, epitope mapping, etc.). Incorporating ND-based platforms in the antibody discovery roadmap enables the generation of various antibody formats (D).

labeling the nanodisc-protein complex. This innovative method ensures equal access to the sides of the target protein's surface during selection. Validation panning experiments were conducted with a library of phage-displayed synthetic antibodies targeting two model membrane proteins: Mj0480 (a small YidC homolog from *M. jannaschii*) and CorA (an ion channel from *T. maritima*) [172].

Another interesting application of NDs is using them not as vehicles for target membrane proteins but as targets themselves. Fuhito Nakagawa et al. developed an antibody, biND5, which binds to various reconstituted nanodisc MSP types with high affinity without requiring additional modifications or tags. Moreover, the binding properties of developed antibodies remain on the same level even after immobilization, which offers prospects for applications in library screening and

antibody discovery research, including for challenging targets such as membrane proteins [173].

1.4. SMALPs

Membrane mimetics such as SMALPs have also been extensively used to generate antibodies against membrane proteins. For instance, enhanced thermostability of the GPCR, particularly human cannabinoid receptor 1 (CB1) incorporated into SMALPs compared to detergent-based methods has been demonstrated. The degree of binding was assessed using yeast display and display of full-length IgG on the surface of mammalian cells. Additionally, the experiments evaluated the degree of preservation of native structure when using the SMALPs system and its compatibility with alternative applications based on fluorescence-activated cell sorting (FACS) and surface plasmon resonance (SPR). The authors note that despite some limitations in the applicability of surface-based methods, the reliability of this detergent-free system in creating membrane protein complexes suitable for screening has been established [174]. With SPR analysis, SMALP technology enabled the study of binding kinetics of therapeutic antibody interactions with diverse ligands [175,176]. Several antibodies against the cytoplasmic domain of the M2 protein of influenza A were engineered and validated utilizing SMALPs. Since M2 is a transmembrane protein, it is crucial for antibody studies to isolate the target domain in its native conformation without structural deviations [177]. Another case of incorporating SMALPs in a validation pipeline is described in the work on the dependence of agonistic or antagonistic activation on ligand valence of human C-type lectin-like receptor 2 (CLEC-2) [178]. A detailed and versatile study of lipid-protein interaction was carried out on the example of CD81, a member of the tetraspanins family. The use of SMALP for purification and solubilization allowed the preservation of the native protein structure for further manipulations to assess stability, yields, lipid environment, etc. [179]

1.5. SapNPs

A noteworthy platform within the framework of lipid nanoparticles is a system based on the saposin A protein family and is termed Salipro®. Since its introduction [120], several studies regarding structural research have been conducted utilizing this platform [125,180,181], including collaboration with AstraZeneca and Thermo Fisher Scientific [180]. However, the antibody discovery pipeline was not left behind. Target membrane protein, reconstituted in Salipro® particles, was incorporated in several steps of the development process. From immunization to in vitro assays to probing for further cell sorting [182].

1.6. Conclusions and future perspectives

Due to rapid advancements in structural biology [183], highly efficient approaches for producing pharmaceutically engaging MPs have become a technical routine [184], along with astounding progress in antibody-based therapeutics development. The steady growth in publications describing functional antibodies identified in pre-clinical research programs and the increased number of antibodies entering clinical studies suggest we are overcoming the challenges inherent in targeting complex integral MPs with antibodies. At the same time, significant resources and diverse strategies are required to achieve high-level expression rates in heterologous systems and extract them as purified, high-quality, and conformationally stable antigens. The discovery of functional antibodies that target complex integral membrane proteins increasingly employs new antibody discovery platforms. Some emerging strategies employ novel antigen formats, alternative immunization schemes (e.g., for chickens and camelids), conformationally constrained antigens, and various microfluidic discovery platforms. Systems designed to aid in expressing transmembrane proteins are essential to various scientific studies. In particular, cell-free synthesis has experienced a renaissance in the last decade [185–187], emerging as a highly effective method for producing membrane proteins for structural studies [188–190] and biopharmaceutical development [191–193]. These versatile systems allow for adding membrane-modeling mediums such as nanodiscs directly to the reaction mixture, which helps solve many of the typical challenges in transmembrane protein production. Continued synergistic co-development of these technologies will undoubtedly lead to new biopharmaceutical products based on antibodies. We anticipate that besides the mentioned biomedical applications, the ND-, SMALP-, and SapNP-based platforms will become ground-breaking nano-therapeutic delivery vehicles for personalized therapy as proposed lately [194–197].

Declaration of generative AI in scientific writing

During the preparation of this work, the authors did not use generative AI in scientific writing.

CRediT authorship contribution statement

Kristina O. Baskakova: Writing – review & editing, Writing – original draft, Validation, Resources, Project administration, Methodology, Formal analysis, Conceptualization. **Pavel K. Kuzmichev:** Writing – review & editing, Writing – original draft, Resources, Formal analysis, Data curation. **Mikhail S. Karbyshev:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors have no conflicts of interest to declare relevant to this article's content.

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