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Review

Advances in nanodisc platforms for membrane protein purification

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Membrane scaffold protein nanodiscs (MSPNDs) are an invaluable tool for improving purified membrane protein (MP) stability and activity compared to traditional micellar methods, thus enabling an increase in high-resolution MP structures, particularly in concert with cryogenic electron microscopy (cryo-EM) approaches. In this review we highlight recent advances and breakthroughs in MSPND methodology and applications. We also introduce and discuss saposin–lipoprotein nanoparticles (salipros) and copolymer nanodiscs which have recently emerged as authentic MSPND alternatives. We compare the advantages and disadvantages of MSPNDs, salipros, and copolymer nanodisc technologies to highlight potential opportunities for using each platform for MP purification and characterization.

Highlights

Nanodiscs have become an extremely valuable tool for membrane protein (MP) research. The first nanodiscs, membrane scaffold protein nanodiscs (MSPNDs), have seen substantial technological development over the past decade, and salipros and copolymer nanodiscs have recently emerged as promising alternatives to MSPNDs.

Recent advances include the introduction of circular MSPNDs, improvements to robust nanodisc method development, and increased breakthrough applications for MP structures and biophysical binding studies.

Salipros are able to adapt to the size of different MPs by using a single scaffold variant. Although very recently established, salipro methodology and applications have seen rapid development.

Copolymer nanodiscs can incorporate MPs in their endogenous lipids via extracting the MP directly from the cell membranes. Three groups of copolymers – styrene–maleic acid (SMA), diisobutylene maleic acid (DIBMA), and polymethacrylate polymer (PMA) – have been employed with varying success to stabilize MPs in an active state.

Nanodisc platforms for membrane protein isolation

MPs are excellent drug targets owing to their favorable location in the cell membrane and their diverse group of binding partners. Previous reports indicate that MPs account for >60% of the current drug targets, highlighting the importance of continued development of expression and characterization methods in this area [1]. One of the major challenges associated with developing MPs as drug targets arises in the preliminary stages of the drug development process where structural or biophysical methods such as cryo-EM [2], surface plasmon resonance (SPR) [3], and nuclear magnetic resonance (NMR) [4] are deployed to obtain in-depth insight into ligand-binding pockets or binding interactions and to evaluate unique therapies targeting MPs of interest. However, a major limitation with these and related techniques is the need for purified proteins. Extracting MPs from cell membranes can be extremely challenging because of their hydrophobic surfaces, flexibility, and lack of stability [5]. Traditionally, an array of detergents has been deployed to purify these proteins [6]. However, as membrane mimetics, detergents are useful only for a small subset of MPs that are not sensitive to changes in their environment, leaving limited options for the purification and subsequent study of many MPs.

In the early 2000s Sligar and colleagues [7–9] introduced a unique approach to improve the stability of purified MPs based on a genetically engineered component – a membrane scaffold protein (MSP) that mimics apolipoprotein A1 from high-density lipoprotein particles. This method then shuttles detergent-purified MP into MSPNDs which are synthetic lipid bilayers encircled by two MSPs [8]. These MSPNDs have been found to better mimic the natural lipid environment of MPs [10] than detergent micelles, and significantly increase MP stability compared to detergents [11]. Since the introduction of this technology, a large array of different MPs have been purified using this method including G protein-coupled receptors (GPCRs) [8,12–14], ion channels [15–17], and other receptors [18,19].

Over the past decade other types of nanodisc-like platforms have been developed that offer some unique advantages over the original MSPNDs. Two of the most prominent MSPND

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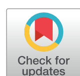

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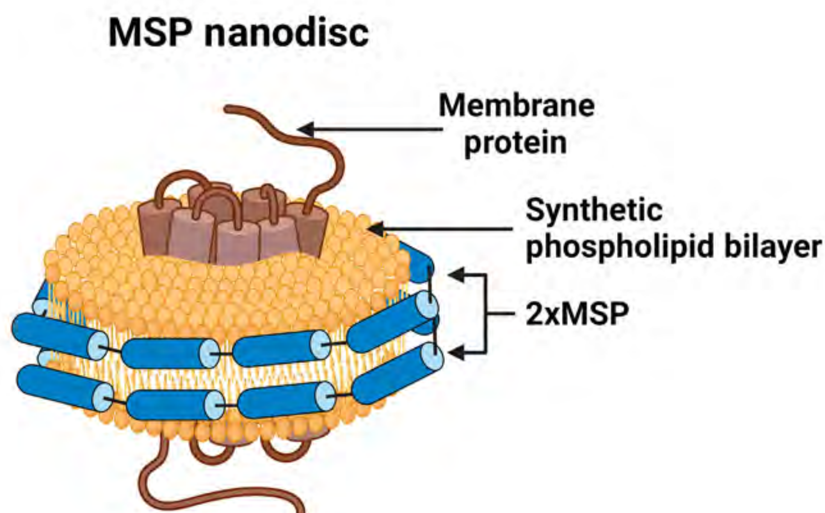
alternatives include salipros [20,21] and copolymer nanodiscs [22–24]. Salipros, another protein-based nanodisc platform, create nanodiscs using saposin protein following a method similar to that for MSPND production. By contrast, copolymer-based nanodiscs offer a unique strategy that allows the proteins to be removed directly from the cell membrane, retaining the native lipid environment [25]. Importantly, this copolymer-based strategy has been valuable in proteomic studies [26,27], although this is not the focus of this review. Both MSP alternatives – salipros and copolymers – have seen rapid development in methodology and applications over the past several years, and this has further cemented their place as true competitors of MSP-based nanodiscs. However, MSPNDs have also seen major advances recently, such as newly engineered MSPs, improved methodology, and new applications, all of which have aided their further development. The past 4 years has seen an explosion in the implementation and use of nanodisc technologies. This growth can be best highlighted by the number of high-resolution structures, where >330 MP structures have been determined using nanodisc technologies (https://www.rcsb.org/). Of these structures, >280 (85%) have been determined over the past 4 years, and 150 have been determined in the past year alone.

This review highlights recent developments and advances regarding the use of MSPNDs, salipros, and copolymer nanodiscs to purify and evaluate MPs. Each of the three nanodisc platforms is briefly introduced before recent advances related to improvements in both methodology and application are discussed, together with the strengths and weaknesses of each platform. Because our focus is on recent advances, this review does not provide in-depth methodology of the three platforms; interested readers can refer to [28,29] for MSPNDs, [30,31] for salipros, and [32–35] for copolymer nanodiscs for in-depth discussion of the methodology.

