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Review

Lock and key: Quest to find the most compatible membrane mimetic for studying membrane proteins in native environment

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ABSTRACT

Membrane proteins play crucial roles in cellular signal transduction, molecule transport, host-pathogen interactions, and metabolic processes. However, mutations, changes in membrane properties, and environmental factors can lead to loss of protein function. This results in impaired ligand binding and misfolded structures that prevent proteins from adopting their native conformation. Many membrane proteins are also therapeutic targets in various diseases, where drugs can either restore or inhibit their specific functions. Understanding membrane protein structure and function is vital for advancing cell biology and physiology. Experimental studies often involve extracting proteins from their native environments and reconstituting them in membrane mimetics like detergents, bicelles, amphipols, nanodiscs, and liposomes. These mimetics replicate aspects of native membranes, aiding in the study of protein behavior outside living cells. Scientists continuously explore new, more native-like membrane mimetics to improve experimental accuracy. This dynamic field involves evaluating the advantages and disadvantages of different mimetics and optimizing the reconstitution process to better mimic natural conditions.

1. Introduction

Biological membranes act as physical barriers, surrounding internal organelles and the entire cell, playing a vital role in cellular functions, particularly in cell survival [1,2]. These membranes consist of various lipids, including phospholipids, glycolipids, and cholesterol, which form the fundamental structure, creating a barrier that separates different cellular compartments. Cholesterol and other sterols contribute to membrane fluidity and stability. Many lipids also have direct roles in cell signaling. Membrane proteins, which make up about half of the plasma membrane surface, depend on the lipid matrix for support and regulation [3]. Lipids not only provide a structural framework but also actively regulate protein activity. Proteins can bind to lipids either specifically, where distinct binding sites are involved, or nonspecifically, where physical properties of the lipids—such as thickness, fluidity, or curvature—affect protein function.

Recent studies show that altering membrane components, such as depleting cholesterol, can soften the tumor matrix, breaking down

Abbreviations: cryoEM, cryo-electron microscopy; smFRET, single-molecule FRET spectroscopy; EPR, spectroscopy Electron paramagnetic resonance (EPR) spectroscopy; CW, continuous wave; NMR, spectroscopy Nuclear magnetic resonance spectroscopy; PDB, Protein Data Bank; CMC, critical micelle concentration; MPs, membrane proteins; SDS, Sodium Dodecyl Sulphate; CTAB, Cetyl-Trimethylammonium Bromide; OmpA, outer membrane protein A; OG, octyl-L-D-glucoside; DDM, Dodecyl-L-D-maltoside; CHAPS, 3-(dimethylammonio)-1-propanesulfonate); LDAO, Lauryldimethylamine-N-Oxide; MNGS, maltose neopentyl glycols; LMNG, Lauryl Maltose neopentyl glycol; DMPC, dimyristoyl phosphatidylcholine; DHPC, dihexanoylphosphatidylcholine; NAPols, nonionicamphipols; SAPols, sulfonate-damphipols; PC-Apols, phosphorylcholineamphipols; IMP, integral membrane protein; MSPs, membrane scaffold proteins; HDL, high-density lipoproteins; GPCRs, G protein-coupled receptors; SMA, styrene-maleic acid; DIBMA, diisobutylene maleic acid; PMA, polymethacrylate; SMALPs, Styrene–Maleic Acid–Lipid Particles; SMAnh, hydrolyzing styrene-maleic anhydride; POPC, palmitoyl-oleoylphosphocholine; ApoA1, apolipoprotein A-I; NSP, nanodisc scaffold peptide; SapA, saposin A; OmpX, outer membrane protein X; pSRII, sensory receptor rhodopsin II; β1AR, β1-adrenergic receptor; POTs, protein-coupled oligopeptide transporters; AcrB, bacterial efflux transporter; SMO, smoothened receptor; LPS, lipopolysaccharides; OMPs, outer membrane proteins; Ail, Attachment invasion locus; SUVs, small unilamellar vesicles; LUVs, large unilamellar vesicles; GUVs, giant unilamellar vesicles; MLVs, multilamellar vesicles; ssNMR, solid-state NMR; ASR, Anabaena sensory rhodopsin; TM, trans-membrane domain; APP, Amyloid Precursor Protein.

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physical barriers to enhance antitumor therapy and improve drug delivery [4]. Membrane proteins, encoded by roughly a quarter of the human genome, occupy specific locations within the lipid bilayer (Fig. 1), performing essential functions such as ion and nutrient transport, signaling, enzymatic reactions, pathogenesis, defense, cell adhesion, metabolism, cellular organization, and bioenergetics [5–10]. In recent decades, the study of membrane proteins has advanced significantly through the development of diverse in-cell and in-vitro functional assays [11], improving structural determination techniques like X-ray crystallography in various environments (detergents, bicelles, nanodiscs, lipidic cubic phases) at resolutions of 3 Å or higher [12–14]. Advances in single-particle cryo-electron microscopy (cryoEM) and single-molecule FRET spectroscopy (smFRET) have also enhanced our understanding of membrane protein structures and conformational dynamics [15–20]. Techniques like EPR spectroscopy (CW and pulse methods), NMR spectroscopy, especially solid-state NMR (ssNMR) and molecular dynamics simulations have further contributed to these insights, alongside site-directed mutagenesis studies to elucidate specific amino acid functions [21,22]. These combined approaches have led to the resolution and deposition of numerous protein structures in the Protein Data Bank (PDB), where out of approximately 225,946 total structures, 7088 (3.1 %) are membrane proteins [23]. Even with this progress, membrane proteins remain under studied due to their complexity and the challenges in characterizing their diverse functional mechanisms. Issues such as poor solubilization efficiency, low protein expression, and the difficulty of finding suitable membrane-like mimetics for protein stabilization complicate research. Membrane proteins' hydrophobic regions make it difficult to isolate without affecting their structural or functional integrity [24,25]. Selecting the appropriate membrane mimetic is essential for obtaining functional proteins in vitro at the required concentrations and purity for further biochemical and biophysical characterization [26]. Given the importance of membrane mimetics in preserving membrane proteins' native states, this review focuses on widely used and emerging mimetics like detergents, liposomes, nanodiscs, bicelles, amphipols, native membrane. We explore their applications, advantages, and limitations in purifying and studying membrane protein structures and functions. Additionally, we review recent innovations that bring these mimetics closer to mimicking native cellular environments (Fig. 2), while recognizing that the scope of this review is limited by the rapid developments in the field.

2. Detergent-based solubilization of membrane proteins mimics as micelles or bicelles protocol and applications

Detergents have traditionally been the most used membrane mimetics for solubilizing membrane proteins [27]. Earlier, scientists employed organic solvent mixtures to mimic membrane properties. However, while membrane proteins generally maintain their secondary structure in organic mixtures, they fail to preserve their tertiary structure [28]. As a result, the use of organic solvents is now considered unfavorable and has been completely phased out. Detergents are amphipathic molecules, consisting of a non-polar hydrophobic tail that act as a surfactant and a polar head group [29,30]. One of their key features is their critical micelle concentration (CMC). Below the CMC, detergent molecules remain soluble in water, but when the concentration exceeds the CMC, their amphipathic nature leads to the formation of aggregates known as detergent micelles, which have a hydrophobic core and a hydrophilic outer surface. These micelles create a clear boundary between the polar and hydrophobic regions, allowing different parts of membrane proteins (MPs) to interact within the micelle and fold into their proper tertiary structure. In the early stages of biochemical research, naturally occurring detergents like saponins and bile salts were used to solubilize and reconstitute MPs in membranes, laying the foundation for understanding their structure and function [31,32]. Over time, advancements in science have led to the development of a wide array of detergent molecules, including non-ionic, zwitterionic, and ionic variants, each offering specific biochemical properties. This diversity provides researchers greater flexibility and precision in manipulating and characterizing membrane proteins in vitro. An effective method for optimizing detergent properties for membrane protein research is the addition of an extra alkyl chain between the two existing ones. This novel biochemical tool and design strategy holds promise for improving membrane protein structure determination, offering new opportunities for more accurate structural insights [33].

Based on their chemical nature, detergents can be classified into three primary classes: First **Ionic Detergents** contain either a positively or negatively charged polar head group. They are commonly referred to as 'harsh' membrane mimetic as they are strong denaturants and may have detrimental effect on the structure of membrane proteins [34–36]. Sodium Dodecyl Sulphate (SDS), (negatively charged) and Cetyl-Trimethylammonium Bromide (CTAB), (positively charged) are the most used ionic detergents (Fig. 3). Strong detergents have tendency of high affinity towards membrane proteins hence affect the structural and functional properties of the proteins however bacterial outer membrane protein A (OmpA) is an exception that its structure is conserved even in ionic detergents such as SDS at room temperature. Second **Non-ionic detergents** are 'mild' and are the predominantly used detergents for solubilizing membrane proteins in their functional form e.g. octyl-L-D-glucoside (OG) and Dodecyl-L-D-maltoside (DDM) (Fig. 3). Non-ionic detergents disrupt the interactions between lipids and proteins without significantly affecting the interactions between molecules of proteins, in contrast to more abrasive ionic detergents [37,38]. This characteristic is especially crucial for solubilizing multimeric membrane protein assemblies, as accurate investigation of their structure and

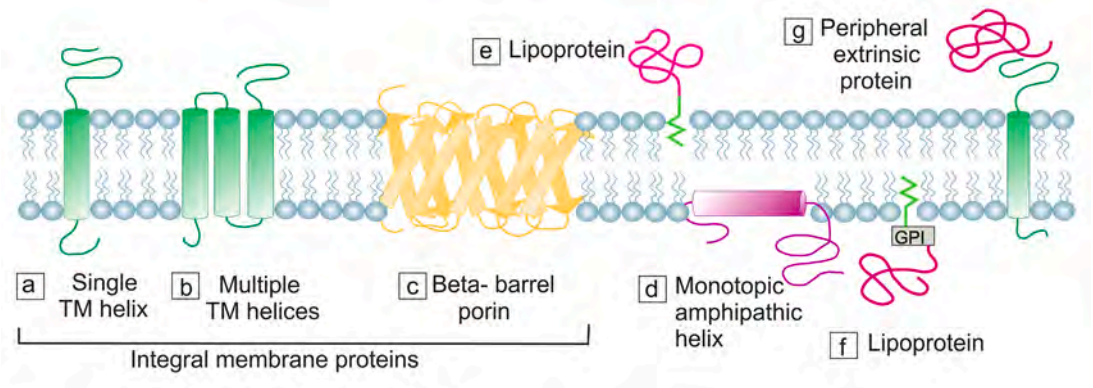

Fig. 1. Membrane Proteins. Integral membrane protein(a). Single transmembrane protein; (b) Multiple transmembrane protein; (c) Beta-barrel porin sweep to the leaflets; (d)Monotopic amphipathic helix that sweep a single leaflet;(e and f) Lipoproteins; and (g)Peripheral extrinsic proteins.

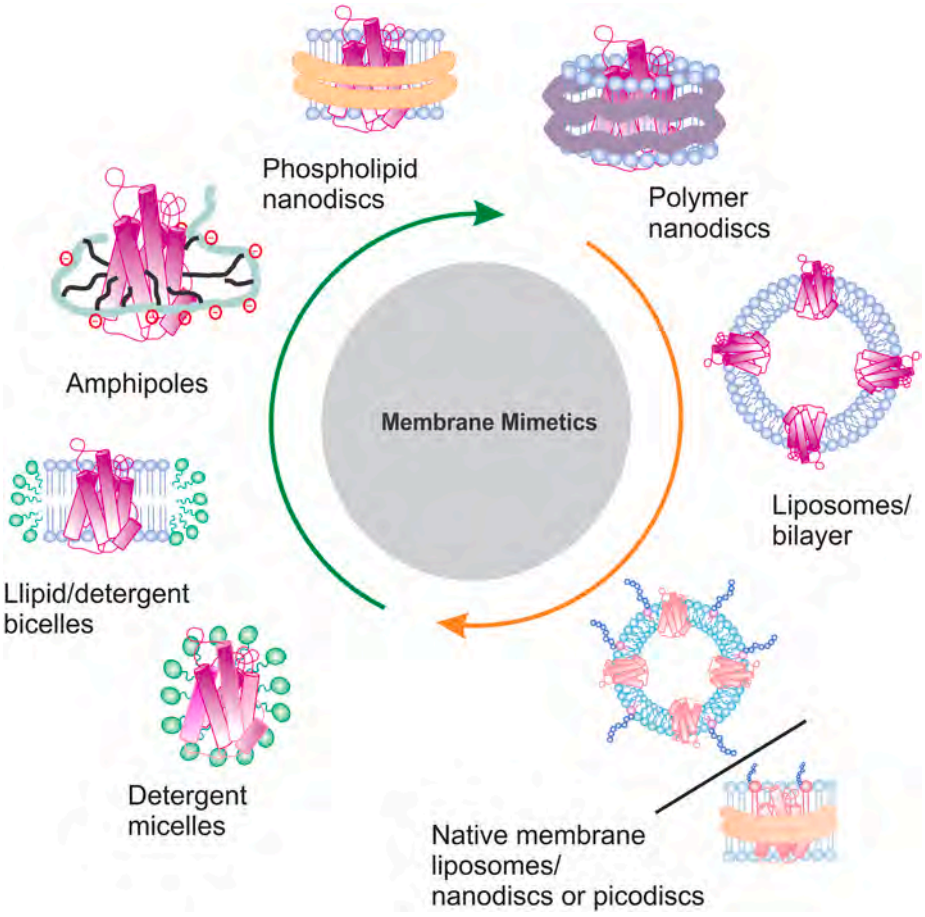

Fig. 2. Schematic representation of various membrane mimetics employed for studying membrane proteins using NMR spectroscopy and other techniques. The membrane mimetics are organized in chronological order, reflecting their emergence in the field of research.