Membrane scaffold protein nanodiscs

Discoidal phospholipid bilayer nanoparticles, dubbed MSPNDs, were engineered by analogy to plasma lipoproteins that produce disc-like structures [36]. MSPNDs are composed of a phospholipid bilayer mimetic – often 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) – that is encircled by two MSPs (Figure 1A) whose size is dependent on the type of MSP that is used. MSPs were designed based on apolipoprotein A1 whose globular region was removed, creating a single α-helical segment that is separated by proline residues [9]. The first MSP was known as MSP1 [36]; further modification of this originally created MSP has resulted in MSPNDs of diverse sizes and styles (Box 1).

Although there are other applications, the primary application of MSPNDs is to create a membrane-mimetic for MPs in which MPs are incorporated into MSPNDs following a simple protocol (Figure 1B). Lipids are solubilized using detergents before being mixed with MSPs in predetermined ratios that are dictated by the MP, MSP, and lipids being used. For example, a typical starting ratio when using MSP1D1 and POPC is 1:50–60; however, this ratio can change based on the MP being incorporated, the type of MSP, and the lipid being used; more detailed information regarding specific ratios is available in the literature [9,29,37]. Micellar purified MPs are then added to the lipid/MSP mixture. Detergents are then removed using Bio-beads® SM-2 adsorbent or dialysis to allow MSPND assembly and MP incorporation [29]. Extensive optimization of the assembly conditions with regard to the lipid:MSP:MP molar ratio is typically necessary to maximize MP incorporation and the formation of homogeneous MSPNDs, making this a lengthy and time-intensive process. Indeed, we have observed the formation of larger structures (possibly MSP:lipid multimers or assemblies) at non-optimal lipid:MSP ratios. Lipid charge can play an important role in protein membrane surface interactions, and thus lipid type and blend may also need to be varied to improve membrane protein stability; detailed nanodisc lipid charge information and the effects of polyelectrolytes on lipid behavior in nanodiscs can be found elsewhere (e.g., [38]).



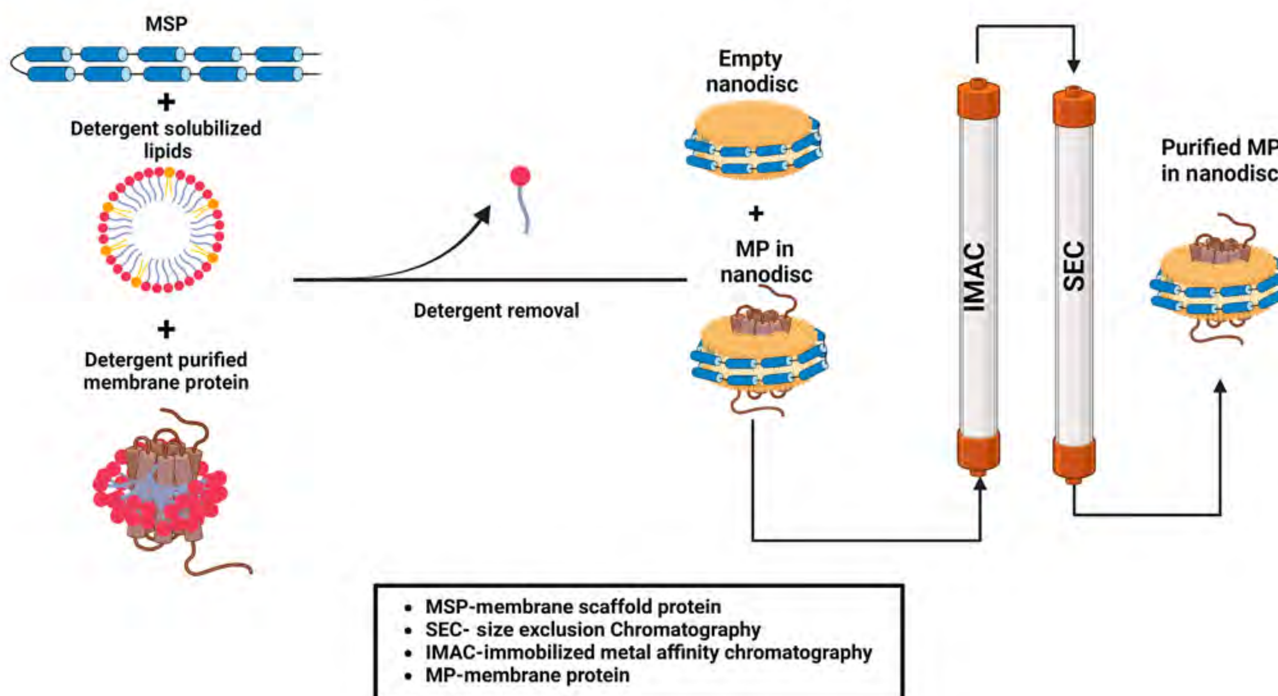


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Figure 1. Schematic of membrane protein (MP)–membrane scaffold protein (MSP) nanodisc assembly. (A) General structure of an MSP nanodisc (MSPND) containing an MP. (B) MP incorporation into MSPND and subsequent purification. MSPs are mixed with detergent-solubilized lipids and detergent-purified MPs. Detergents are then removed using Bio-beads® SM-2 adsorbent or dialysis, producing empty MSPND and MSPNDs containing MPs. Empty MSPNDs are then separated from those that contain MP by immobilized metal affinity chromatography (IMAC). Size-exclusion chromatography (SEC) is then used to further purify MSPNDs that contain MPs. Figure created with BioRender.com.

Recent work has aimed to reduce the time necessary to incorporate MPs into MSPNDs because the optimization of conditions for a new protein of interest can often take months to complete when starting from scratch. One case study streamlined the optimization process to reduce this initial time investment [12]. The method used *in situ* dynamic light scattering (DLS) as a screening tool to determine the size and dispersity of the MSPND under varying assembly conditions. Using crystallization microplates requiring volumes as low as 0.5–2 μl, many different assembly conditions were evaluated simultaneously with a limited initial sample, in contrast to traditional DLS and size exclusion chromatography (SEC) methods. The authors demonstrated proof of concept for this method by optimizing nanodisc incorporation of a model GPCR, the adenosine A_{2A} receptor ($A_{2A}R$), where optimal conditions were confirmed using SEC.

Improvements related to the empty MSPND assembly protocol have included the use of a Sephadex G-25 resin column for association in as little as 15 minutes, in contrast to the hours needed in the traditional method [9,39]. Although no MPs have been incorporated into MSPNDs using this approach, it provides a potential groundbreaking alternative to the use of Bio-beads® SM-2 adsorbent and dialysis where protein losses typically occur.