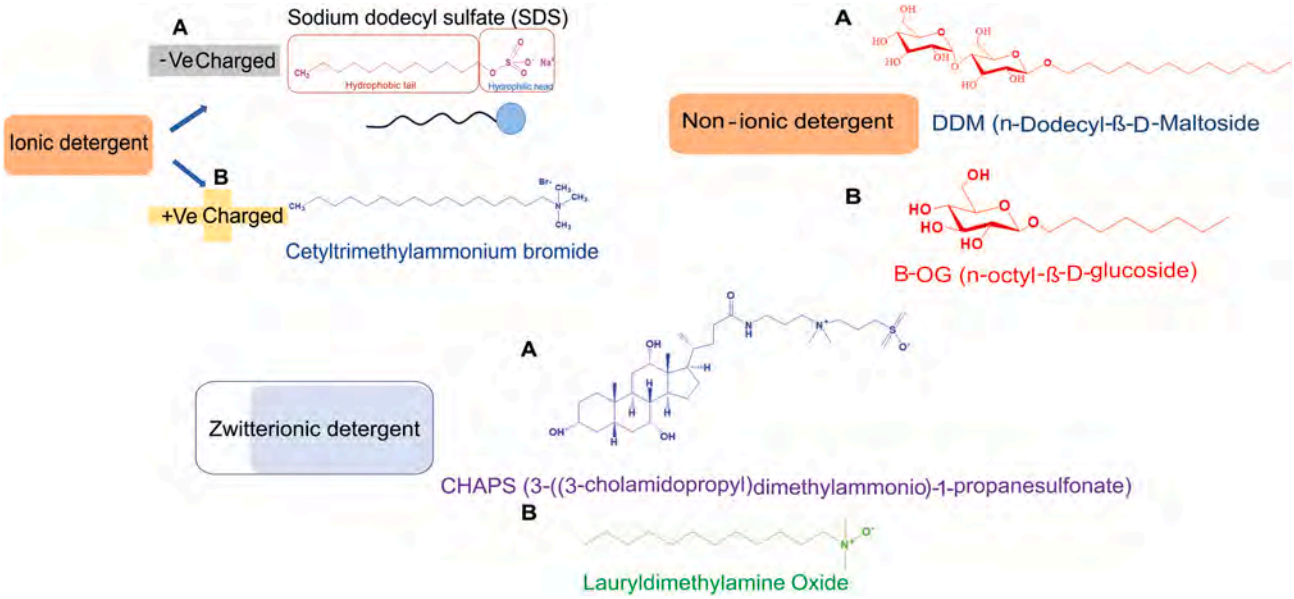

Fig. 3. Structure of detergent commonly used for membrane mimetics for solubilizing membrane proteins these are ionic (A. positive charged ionic detergent SDS, B. negative ionic charged Cetyltrimethylammonium bromide), non-ionic (A.DDM and B. B-OG (n-octyl-β-D-glucoside)) and sometime zwitterionic detergent (A. CHAPS and B. Lauryl dimethylamine Oxide).

function depends on maintaining the native protein-protein interactions. Non-ionic detergents aid in the selective disruption of lipid-protein connections while protecting protein-protein interactions, allowing membrane proteins to be extracted from biological membranes while preserving their original shape and functionality (because of mild nature of non-ionic detergents). Because of their complex structure and hydrophobic nature, membrane proteins are typically difficult to purify and analyze in biochemical research, making detergents useful tools. Non-ionic detergents can also play in terms of downstream processing, including protein purification, crystallization, and functional tests. and third **Detergents with zwitterionic properties**, such as 3-(dimethylammonio)-1-propanesulfonate) [CHAPS] and Lauryldimethylamine-N-Oxide (LDAO) have overall zero charge. These have comparatively less denaturing and strong solubilization potential than ionic detergents [39,40]. These are structurally closer to lipids and believed to provide better membrane mimicking environment due to matching chemistry of head group and length of the hydrophobic tail. So, they are placed in between ionic and non-ionic detergents as shown in Fig. 3.

There are two stages in the solubilization protocol of MPs (i) At lower concentration, the detergent molecule inserts itself into the bilayer and causes its destabilization and fragmentation. (ii) At high detergent concentrations above the CMC, the lipid bilayer dissolves and different complexes are formed like protein-detergent, lipid detergent as well as the ternary protein-lipid-detergent complexes [41] (Fig. 4).

Prior to performing additional downstream applications, selecting the optimal detergent is crucial since different detergents have varying capacities for soluble biological membranes [42]. This is usually carried out by adding various detergents to membrane fractions at critical concentrations and the solutions are incubated at 0–4 °C for 30–60 min, then centrifuged at 4 °C at range 1000 g to 16,000 g for 1 h. After that, activity of the protein which is to be studied is measured in both the pellet and supernatant fraction. The best detergent for the protein of interest will effectively solubilize the membrane protein and hence, there would be considerable activity of that protein in the supernatant fraction. As membrane proteins can perturb the well-ordered arrangement of lipids in the membrane and thus promote easy access for detergent micelles to insert themselves into the bilayer and solubilize them, few rigidly packed bilayers can be detergent resistant which can result in lipid portions still being associated with the extracted membrane proteins. Hence, optimal detergent/ protein and detergent/ lipid ratio is essential for effective solubilization of membranes and thereby, their correct extraction [43]. The type of membrane that is targeted for solubilization is also a deciding factor for the selection of detergent for ex, Triton-X 100 can solubilize the inner membrane of gram-negative bacteria but not the outer membrane.

The high concentration of detergent used for solubilization can hamper further purification processes and thus removal of excess detergent is essential. It is performed by carrying out size exclusion chromatography, adsorption chromatography or dialysis [44]. After solubilization and excess detergent removal, standard protein purification protocols can be employed for the purification of membrane protein. Detergents are also used as intermediate anchors of membrane proteins before their reconstitution into more lipid like membrane mimetics such as bicelles, nanodiscs and liposomes for additional biophysical and biochemical studies.

Currently, newer detergents which mimic the lipid bilayer like maltose neopentyl glycols (MNGS) with two maltose residues as polar head group and two alkyl chains as non-polar tail and calixarenes have

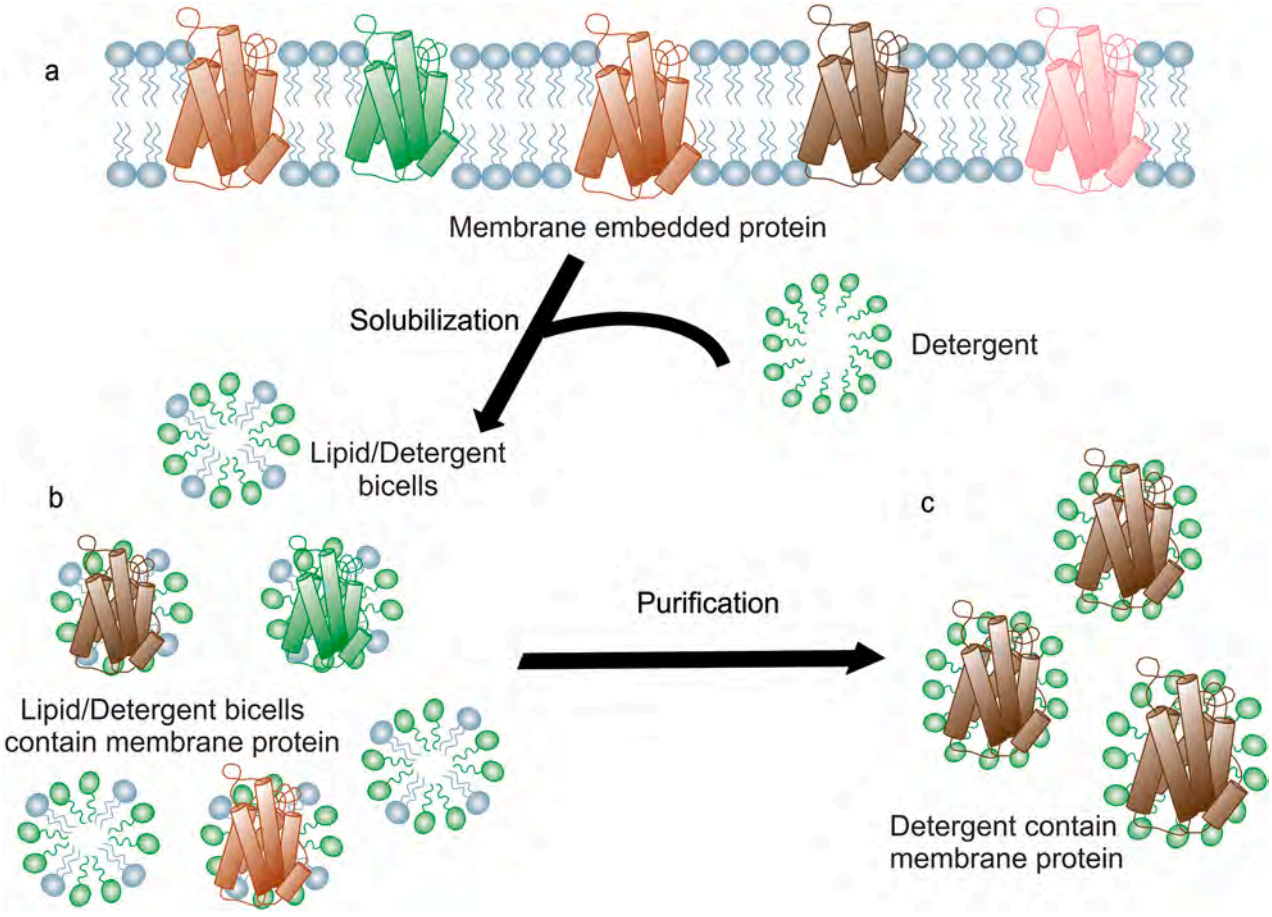

Fig. 4. Steps involve in the process of purification of membrane protein from biological membrane by solubilizing it. (a) Membrane protein present in the native environment in lipid bilayer, (b) Solubilization of membrane protein using detergent, (c) Purified membrane protein got from biological membrane.

resulted in reduction of flexibility and increase in hydrophobicity in the micelle interior. They confer better stability and it was also shown that Lauryl Maltose neopentyl glycol (LMNG) also assist in improving crystallization of the membrane protein. Detergent molecules are usually much more curved in comparison to the lipid bilayer [45]. Due to the polar bulky head groups, the arrangement of detergent monomers is not well ordered. The high solubility of detergents in aqueous medium coupled with this loose arrangement results in a continuous exchange of detergent monomers between the binary protein-detergent complex and detergent monomers. Due to this unstable presence of polar groups at the interface, their shielding effect decreases and water molecules penetrate the nonpolar core of the micelles very easily. As the hydrophobic tail of detergent molecules are generally shorter in length to the phospholipid tails of the bilayer, flexibility rises, which may have an impact on the membrane protein's structure and functional dynamics. The micelles which are formed by longer alkyl chain hydrophobic tails exhibit lower permeability into the hydrophobic core and additionally, they show greater lateral strain on the membrane proteins' transmembrane section, thereby helping in maintaining their stability.

Detergent micelles are widely used for the solubilization and purification of membrane proteins, making it possible to study their structure and function in solutions. Solubilized membrane proteins in detergent micelles are often utilized for structural analyses using techniques such as X-ray crystallography, cryo-electron microscopy (cryo-EM), or nuclear magnetic resonance (NMR) [46]. These studies can provide valuable insights, but the detergent environment may not perfectly mimic the native lipid bilayer, potentially impacting the protein's structure or function. While functional assays, such as ligand binding or enzymatic activity studies, can also be performed in micelles [47], researchers frequently convert solubilized proteins into other model systems for more physiologically relevant analyses. For example, reconstitution into liposomes allows membrane proteins to be studied within a lipid bilayer environment, ideal for investigating protein-lipid interactions, ion transport, and channel activity [48]. Similarly, nanodiscs, which use scaffold proteins or polymers to stabilize membrane proteins in a nanoscale lipid bilayer, offer a more native-like environment for structural and functional studies. Amphiphilic polymers, such as amphipols, provide an alternative to detergents, enhancing protein stability in solution. Ultimately, while solubilized proteins in micelles are used for initial analyses, they are often transitioned to model systems like liposomes, nanodiscs, or amphipols for more accurate and physiologically relevant studies [49].

3. Detergent-assisted bicelles provide fine-tuning lipid bilayer composition and size

Bicelles (bilayered micelles) are discoidal lipid nanoaggregates. They are acknowledged as the first membrane mimetic with the capability to integrate considerable amount of lipids to mimic the lipid bilayer for membrane proteins. They are created by combining either short-chain phospholipids in an aqueous solution or long-chain phospholipids with detergents [50–52]. Unlike micelles, they contain a planar region (made up of the long chain phospholipids) which is surrounded by a detergent rim having a protective function to shield the tails of long chain phospholipids from water. During the formation of bicelles, the long chain phospholipid 1,2-dimyristoyl phosphatidylcholine (DMPC) is usually used with the detergent CHAPS or with short chain phospholipid dihexanoylphosphatidylcholine (DHPC) [53]. Cholesterols and sphingolipids are often doped in bicelles to produce a membrane model which can have close resemblance to the native environment of the membrane protein of interest.

When lipids in bicelles comprise charged groups such as negative charge bearing lipids, the membrane proteins get inserted at varying depths inside the membrane. This depth depends on the type and charge of the lipid present at the interface. A primary advantage of bicelles is the opportunity to fine tune their sizes by varying a parameter q (long chain to detergent/short chain lipid ratio) [54]. The radius (R) of their bilayer region can be calculated by the equation,

$$R = \frac{1}{2} rq\left[\pi + \left(\pi^2 + \frac{8}{q}\right)^{\frac{1}{2}}\right] \quad (1)$$

where, r = half the bilayer thickness. Their overall diameter can therefore be expressed as D = 2R + 2r8. Higher q value bicelles have lower concentration of short-chain lipids than the long-chain lipids and are bigger in size than bicelles with low q value. Membrane protein perturbation can occur when they are embedded in small q-value bicelles, as these bicelles tend to be lipid-poor and detergent-rich. It is important to note that the q-value alone does not determine bicelle morphology; the total lipid concentration also plays a critical role. Therefore, dilution of the bicelle solution can significantly affect their size and morphology. Consequently, isotopically labeled membrane proteins reconstituted in small, mobile bicelles are studied using solution NMR spectroscopy. Some of the bicelles can align in presence of magnetic field and the embedded MPs align along with the bilayer are suitable to be studied using ssNMR and other methods used to examine bicelles [55,56]. Oriented bicelles have proven to be a valuable model system in ssNMR studies for simulating membrane environments with varying curvatures. This innovative approach enables researchers to mimic the structural diversity of biological membranes and investigate the behavior of proteins and peptides in response to distinct curvature preferences. By demonstrating the applicability of DNP signal enhancement techniques to oriented membrane samples, this method also provides a foundation for further advancements in sample preparation protocols [57]. The study of biomolecule partitioning within bicelles offers crucial insights into their interactions with specific membrane geometries. These findings play a pivotal role in understanding membrane-protein dynamics, shedding light on functional mechanisms involved in processes such as membrane remodeling, signaling, and molecular transport.

In ssNMR investigations, oriented bicelles have shown themselves to be an effective model system for replicating membrane environments with different curvatures. With this novel method, scientists may replicate the structural variety of biological membranes and study how proteins and peptides react to different curvature preferences. This approach also lays the groundwork for future developments in sample preparation procedures by proving that DNP signal enhancement techniques can be applied to orientated membrane samples [57,58]. Understanding how biomolecules partition within bicelles provides important information about how they interact with particular membrane geometries. These discoveries are essential for comprehending membrane-protein dynamics because they provide insight into the functional mechanisms behind processes like molecular transport, signaling, and membrane remodeling.

4. Amphipols

Amphipols (amphipathic polymers) are a group of non-detergent surfactants. They are short polymers which consist of polar groups separated by hydrophobic chains along the length of the polymer. The primary goal in creating amphipols was to offer a medium that would aid in the target membrane protein's ability to hold onto its cofactors, subunits and lipids associated with it [59]. Jean-Luc Popot, C. Tribet and R.Andebart designed amphipols to carry lot of hydrophobic chains so that their interaction with the membrane protein is through multiple points of contact. Amphipols dissociate from the amphipol/membrane protein complex very slowly or in some cases, dissociate only when subjected to exchange with other surfactants and thus result in an increased stability of the membrane protein in solution. The mechanism of action of amphipols was validated by a study on four integral membrane proteins namely bacteriorhodopsin (BR), a bacterial photosynthetic reaction center, matrix porin, and cytochrome B6f. It is shown

that BR has been stable in amphipols at 40 °C for 7 days [60]. On the contrary, solubilization with any detergent leads to protein aggregation in a few hours.