An express method for MP incorporation into MSPNDs using endogenous lipids has been reported using crude detergent-solubilized MPs extracted from whole human cell lysates [40]. This express method improves upon previous MP endogenous lipid MSPND preparation methods [41–43] by removing the membrane isolation step, thus saving valuable time [40]. MPs from mammalian-produced basigin, stomatin from red blood cells, and glycoprotein IX from platelets were successfully incorporated into MSPNDs using this express method, indicating proof of concept. This simplified method has the potential to reduce incorporation time and increase overall MP MSPND incorporation through reduced MP losses, and better simulates the cell membrane owing to the use of endogenous lipids. Some of the disadvantages of this express method include MSPND lipid inhomogeneity and organelle-specific proteins that can interfere with or disrupt MSPND incorporation.

Recent MP-MSPND applications have focused on the use of cryo-EM- and nuclear magnetic resonance (NMR)-based assays to determine novel structural information related to specific MPs.

Box 1. MSP variants – development and implementation

MSP1 and MSP2 were the first MSPs created [36]. Since the original two MSPs, many variations have been generated (Figure IA); only the most prominent and recently constructed variants are highlighted here. One of the most popular constructs, MSP1D1, which creates assembled nanodiscs 9.5 nm in diameter, was created by removing the first 11 N-terminal amino acids from MSP1 and replacing the 6×His tag and X linker with a 7×His tag and tobacco etch virus protease (TEV) linker to allow affinity tag removal [9]. MSP1D1 has been modified further to create MSP1E1D1, MSP1E2D1, and MSP1E3D1 [91] to enable MSPND of increased sizes (10.5 nm, 11.1 nm, and 12.1 nm in diameter, respectively). Even larger MSPNDs (16–17 nm) have been made using MSPs MSP2N2 and MSP2N3 [9,92]. Smaller MSPs, MSP1D1ΔH4, MSP1D1ΔH4–5, and MSP1D1ΔH4–6, have also been designed to create MSPNDs of 9.1 nm, 7.8 nm, and 6.8 nm in diameter, respectively [93]. These size variations offer the ability to scale the platform to the size of the individual membrane protein, a large protein complex, or perhaps even to investigate the effect of membrane asymmetry and curvature on the function of individual proteins or complexes.

MSPs have recently been covalently linked at their N and C termini to create circularized nanodiscs (cNDs) that are more stable than normal MSPNDs [50]. This increase in stability is more pronounced in the smaller MSPs constructs (MSP1D1ΔH4, MSP1D1ΔH4–5, and MSP1D1ΔH4–6), where all circularized MSPs (cMSPs) variants showed significant increases in stability compared to their linear counterparts [94]. Smaller cMSPs – cMSP1D1ΔH45 and cMSP1D1ΔH4–6 – have been used successfully with solution NMR to generate high-quality spectral data such as that obtained with larger discs which linear MSPs of the same variants were unable to deliver. cMSPs variants cNW30 and cNW50 have also allowed the formation of larger MSPNDs – 30 nm and 50 nm in diameter – which may be useful for larger protein complexes [50]. cMSPs can be produced extracellularly using sortase-mediated ligation (Figure IB) [95]; they can also be produced intracellularly through intein splicing (Figure IC) [94] or via the Catcher-SpyTag system [96]. A potential issue with cMSPs is lower protein yield (<20 mg of cMSP protein/l of cell growth) compared to their linear forms (100 mg of protein/l of cell growth) [51]. However, recent work with circular MSP1E3D1 (csMSP1E3D1) has demonstrated that introduction of negative charges can lead to significantly increased yields (75 mg of protein/l of cell growth). Many of the common MSP variants are likely to be transformed into circularized variants over the next few years as cMSP usage increases because of the favorable stability and versatility.

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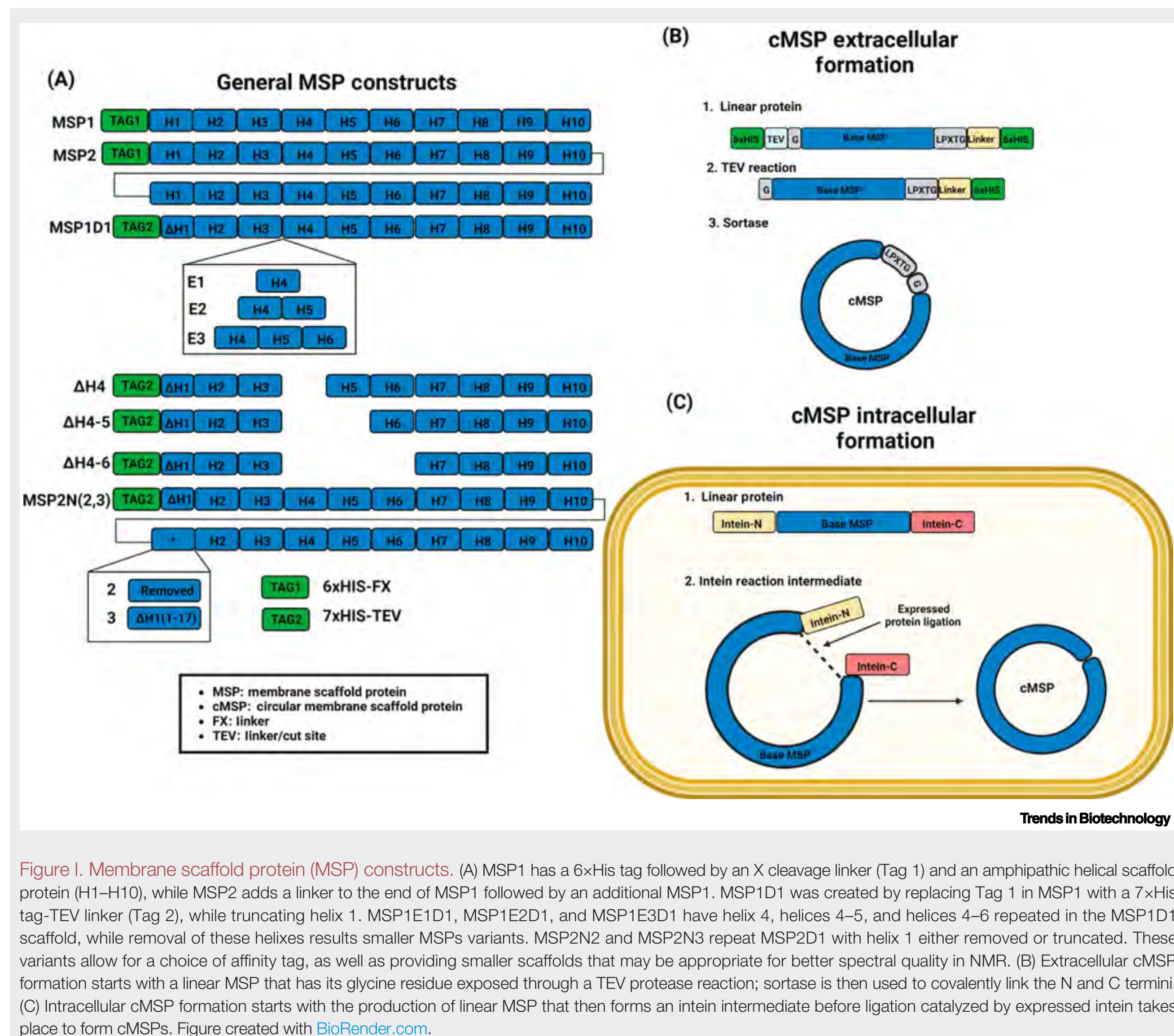