Though several types of amphipols have been studied, A8–35 remains the most used one [61]. Its nomenclature is based on the existence of a brief polyacrylate chain, which is followed by a number representing the molecular weight of the polymer's modified monomer. The content of the charged monomers is indicated by the number following the hyphen. The short polyacrylate chain in A8–35, which includes roughly 70 residues, has had octylamine and isopropylamine randomly grafted at residues 17 and 28, respectively. The charge of amphipol is derived from the free acid groups and they make the polymer hydrophilic and the octylamine moieties render the molecule amphipathic. Recently developed other amphipols include glycosylated nonionicamphipols (NAPols), sulfonatedamphipols (SAPols) or phosphorylcholineamphipols (PC-Apols). SAPols are derived from A8–35 but they lack the isopropyl grafts which are replaced by taurine. Taurine contains sulfonate groups which keeps SAPols water soluble at acidic pH as the sulfonate groups are not protonated even at zero pH, whereas A8–35 aggregates at such conditions.

Table 1
Most frequently used lipid membrane mimetics in the functional and structural studies in MPs.

System /Type	Applicable Techniques to Study MPs	Advantages	Disadvantages
Micelles	Solution NMR, X-ray crystallography, Single-particle Cryo-electron microscopy, Neutron solution scattering, MS/MS, EPR spectroscopy, Fluorescence spectroscopy, smFRET, Isothermal titration calorimetry (ITC) for ligand binding/protein interactions functional assays	Effortless of the system makes it an ideal starting point for downstream applications. Additionally, there is a wide availability of a large variety of detergents.	The propensity of IMP denaturation and the chances of non-physiological IMP conformations rise due to mismatched 'IMP-micelle' hydrophobic stiffnesses. It is necessary to consider the CMC (Critical Micelle Concentration) of the detergent in such cases
Bicelles	Solution NMR, Solid-state NMR, X-ray crystallography, EPR spectroscopy	Convenient preparation, ensuring the provision of homogeneous and translucent suspensions, creating a true lipid environment under physiological conditions. Myriad forms of lipids can be incorporated to match specific requirements, and bicelles of different sizes can be prepared	The size and geometry of bicelles can be affected by the total lipid concentration. There is a risk of IMP perturbation in case of an insufficient bilayer size
Nanodiscs	Solution NMR, Single particle Cryo-EM, Cryo-electron tomography, atomic force microscopy (AFM), Fluorescence spectroscopy, smFRET, Isothermal titration calorimetry (ITC) for ligand binding/protein interactions functional assays	The IMP maintains its integrity and shape even upon dilution, and the soluble domains in IMPs are easily accessible. Additionally, there is the possibility of adjusting the size to accommodate either a monomeric IMP or a larger IMP complex.	Optimizing assembly conditions can be time-consuming, and it is not suitable for large MP (Membrane Protein) oligomers. The dynamics of lipids are affected by the protein 'belt', limiting the size range to relatively small structures
Liposomes	Electron crystallography, solid-state NMR, Atomic force microscopy, electrophysiology, fluorometry	Due to its large size, it can accommodate large and multicomponent systems, providing a continuous membrane that closely resembles the native environment for IMPs. The diffusion behavior is like that of a native phospholipid membrane, offering a broad range of possible lipid compositions.	The orientation of IMP is frequently non-native, and the system can be relatively expensive compared to traditional methods due to its low solubility
Native membrane	Electron cryotomography, solid-state NMR, Atomic force microscopy	Nearer to the natural environment and more authentic.	It is often challenging to handle due to the low content of the protein of interest compared to the presence of contaminants

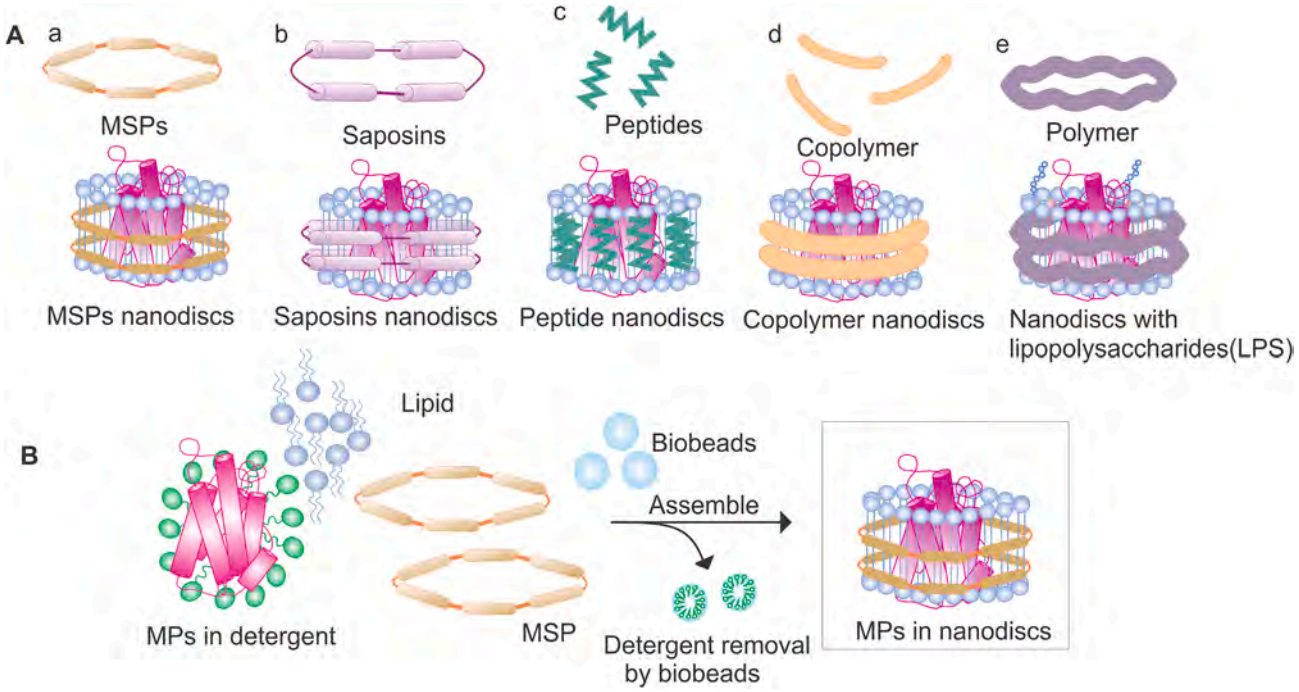

Fig. 5. A. Nanodiscs and their modifications based on different types of wrapping belts surrounding the Nanodiscs a. MSPs Nanodiscs, b. Saposins Nanodiscs, c. Peptides Nanodiscs, d. Copolymer Nanodiscs, e. Nanodiscs with LPS. B. Formation of Nanodiscs containing membrane proteins by removal of detergents and assembling with MSP (membrane scaffold protein).

5. Nanodiscs and their modifications

Sligar and colleagues demonstrated nanodisc technology for the first time on the liver microsomal NADPH-cytochrome reductase enzyme, also known as CYP450 reductase in 1998 [62]. Proteolipid systems, comprising fragments of lipid bilayers enclosed in high-density lipoprotein (HDL), were the precursors of nanodiscs. After that, lipoprotein belt (membrane scaffold protein, or MSP), peptide, saposin, or copolymers held lipid nanostructures in place while nanodiscs diversity increased which can help in drugs delivery at targeted spot and also membrane protein mimetics. All of these membrane-like nanoscale lipid bilayer structures are self-assembled and have a disc form. Two key benefits of nanodisc technology are its lack of detergent chemicals and its capacity to retain integrity and shape even after dilution. Additionally, by adjusting the nanodisc size, nanodiscs can separate IMP oligomeric forms and provide easy access to soluble domains in integral membrane proteins (IMP) [63]. In Table 1 displays the most recent methods used to examine nanodiscs, along with their benefits and drawbacks. Today, nanodisc systems are mostly categorized based on the belt they use (Fig. 5 A), such as:

5.1. MSP nanodiscs

MSP nanodiscs are a widely used system for stabilizing and studying membrane proteins in a native-like environment. These nanodiscs employ amphipathic membrane scaffold proteins (MSPs), derived from truncated forms of apolipoprotein A-I (a primary component of high-density lipoproteins, HDL), to encircle and stabilize a discoidal phospholipid bilayer [64,65]. This bilayer embeds the target transmembrane proteins, enabling their solubilization and characterization in aqueous solutions.