Figure I. Membrane scaffold protein (MSP) constructs. (A) MSP1 has a 6×His tag followed by an X cleavage linker (Tag 1) and an amphipathic helical scaffold protein (H1–H10), while MSP2 adds a linker to the end of MSP1 followed by an additional MSP1. MSP1D1 was created by replacing Tag 1 in MSP1 with a 7×His tag-TEV linker (Tag 2), while truncating helix 1. MSP1E1D1, MSP1E2D1, and MSP1E3D1 have helix 4, helices 4–5, and helices 4–6 repeated in the MSP1D1 scaffold, while removal of these helixes results smaller MSPs variants. MSP2N2 and MSP2N3 repeat MSP2D1 with helix 1 either removed or truncated. These variants allow for a choice of affinity tag, as well as providing smaller scaffolds that may be appropriate for better spectral quality in NMR. (B) Extracellular cMSP formation starts with a linear MSP that has its glycine residue exposed through a TEV protease reaction; sortase is then used to covalently link the N and C termini. (C) Intracellular cMSP formation starts with the production of linear MSP that then forms an intein intermediate before ligation catalyzed by expressed intein takes place to form cMSPs. Figure created with BioRender.com.

Cryo-EM was recently used to determine the structure of a rhodopsin dimer/tetramer ncorporated into MSPNDs, and resolved the interdimeric interface that involves transmembrane segment TM1 and helix 8/TM4 in this class A GPCR. This revealed valuable information about how rhodopsin and potentially other GPCRs dimerize [44], expanding upon the first observation of dimeric rhodopsin in a nanodisc complex [45]. Cryo-EM has also been used recently to solve the structure of the M_2 muscarinic receptor β-arrestin (βarr1) complex in MSPNDs [14]. Upon GPCR agonist activation, βarr1 destabilizes and blocks G protein binding to the GPCR, and was found to interact with both the lipid bilayer and the receptor. This breakthrough observation was made possible only through incorporation of the M_2 receptor into MSPNDs which could accommodate the size of the complex. Although MSPNDs provide a valuable approach for examining GPCR interactions with other proteins, cryo-EM of complexes in detergents has also provided valuable knowledge. For instance, the structure of the neurotensin receptor 1 (NTSR1) in complex with preactivated

βarr1 in detergents was determined recently using cryo-EM and compared to the M_2–βarr1 cryo-EM structure [46]. When compared, βarr1 was observed to interact with the receptors though the same regions, but in unique and different ways, indicating the possibility that two β-arrestin isoforms could interact with many GPCRs [46]. A cryo-EM structure of nanodisc-supported β_1-adrenoceptor (β_1AR) in complex with βarr1 in the presence of a biased agonist revealed that the agonist activated only a portion of the GPCR signaling pathway compared to β_1AR coupled to the G protein-mimetic nanobody bound to the same ligand [47]. This contrast suggests that the biased agonist can obtain different binding interactions and subsequent activation, thus opening the door for potential biased agonist design. More information on GPCR–βarr1 interactions and related structural work can be found in a recent review [48].

Another cryo-EM MSPND structure determined in the past few years was of neurotensin receptor 1 (NTSR1) bound to G protein $G_i\alpha1\beta1\gamma1$; this represents one of only a few receptor–G-protein structures in a native-like lipid bilayer [49]. Structural data from NTSR1–G_i present in circular MSPNDs led to a key observation on how the lipid bilayer affects G protein coupling and how the G protein interacts with NTSR1. Circular MSPNDs have also led to improved NMR signal intensities and spectral resolutions of VDAC-1 and NTR1, with significant enhancement at increased temperatures [50]. Circular nanodiscs have allowed samples to be stable at 45°C for 10 days, which is advantageous for NMR studies where resolution is typically improved at higher temperatures [51].

Although MSPND structure-based applications are the most common, unique applications related to antibodies and drug development have been reported in recent years. For instance, anti-β_1AR autoantibody-induced dilated cardiomyopathy (DCM), a cause of heart failure, makes up an estimated 25–75% of DCM cases [52]. Anti-β_1AR autoantibody-induced DCM could be treated by immunoadsorption of anti-β_1AR antibodies; however, there is currently no reliable diagnostic method to determine whether the DCM is caused by the presence of anti-β_1AR autoantibodies. In this study, an enzyme-linked immunosorbent assay (ELISA)-based detection system used β_1AR MSPNDs that were immobilized on a GFP trap (typically an anti-GFP nanobody coating) to detect serum levels of anti-β_1AR antibodies reliably, thus enabling potential DCM diagnosis.

Another recent application looked at streamlining the identification of multi-pass receptor antigen-specific hybridomas, a key step in developing monoclonal antibodies [53]. The study incorporated the ion channel chimera of voltage-sensor domain 4 linked to the sodium channel from *Arcobacter butzleri* (VSD4-NavAb) into MSPNDs for use in conjunction with fluorescence-activated cell sorting (FACS) to determine antigen-specific hybridoma binding. Although FACS is commonly used for the identification of antigen-specific hybridomas, this technique previously could not be used for multi-pass receptors because of the instability associated with purified receptors. Using MSPNDs resulted in increased stability, offering a breakthrough in using FACS for multi-pass receptor monoclonal antibody development which had been previously bottlenecked by a tedious screening process. Thermally stabilized A_{2A}R incorporated into MSPNDs also provided increased stability for SPR studies to determine kinetic binding parameters for A_{2A}R-selective ligands [13], laying the groundwork for potential high-throughput use in drug screening.

Saposin–lipoprotein nanoparticles

An alternative protein-based belt system, sphingolipid activator proteins, known as saposin proteins, are small, non-enzymatic proteins that are known to modulate lysosomal breakdown in their active state [54]. There are four different saposin proteins, saposins A–D, named based on their placement in the precursor protein prosaposin, and that differ only slightly in amino

acid sequence and structure [55]. The lipid-bound state of saposin A, which was first characterized in 2011, was found to solubilize liposomes to produce salipros that were 3.2 nm in diameter [56]. Based on this observation, X-ray crystallography of saposin A–lauryldimethylamine-*N*-oxide (LDAO) structures, and coarse-grain molecular dynamics simulations of lipid-bound saposin A [56], together supported a model of the ability of saposin A to produce discoidal high-density lipoprotein particles [20] that have been used to stabilize and purify many different MPs over the past decade, including GPCRs [30,57], peptide transporters [20], ion channels [58], and solute carriers [59].

Salipros have a unique flexible nature and lipid-to-saposin size-dependency that allows them to form nanoparticles that adapt to the size of the MPs being incorporated by adjusting the number of saposin scaffold proteins that are used to form the belt around the MP–lipid complex. This interaction is unlike the MSPNDs whose size is dependent on the length of the scaffold protein itself [21]. This feature allows salipros to be used for a wide range of differently sized MPs without the need to optimize the scaffold protein to ensure that it produces a disc sufficiently large to fit the protein of interest [20].

Salipros are produced in a very similar way to MSPNDs (Figure 2A). Lipids are first solubilized in detergents before being added to purified saposin proteins in predetermined stoichiometric ratios. Salipro formation is then initiated by detergent removal or dilution, where the goal of both approaches is to bring the detergents below the critical micelle concentration (CMC) to drive formation of the complex (Figure 2A). During this step the lipids spontaneously rearrange themselves into a lipid bilayer that is then stabilized by saposin scaffold proteins. MPs are incorporated into salipros by adding detergent-purified MPs to the lipid–saposin mixture before detergent removal or dilution. During the removal or dilution step the MPs self-assemble in the native-like lipid environment provided by the salipros (Figure 2A). Salipros containing MPs can be isolated from empty salipros using immobilized metal affinity chromatography (IMAC) and SEC (Figure 2C). Saposin A is the recommended first choice of saposin protein owing to its ability to bind the largest range of different lipids, although the choice of lipid should be made based on the MP of interest because different MPs appear to favor different lipids [21].

DirectMX has recently appeared in the literature as an alternative method to that discussed above [30]. DirectMX focuses on incorporating non-detergent-solubilized MPs into salipros by utilizing endogenous lipids. The method uses crude solubilized mammalian membrane extract in the presence of a low amount of digitonin, which is a mild detergent that helps to increase fluidity in the membrane and can be used to increase saposin open-state activity. The material is first prepared by lyzing the cells and removing any insoluble material from the lysate via an ultracentrifugation step. The crude membrane material is then mixed with saposin A, where saposin A utilizes endogenous lipids to form salipros (Figure 2B). Salipros containing the MP of interest are then purified using IMAC and SEC as in the traditional approach (Figure 2C). DirectMX has been used successfully to purify an active chemokine receptor and an active human solute carrier family 1 transporter [30]. Although the implementation and use of DirectMX are still at early stages, DirectMX offers the potential to omit the step of MP purification in detergents before salipro incorporation, thus decreasing the time and material handling required while also offering the ability to incorporate MPs that are poorly solubilized in detergents. Both methods have been used to successfully incorporate MPs into salipros; however, individual MP applications still require empirical method optimization.

Owing to its relatively new development in comparison to MSPNDs, the applications of salipro technology have been limited mostly to structure-based analytical techniques such as NMR