MSPs are arranged in an antiparallel conformation, providing a hydrophobic surface facing the lipid tails and a hydrophilic surface outward, which confers high solubility to the nanodiscs. Assembly involves incorporating detergents during membrane protein purification and nanodisc formation, followed by detergent removal using bio-beads (Fig. 5 B). Once assembled, the nanodiscs eliminate the need for detergents, maintaining the embedded proteins in a stable, native-like state [66–68].

The size of MSP nanodiscs ranges from 7 to 17 nm, depending on the specific MSP variant used. MSP constructs are engineered by modifying the number of amphipathic α-helical repeats. For example: MSP1 contains a single repeat of 10 helices, forming ~10 nm discs. MSP2, with two repeats, produces larger discs. Extended versions such as MSP1E1, MSP1E2, and MSP1E3 yield even larger nanodiscs. Shortened constructs like MSP1D1 and MSP1D2 result in smaller nanodiscs. This tunability allows researchers to customize nanodisc dimensions (8.4–17 nm diameter, ~150 kDa molecular mass) to suit specific experimental needs [65,69–71].

MSP nanodiscs are versatile tools for studying membrane proteins from prokaryotic and eukaryotic systems. They have been successfully used to characterize a variety of proteins, including transporters, ion channels, and G protein-coupled receptors (GPCRs). By providing a detergent-free, biocompatible lipid environment, MSP nanodiscs facilitate structural and functional analyses through techniques such as cryo-electron microscopy (cryo-EM), NMR spectroscopy, and ligand-binding assays.

5.2. Copolymer nanodiscs

Synthetic copolymer nanodiscs represent a significant advancement in membrane protein research, allowing the direct extraction and stabilization of membrane proteins in their native state. These nanodiscs are synthesized from intact cell membranes using polymers that serve dual roles: solubilizing and stabilizing membrane proteins and their surrounding lipid bilayers. This innovative approach bypasses the need for traditional detergents, thereby preserving the native structure and function of membrane proteins [72–75].

The assembly of copolymer nanodiscs involves the spontaneous encapsulation of membrane proteins directly from native membranes. This process generates nanosized “slices” of the lipid bilayer, stabilized by synthetic polymer rings such as styrene-maleic acid (SMA), diisobutylene maleic acid (DIBMA), or polymethacrylate (PMA) copolymers. These polymers interact with lipids to form stable, discoidal structures in aqueous environments. Unlike membrane scaffold protein (MSP)-based systems, copolymer nanodiscs are non-proteinaceous, have higher purity, and are versatile in their applications, including structural biology, drug delivery, and biosensing.

SMA-based nanodiscs, known as SMALPs (Styrene–Maleic Acid–Lipid Particles), were established by Knowles et al. and Jamshad et al. These nanodiscs are formed by hydrolyzing styrene-maleic anhydride (SMAnh), with varying styrene-to-maleic acid ratios [76]. SMA copolymers selectively excise portions of lipid bilayers, stabilizing them through hydrophobic interactions between the lipid bilayer edges and the styrene phenyl rings [77]. The resulting monodisperse nanodiscs, typically 10–11 nm in diameter and containing around 140 lipid molecules, are well-suited for isolating and studying integral membrane proteins.

Researchers have also synthesized and utilized Lipodisqs incorporating various lipids such as palmitoyl-oleoylphosphocholine (POPC) or dimethylphosphocholine (DMPC). The pH-dependent stability of Lipodisq is an important factor to consider when dealing with them; because of the maleic acid moiety's tendency to protonate at pH values below 6.5, this can be a limitation when examining IMPs at lower pH levels [78].

Copolymers compared to amphipols are synthetic polymers designed to wrap around and stabilize purified membrane proteins in aqueous environments, eliminating the need for detergents or lipids. They are amphiphilic in nature, with a hydrophobic backbone that interacts with the transmembrane regions of membrane proteins and short hydrophilic side chains that ensure solubility in water. This unique structure allows amphipols to maintain the functional integrity of membrane proteins without requiring a lipid-based environment.

In contrast, copolymers (polymers comprising more than one chemically distinct monomer) are used in nanodiscs comprising distinct hydrophobic and hydrophilic segments. The hydrophobic block, such as polystyrene, embeds within the lipid bilayer to stabilize it, while the hydrophilic block, often polyethylene glycol (PEG), interacts with the surrounding aqueous environment to maintain solubility [79]. These copolymers self-assemble into nanodiscs that replicate the structure and dynamics of natural lipid bilayers, creating an optimal environment for the study and stabilization of membrane proteins. While amphipols directly stabilize proteins, copolymers for nanodiscs provide a lipid-mimicking matrix that supports more native-like protein behavior [80].

5.3. Peptide nanodiscs

Peptide nanodiscs, or peptidiscs, represent a different and adaptable tool in membrane protein research, providing a streamlined and flexible alternative to traditional membrane scaffold protein (MSP) nanodiscs and Saposin nanodiscs [81]. Peptidiscs are constructed using short synthetic amphipathic peptides inspired by apolipoprotein A-I (ApoA1). These peptides arrange themselves antiparallelly around the hydrophobic edges of the lipid bilayer, stabilizing the embedded membrane proteins without requiring conventional detergents, which often disrupt protein structure and function.

The foundational component of peptide nanodiscs is the nanodisc scaffold peptide (NSP), an ApoA1-mimetic peptide. Initial NSPs were 37 amino acids long, but shorter variants, as small as 18 amino acids, have since been developed. These peptides displace detergent molecules and form a protective, flexible scaffold around the membrane protein, maintaining its native structure and function. This adaptability allows

the peptidisc to conform to the size and shape of transmembrane protein complexes, making it particularly suitable for stabilizing multi-subunit proteins that may dissociate in prolonged detergent conditions [82–84].

Peptide nanodiscs are advantageous due to their simplified assembly process, which does not necessitate the addition of specific phospholipids. Membrane proteins can be reconstituted directly after mild detergent extraction, preserving their integrity. While peptidiscs are effective for purifying affinity-tagged membrane proteins under detergent-free conditions, high-resolution structural data from such systems remain limited. Applications have primarily focused on bacterial membrane proteins, with eukaryotic membrane protein systems still largely unexplored [85].

Peptidiscs offer several key benefits. The peptide-to-lipid ratio can be adjusted to control the nanodisc diameter, allowing researchers to tailor the system to specific experimental needs. This customizability makes them a valuable resource in structural biology, drug discovery, and other fields requiring stable membrane protein systems.

5.4. Saposin nanodiscs

The saposin protein family, comprising four members (saposin A–D, each with a molecular weight of approximately 10 kDa), has emerged as a adaptable tool in the study of membrane proteins. Among these, saposin A (SapA) has been predominantly utilized for assembling saposin nanodiscs. These nanodiscs, also referred to as salipro nanodiscs, are self-assembled structures formed by saposin proteins, phospholipids, and membrane proteins [86]. Frauenfeld et al. pioneered the use of saposin proteins as scaffolds for reconstituting various membrane proteins in phospholipid environments, demonstrating their utility in stabilizing integral membrane proteins (IMPs) for structural and functional studies.

One of the primary advantages of saposin nanodiscs lies in their adaptability to membrane proteins of varying sizes. Unlike traditional systems, such as membrane scaffold proteins (MSPs), saposin nanodiscs do not require extensive optimization of protein-to-lipid ratios or scaffold constructs. This flexibility enables their application across a wide range of membrane proteins without the need for laborious screening processes [87].