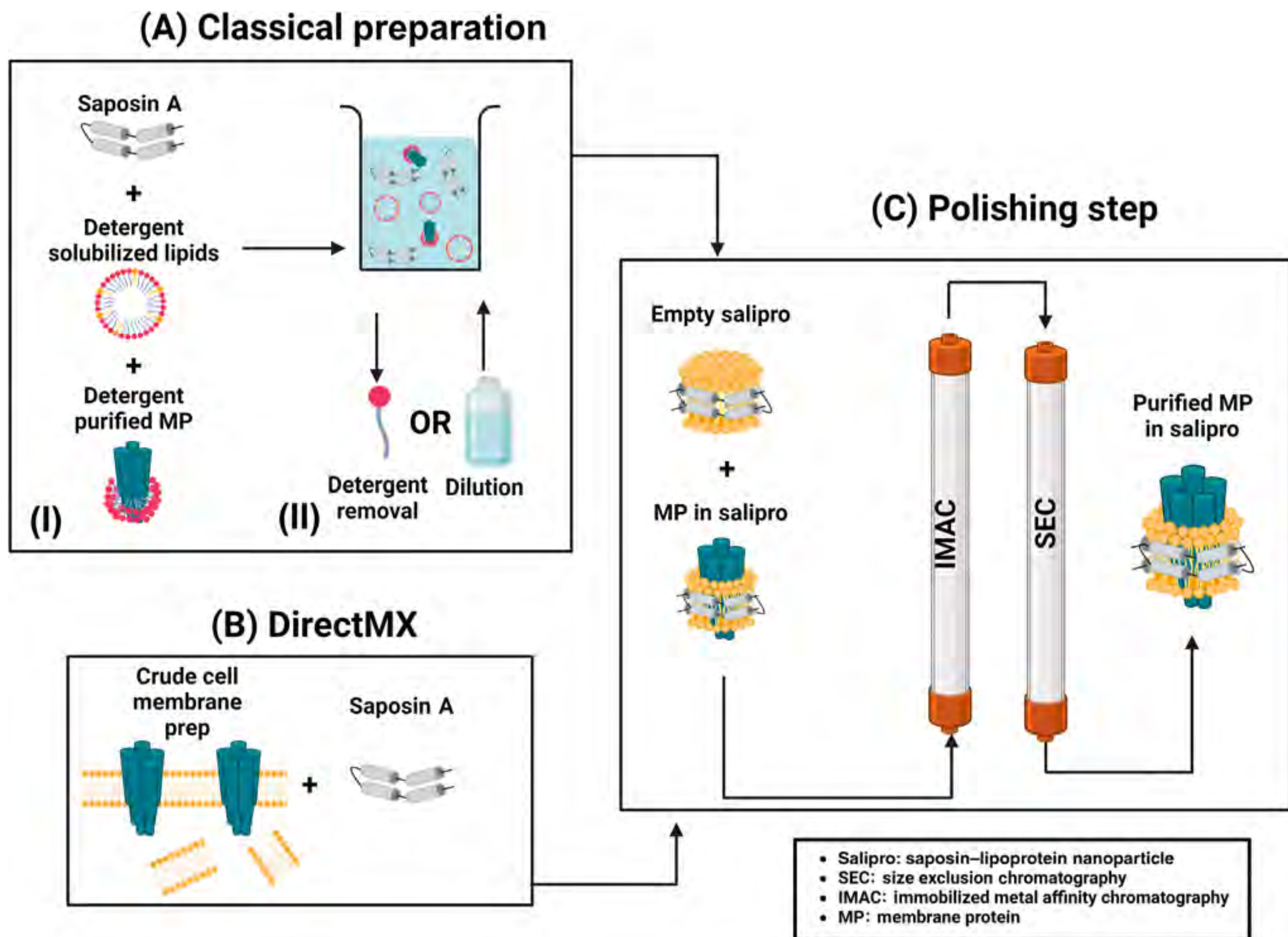

Figure 2. Membrane protein (MP) incorporation into saposin–lipoprotein nanoparticles (salipros). (A) Classic salipro preparation: (i) saposin A is mixed with detergent-solubilized lipids in predetermined stoichiometric ratios before the addition of a stoichiometric amount of detergent-purified MP. (ii) The detergents are removed from solution using either dialysis or Bio-beads® SM-2 adsorbent or the detergents can be diluted to below the critical micelle concentration by the addition of buffer. (B) DirectMX preparation. Crude cell membrane preparations are mixed directly with saposin A in stoichiometric ratios. (C) Empty salipros are separated from MP-incorporated salipros by using by immobilized metal affinity chromatography (IMAC) followed by size exclusion chromatography (SEC). Figure created with BioRender.com.

and cryo-EM. Nevertheless, salipros offer some unique applications regarding each of these technologies. In one solution-based NMR study [57], three different MPs were incorporated into salipros: bacterial outer MP X (OmpX), sensory receptor rhodopsin II (pSRII), and β_1AR [57]. Analysis of the solution NMR spectra indicated successful incorporation of functional receptors into salipros; however, salipros failed to provide spectra as clean as those of OmpX in MSP1D1ΔH5 and pSRII in detergent micelles. Future improvement to NMR structures of these three MPs in salipros would most likely improve with perdeuteration, and offers an attractive alternative to MSPNDs for solution NMR owing to their ability to adjust to the size of the MP and the ability to form lower molecular weight complexes than MSPNDs [57].

Cryo-EM studies have been performed for several different receptors stabilized in salipros: protein-coupled oligopeptide transporters (POTs) [20], AcrB [60], smoothened receptor (SMO) [61], and nicotinic receptor [58,62–64]. The cryo-EM map constructed for salipro–nicotinic

receptor bound to α-bungarotoxin yielded the highest-resolution map of eukaryotic Cys-loop receptors to date [62], leading to many insights into ligand recognition interactions and the effect of receptor mutations that lead to disease. Cryo-EM of salipro SMO revealed information about how fusion proteins yield improved cryo-EM structure determination through the discovery of previously undescribed SMO structural information [61]. Finally, cryo-EM structures for apo AcrB and AcrZ-bound AcrB protein incorporated into native lipid salipros offered new insights regarding the native and bound conformations of AcrB [60]. The use of salipros in cryo-EM studies has thus enabled important structural information not available via other options owing to its ability to mimic a native-like cellular membrane.

Copolymer-based nanoparticles

MSPND and salipros still rely heavily on using detergent-purified MPs as a starting point for MP nanodisc incorporation. Although MPs must remain stable in detergents for only a short period of time, protein destabilization can still occur [65]. Alternatively, the polymerization of two different types of monomers creates copolymers which extract MPs directly from the cell membrane (Figure 3) through the formation of copolymer nanodiscs, and enabling the inclusion of the endogenous lipid environment [25]. Styrene–maleic acid (SMA) copolymers (referred to here as SMAs), which are formed from hydrophobic styrene and hydrophilic maleic anhydride monomers, were the first copolymers used to for this process [66]. As the most widely used copolymer, SMAs have been used to purify many types of MPs, including GPCRs [33,67], ABC transporters, cell division proteins [68], and ion channels [69], among others.

SMAs form nanodiscs by inserting themselves into the cell membrane through a process that is driven by the hydrophobic portions of the copolymer. Once inserted into the cell membrane, the copolymer creates small nanodiscs out of the lipid bilayer, called SMA lipid particles (SMALPs) [65,70]. MPs encapsulated in SMALPs are then purified further using SEC and affinity chromatography [67]. Although the success of SMA protein extraction depends on the MP of interest and the expression platform being used, SMAs solubilize protein generally equally efficiently as, or better than, detergents [68]. MPs have been shown to be often more stable in SMALPs than in their detergent counterparts [69], following the general pattern of increasing stability provided by nanodisc platforms.

Owing to the relatively recent development of SMALPs, proof-of-concept research has dominated the literature. However, over the past several years a handful of cryo-EM structural studies have been completed for AcrB [71,72], respiratory cytochrome bo_3 ubiquinol oxidase (cytbo$_3$) [73], and alternative complex III (ACIII) [74], and we expect this number to increase. Some of the disadvantages of using SMALPs include sensitivity to low pH [75] and divalent cations [68] which cause the SMA to precipitate from solution, while also causing UV spectral interference. However, modified SMAs have been designed to combat pH- and cation-sensitivity issues. For example, SMA-EA [76] has a reduced number of carboxylic groups, and SMA-QA [77] has a quaternary ammonium group instead of a carboxylic/carboxylate group as the hydrophilic portion; both show improved stability in low pH and reduced sensitivity to divalent cations compared to SMAs. SMA-EA and SMA-QA have also allowed investigation of the effect of copolymer charge on membrane protein stability because SMA-EA is negatively charged whereas SMA-QA is positively charged. The charge of the membrane protein and the charge of the copolymer have been shown to play essential roles in the solubilization and stability of the copolymer membrane protein complex [78], indicating that charge–charge interactions need to be considered when working with copolymer nanodiscs. SMA-EA and SMA-QA can also form macro-nanodiscs, which are nanodiscs >20 nm in diameter [76,77]. These macro-nanodiscs can be used as an alignment medium using a magnetic field for the measurement of residual dipolar couplings (RDCs), which can be used to provide high-resolution structures with NMR [79,80].

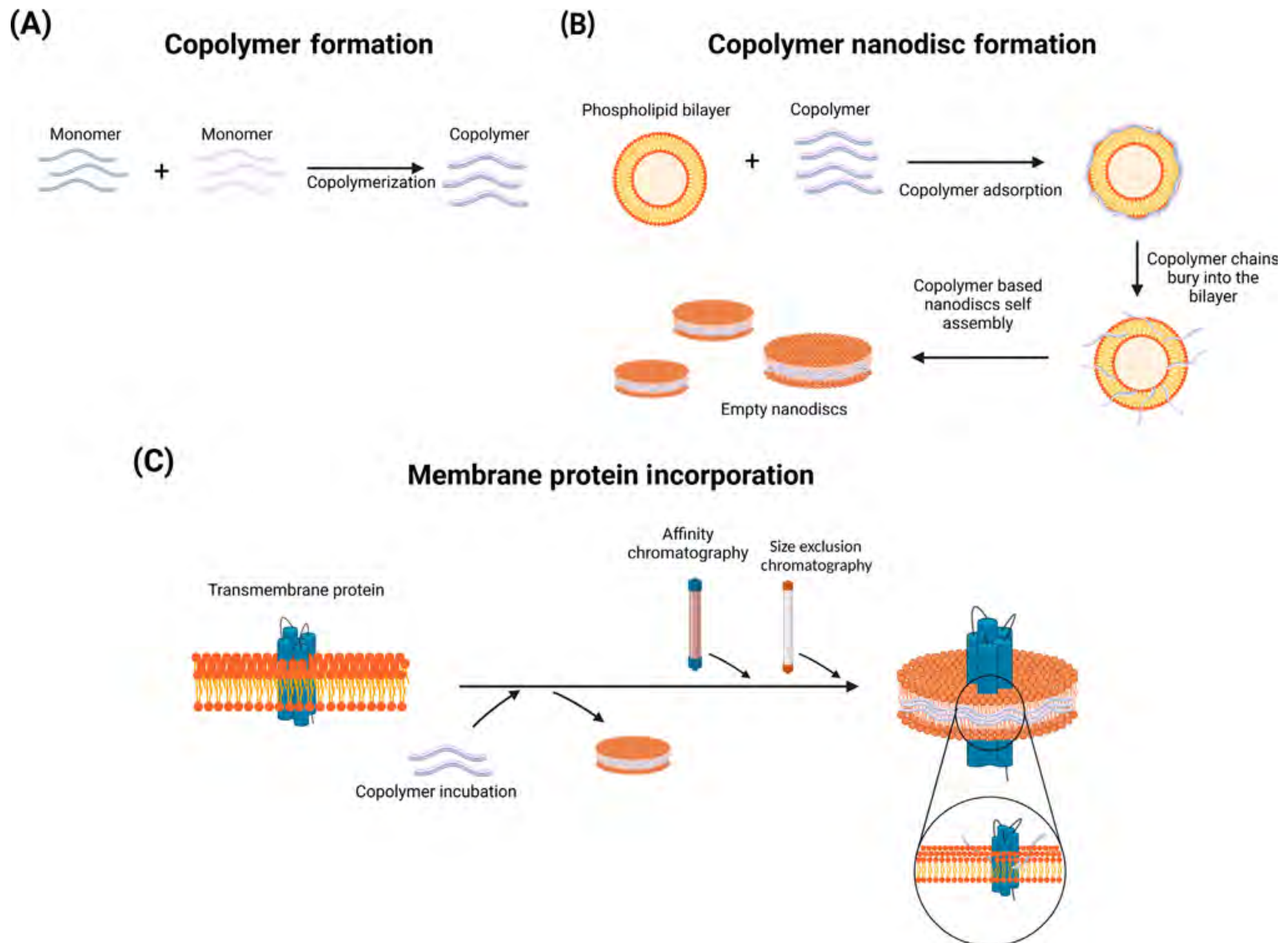

Figure 3. Membrane protein (MP) incorporation into copolymer nanodiscs. (A) Formation of copolymer via copolymerization of two different types of monomers. (B) Self-assembly of copolymer-based nanodiscs takes place upon lipid addition. (C) Extraction of transmembrane protein from its native or synthetic lipid environment occurs with copolymer incubation, followed by further purification of formed MP–copolymer nanodiscs with affinity chromatography and size exclusion chromatography (SEC). Figure created with BioRender.com.

Since the introduction of SMAs, other alterative copolymers including diisobutylene maleic acid (DIBMA) [81], polymethacrylate polymer (PMA) [82], and non-ionic inulin polymer [83] have been designed to overcome the perceived limitations of SMAs. DIBMA is an alternating styrene-free copolymer composed of maleic acid and diisobutylene [81]. DIBMA was first used to solubilize outer membrane phospholipase A (OmpLA) [81], but has since been used to solubilize a variety of membrane proteins including $A_{2A}R$, calcitonin gene-related peptide receptor (CGRP), ATP-binding cassette (ABC) transporter BmrA [84], ZipA [32], rhodopsin II from *Natronomonas pharaonic* (NpSRII) [85], and $\beta_{2A}R$ [86].

A recent study compared DIBMAs and SMAs directly in their ability to solubilize, stabilize, and characterize several different proteins [84]. The study found that SMAs were more effective at solubilizing BmrA and ZipA (both expressed in *Escherichia coli*) compared to DIBMA, while also yielding more purified protein. However, for both $A_{2A}R$ (expressed in *Pichia pastoris*) and CGRP (expressed in Cos7 cells), DIBMAs and SMAs solubilized the receptors similarly. As

predicted, BmrA and ZipA DIBMA lipid particles (DIBMALPs) had much greater stability to the presence of divalent cations than SMALPs. DIBMAs also were less stable over time compared to SMAs, as seen with $A_{2A}R$ and BmrA. In an alternative study, DIBMAs generated using reversible addition-fragmentation chain-transfer polymerization (RAFT) – a method used to precisely control polymer size – were capable of solubilizing ZipA with a similar efficiency to SMAs [32], indicating that polymer size and/or heterogeneity can play essential roles in DIBMA solubilization. The main advantage that DIMBAs have over SMAs is that they are more amenable to biological application because of their increased stability in divalent cations, far-UV compatibility, and decreased lipid order [81,87], making DIBMAs an improved alternative to SMAs.

Another SMA alterative is an entirely styrene- and maleic acid-free copolymer, PMA, that has been developed based on the amphipathic helical structure of MSPs [82]. PMAs are generated from a combination of hydrophobic butylmethacrylate and cationic methacroylcholine chloride through free radical polymerization. PMAs form nanodiscs by interacting with lipid acyl chains and phosphate headgroups from the cell membrane [34,82]. PMAs have been used to extract and purify NTSR1 directly from Sf9 membranes, marking the first MP purified using PMAs [34]. NTSR1 was successfully extracted using PMAs under non-denaturing conditions (26°C, 500 mM NaCl, pH 7.0–7.4), while yielding a similar amount of protein to that of the traditional detergent purification scheme [34]. NTSR1 in PMA nanoparticles also showed increased G_{il} and G_q stimulation compared to detergent micelles, which can be attributed to its ability to preserve the native lipid bilayer. PMAs were also extremely tolerant of the presence of divalent cations and lack the strongly UV-absorbing styrene, resulting in increased compatibility with spectroscopy techniques [82]. These key improvements over SMAs make PMAs an extremely interesting alternative despite their recent development.