Applications of saposin nanodiscs are predominantly in structure-based analytical techniques, such as solution-based NMR and cryo-electron microscopy (cryo-EM). For instance, NMR studies have successfully incorporated proteins such as bacterial outer membrane protein X (OmpX), sensory receptor rhodopsin II (pSRII), and β1-adrenergic receptor (β1AR) into saposin nanodiscs. Cryo-EM has further expanded the utility of saposin nanodiscs, enabling high-resolution characterization of proteins including protein-coupled oligopeptide transporters (POTs), bacterial efflux transporter AcrB, smoothened receptor (SMO), and various nicotinic receptors.

Structurally, saposin nanodiscs are formed by the arrangement of two or more SapA proteins in a V-pattern around a core lipid disc. This architecture provides remarkable flexibility, allowing the system to accommodate IMPs of diverse sizes and configurations. The lipid composition can also be fine-tuned to better mimic the native environment of the membrane protein, further enhancing the stability and relevance of experimental findings [88,89].

The broad tolerance of saposin nanodiscs to varying lipid-to-saposin ratios and their ability to preserve biocompatibility make them an indispensable tool for membrane biology research. Their robustness and flexibility facilitate the study of proteins with diverse structural and functional properties, making them valuable for drug discovery initiatives targeting membrane-associated diseases.

5.5. Nanodiscs with lipopolysaccharides (LPS) inserted inside

Preserving the integrity of the lipid environment is essential for correct understanding of protein structure and function in membrane protein research. This is where lipopolysaccharides (LPS)-filled nanodiscs offer a breakthrough. The LPS is a major component in outer membrane of Gram-negative bacteria and has crucial structural and functional contribution of in coordination with outer membrane proteins (OMPs). Hence a better understanding of the OMPs from Gram-negative bacteria can be achieved by studying these proteins in the membranes having LPS incorporated inside it, which is a challenging task [90]. All things considered, LPS-loaded nanodiscs provide a potent tool for studying membrane proteins in conditions that more closely resemble their native habitats, enhancing our knowledge of cellular functions and illness mechanisms. It was shown that (attachment invasion locus) Ail, an OMP from *Yersinia pestis* has mutually constructive interaction with LPS that producing an extended conformation of Ail towards the surface of membrane causing effective thickening and promoting rigidification of the LPS membrane. Overall, this interaction promotes survival of *Y. pestis* survival inside human serum, imparting antibiotic resistance, and cell envelope integrity [91,92]. Lipopolysaccharides (LPS) are large amphipathic glycoconjugates composed of a hydrophobic lipid domain, known as Lipid A, attached to a core oligosaccharide and a distal polysaccharide. Often referred to as lipoglycans due to their lipid and sugar components, LPS molecules consist of three key regions: Lipid A, which serves as the hydrophobic endotoxin and primary virulence factor; the O-antigen, a hydrophilic and repeating oligosaccharide at the distal end; and the hydrophilic core polysaccharide linking these domains. LPS play a crucial role in protein interactions, aiding in the isolation or separation of specific target proteins and determining protein structure in membrane model using ssNMR and other techniques. Determine the protein structures for LPS interactions is particularly relevant to identifying proteins associated with Gram-negative bacterial membranes. In contrast, nanodiscs, such as those formed using membrane scaffold proteins (MSPs), lack LPS and are utilized to study proteins from organisms other than Gram-negative bacteria in an environment free from LPS interference.

6. Liposomes

Liposomes, first described by Bangham et al. in 1961, are resourceful vesicles made up of one or more lipid bilayers that encapsulate aqueous compartments [93]. These structures have proven invaluable in biological and pharmaceutical research due to their ability to mimic cellular environments. Liposomes are classified based on their size and structure into unilamellar and multilamellar vesicles. Unilamellar liposomes include small unilamellar vesicles (SUVs, 20–100 nm), large unilamellar vesicles (LUVs, >100 nm), and giant unilamellar vesicles (GUVs, >1 μm). In contrast, multilamellar vesicles (MLVs) contain multiple bilayers and typically exceed 500 nm in size [94,95]. These aqueous compartments allow liposomes to encapsulate hydrophilic and hydrophobic substances, making them effective carriers for drug delivery and excellent tools for studying biological systems.

Liposomes are primarily composed of phospholipids, which form synthetic bilayers that closely resemble biological membranes. These bilayers can be customized by altering lipid composition, cholesterol content, and preparation methods, enabling researchers to tailor liposomes for specific applications. Natural lipids from biological membranes provide precision, while synthetic lipids offer greater versatility. Techniques for preparing liposomes include extrusion through polycarbonate filters for LUVs, sonication for SUVs, and low-frequency electric fields for GUVs. Additional methods, such as freeze-thaw cycles, detergent extraction, and hydration of lipid films, are used to create MLVs [96]. These preparation techniques ensure liposomes of varying sizes and properties to meet diverse research needs.

Liposomes are extensively utilized in drug delivery systems due to their biocompatibility and ability to encapsulate both hydrophobic and hydrophilic compounds, effectively mimicking biological membranes [97]. Additionally, they serve as simplified systems for reconstituting membrane proteins into artificial bilayers, enabling functional and