Inulin – a naturally produced fructose polysaccharide – was very recently developed as a non-ionic amphiphilic polymer [83]. Non-ionic inulin was synthesized by hydrophobic functionalization of inulin derived from chicory, a type of herbaceous plant. Non-ionic inulin was found to be stable between pH 2.5 and 8.5, as well as in the presence of divalent cations up to 100 mM. Its lack of aromatic groups also makes it more compatible with UV spectroscopy methods. Non-ionic inulin was found to have an *E. coli* solubilization efficiency of >80%, the highest seen for pentyl inulin, compared to 100% with DDM and <50% with charged copolymers SMA, SMA-EA, and SMA-QA (percentages assume that DDM is 100%) [83]. This increase in *E. coli* solubilization efficiency can be attributed to the hydrophobicity of non-ionic inulin. Charge can be added to stabilize non-ionic inulin nanodiscs using pentyl-inulin, where liposomes with at least 20% charged lipids improved stability [88], and the resulting nanodiscs were then used to reconstitute redox complex CYP450 and CYP450 reductase (CPR) from purified proteins [89]. The membrane protein applications related to these non-ionic polymers should provide an interesting avenue of research and structures over the next several years.

Advantages/disadvantages of nanodisc platforms

MSP-, saposin-, and copolymer-based nanodiscs can be broken down into two categories: protein-based and amphiphilic detergent molecules. MSP and saposin both fit into the protein-based scaffold category, while copolymers fit into the amphiphilic detergent molecules category. In general, each of the scaffold systems has its own unique challenges and tradeoffs. However, at present each system takes an extensive amount of trial and error around the reaction conditions for an MP of interest, which can be extremely time-consuming, although they provide a significant increase in the stability of purified MPs compared to traditional detergent purification methods. MSPs – the original nanodisc scaffold proteins – are the easiest of the three types of scaffold proteins to use owing to the expansive amount of research completed

to date. This database of information gives the user an important starting place compared to other systems, including reliable protocols for incorporating MPs, a variety of different MSPs including new and more stable cMSPs, and many advanced applications including structure-based assays such as NMR and cryo-EM, as well as biophysical applications relating to SPR and ELISA. MSPND use may require testing of different MSP constructs, as well as the need to use detergent-purified MPs as a starting point.

Despite their recent development, salipros have shown exceptional promise as alternatives to MSP-based nanodiscs. The ability of salipros to produce nanodiscs of different sizes without the need to change scaffold protein variants and the recent introduction of the DirectMX method help to showcase the important features of this type of scaffold protein. However, because of its relatively recent development, extensive protocols and advanced applications are currently not available, leaving implementation and characterization mostly up to the user. So far only one study has directly compared MSPNDs and salipros [90]. This study concluded that both systems could form highly stable empty nanodiscs which remained stable for 70 days. However, they found that this stability was lipid-dependent, where salipros were the least sensitive to lipid type. They found that both systems successfully incorporated the ABC transporter MsbA, and salipros showed slightly higher sample homogeneity. MsbA activity was much higher in both systems compared to that of amphipathic polymers; however, MsbA in MSPNDs showed higher activity in a greater number of different lipids than salipros. Both systems showed vary similar stability in all but one tested lipid, where MSPNDs provided increased stability in POPC.

Copolymers can extract MPs directly from cell membranes without the need to use detergents, thus offering a unique advantage over MSPNDs and salipros. Although copolymers have been used so far to extract many different MPs including GPCRs, ion channels, and ABC transporters, several limitations remain because of their recent development. Challenges associated with SMALPs, the first copolymer nanodiscs, related to UV interference and stability at non-neutral pH and in the presence of divalent cations have been addressed with the addition of SMA alternatives PMAs and DIBMAs, and more studies with these alternatives should flesh out their value in biophysical and structural studies.

Concluding remarks

Nanodisc platforms offer a valuable resource for the investigation and characterization of individual MPs and their complexes because of their enhanced ability to mimic the cell membrane compared to detergents. In this review we have presented the reader with recent developments and advances regarding MSPNDs, salipros, and copolymer nanodiscs to purify and evaluate MPs. We have also highlighted the advantages and disadvantages of using each of the different platforms, allowing the reader to make an informed decision about which platform is right for their application. To further advance nanodisc platform development, a systematic comparative study needs to be completed, focusing on the ability of different nanodisc platforms to support MP stability, ease of use, and overall effectiveness, to better inform the user on what platform is most applicable for their needs. Further investigations should also help to define the use of MSPND and copolymer nanodisc variants where size may be relevant in different MP applications, such as in the reconstruction of protein complexes or for NMR. Ongoing development of variants of each of the different nanodisc platforms will also be important to overcome the many challenges associated with each platform, including ease of use. We look forward to the further development of these three unique nanodisc platforms, as well as their implementation in MP purification and new applications (see Outstanding questions) in the years to come.

Outstanding questions

Are circular MSPNDs poised to replace conventional MSPNDs because of their increased stability?

Are there approaches to streamline the process steps to facilitate decision-making for naïve users?

Will salipros become a true competitor to MSPNDs owing to their size flexibility?

Will the salipro DirectMX method be broadly useful for many different cell types or applications?

Can we achieve better or broader head-to-head comparisons (including structural detail and long-term stability) of different nanodisc methods to evaluate their promise for a MP of interest given the start-up time needed for each in-house application?

How much does lipid composition affect nanodisc choice or the need for broader scaffold choices or screening methods?

Will salipros and copolymer scaffolds also fuel an explosion in MP structural determination as seen for MSPNDs?

Which of the three copolymer technologies (SMA, DIBMA, PMA) will become the most broadly used?

Are PMAs poised to replace SMAs as the most widely used copolymer to create nanodiscs in more practical biological applications, or are better polymer building blocks needed?

Can improved copolymer understanding facilitate enhanced copolymer design leading to increases in homogeneity, stability, and extraction efficiency?

Are there broader applications of nanodiscs beyond structural or biophysical studies (e.g., biomarker discovery)?

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Declaration of interests

The authors declare no conflicts of interest.

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