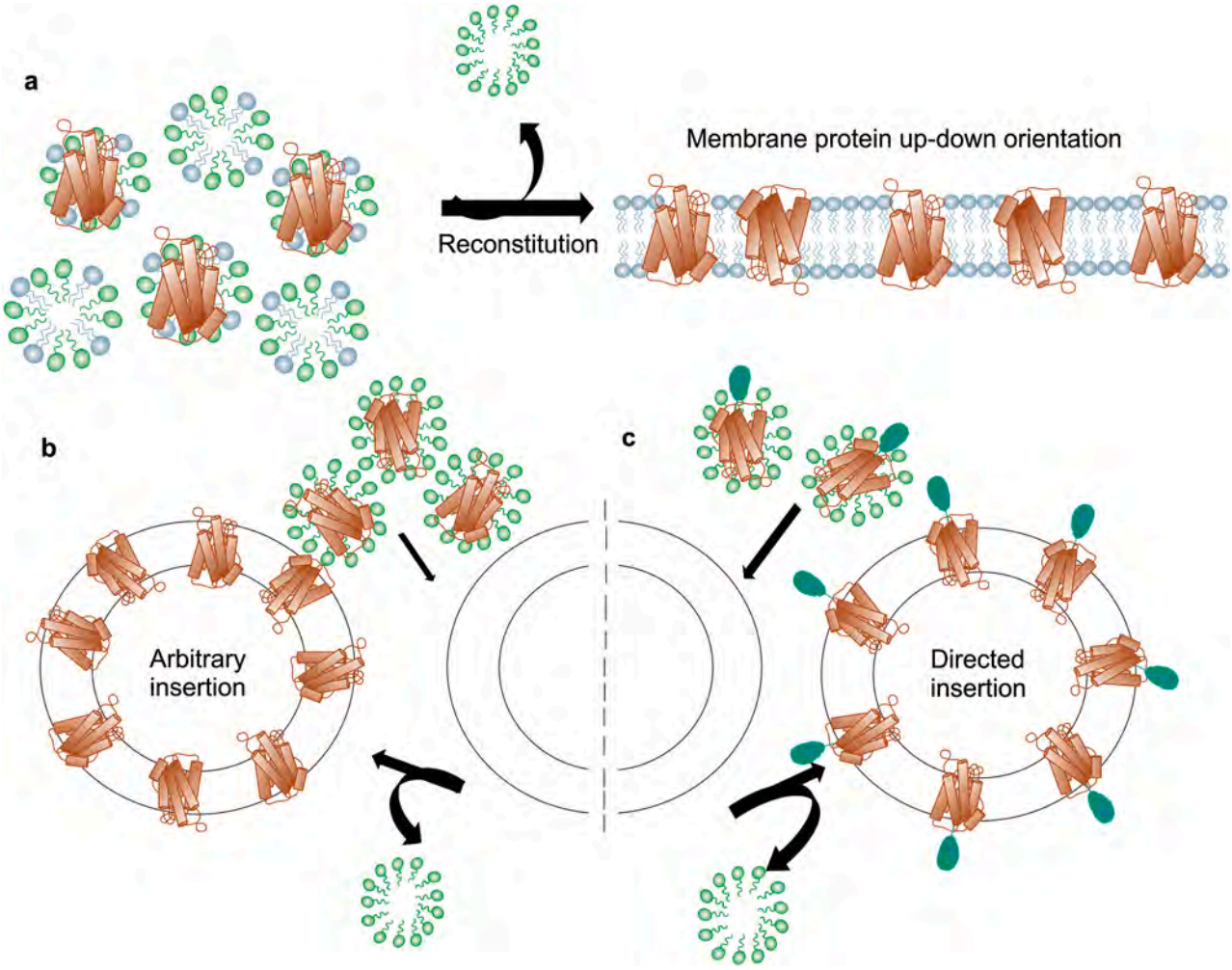

Fig. 6. Reconstitution of membrane protein into liposomes. (a) In the bottom-up approach, achieved by removing detergents from a ternary mixture (left), membrane proteins are typically arranged in an alternating up-down orientation within the liposomes (right). (b) Alternatively, membrane proteins can be inserted into preformed liposomes without specific orientation. (c) For a more controlled orientation, membrane proteins can be directionally inserted into preformed liposomes, ensuring that their soluble domains face outward.

structural studies. The reconstitution process typically involves solubilizing membrane proteins in mild detergents, integrating them into lipid bilayers, and removing detergents through methods like dialysis, size-exclusion chromatography, or hydrophobic adsorbents such as Bio-Beads [98,99]. Membrane proteins can be inserted into liposomes either randomly or directionally. Random insertion leads to mixed orientations, complicating activity analysis. Directed insertion techniques include fusing a soluble domain for unidirectional integration or using functionalized substrate surfaces, like nitrilotriacetic acid monolayers, to orient hexahistidine-tagged proteins specifically within the liposomal membrane. This approach ensures the proper insertion of membrane proteins into the liposomal membrane, producing proteoliposomes that simulate native cellular conditions [100].

The process begins with dissolving lipids in an organic solvent, followed by solvent evaporation using a gas stream (e.g., nitrogen or argon) and vacuum drying to form a thin lipid film. This film is subsequently hydrated with a suitable buffer, leading to the formation of multilamellar vesicles (MLVs). Proteoliposomes are then created by removing detergents while maintaining specific pH, ionic, or chemical gradients within and outside the liposome. Techniques like fluorometry are often employed to study liposomal systems, providing insights into their properties and functionality. For enhanced sensitivity, organic solvents can be used to minimize noise when liposomes are extracted from biological media [101]. This versatile methodology makes liposomes an invaluable tool for both drug delivery and membrane protein research.

Table 1 provides an overview of contemporary liposome analysis methods, detailing their advantages and limitations. These methods are especially beneficial for in vitro functional assays of membrane proteins. In such studies, radioactive substrates, often ^{3}H-labeled, are commonly used to investigate transporter activity in liposomes [102]. Additionally, Samit et al. incorporated lipopolysaccharide (LPS) into liposomes to model the outer membrane of gram-negative bacteria. This modification was evident in the ssNMR spectra of the outer membrane protein Ail, showing improved conformational order and reduced ^{15}N transverse relaxation in the presence of LPS.

Natural membranes typically contain numerous other components that complicate the biophysical study of membrane proteins. However, there are exceptions, such as the purple membranes from Halobacteria and vertebrate rod cell disc membranes, where proteins can be analyzed in their native environment [103]. For most membrane proteins, a bottom-up approach involving artificial membrane reconstruction is necessary. This involves solubilizing and isolating the protein from the membrane using detergents, following similar principles as detergent micelles. Once purified, the protein is reconstituted into an artificial lipid bilayer for further analysis.

Membrane protein reconstitution can be achieved through two primary approaches. In one, lipids self-assemble into bilayers as detergent is removed from a mixture of protein and detergent-solubilized lipids

(Fig. 6). In another, the solubilized protein is inserted into preformed liposomes. In both cases, detergent removal, typically via dialysis, size-exclusion chromatography, or rapid dilution, facilitates membrane insertion. Dialysis can yield homogeneous vesicles but is slow and works best with detergents having high critical micelle concentrations (CMC). Faster methods, such as those using hydrophobic adsorbents, may lead to uneven protein distribution or incomplete detergent removal.

7. Native membranes

The utilization of native membrane models for the study of proteins represents an advanced approach that has significantly enriched the realm of scientific research. While alternative mimetic membranes have shown promise, they have not yet achieved the level of fidelity required to fully emulate the intricate demands of membrane proteins at a physiological level. Native membranes, in most cases, do not seamlessly align with the specific experimental requirements of contemporary biophysicists [104]. One of the central challenges in working with native membrane is their notorious reputation for being exceptionally challenging to manage. Usually, these membranes confine the protein of interest in only trace amounts amidst a vast backdrop of other membrane proteins, complicating isolation, and study. However, the persistence of researchers has yielded valuable insights through native membrane preparations, particularly those derived from *E. coli* and aptly named Kabackosomes. These isolated membrane vesicles have provided a unique opportunity to delve into the biochemical properties of transport processes within the cytoplasmic membrane, all this transpiring well prior to the discovery of the first membrane protein structures [105]. Furthermore, specialized cellular membranes, by their very nature, possess unusually high concentrations of certain membrane proteins. Unusually, these specialized membranes offer a degree of purity that is otherwise challenging to attain, thereby facilitating direct biophysical studies in their unaltered native state. Despite the challenges linked to working with native membranes and their limited protein content, certain studies have delved into crystalline assemblies of porins directly within bacterial outer membrane sacculi [106].

An innovative approach to the in-situ study of membrane proteins involves the enhancement of secreted extracellular vesicles with proteins of interest, offering promising applications for the examination of integral type I membrane proteins derived from both *C. elegans*, and Herpes simplex virus [107]. Beyond electron microscopy, ssNMR spectroscopy has proven to be a successful method for investigating membrane proteins within their native lipid bilayers [108–111]. For instance, Ail and its virulence traits can be manifested within a bacterial outer membrane, enabling concurrent in situ microbiological assessments and structural investigations [91], native *E. coli* inner membranes were utilized as a surrogate system to depict Anabaena sensory rhodopsin (ASR). The study presents an in situ structural analysis of the trans-membrane domain (TM) of LR11 (sorLA) within native *Escherichia coli* membranes. LR11 is known to interact with the human amyloid precursor protein (APP), an important participant in Alzheimer's disease pathology [112]. These studies have proven instrumental in advancing our understanding of membrane protein structure and function. Whether through the exploration of transport processes or the examination of highly specialized cellular membranes, native membrane preparations have provided invaluable insights into the world of biophysics and structural biology. The latest methods used to explore native membrane, along with their benefits and drawbacks shown in Table 1.

8. Criteria and methodologies to evaluate the success of reconstitution strategies of protein in membrane model

Membrane proteins play crucial role in cellular processes, making their structural and functional analyses essential for understanding biological mechanisms. Various approaches, such as X-ray crystallography, cryo-electron microscopy, NMR spectroscopy, and computational modeling, have been developed to study their structures. Functional analyses typically involve activity assays, ligand-binding studies, and transport assays to determine their physiological roles.

The reconstitution of membrane proteins into model systems that mimic their natural surroundings, such as detergents, liposomes, nanodiscs, bicelles, amphipols or native membrane, is a crucial component of these studies.

A few criteria are used to assess the effectiveness of reconstitution strategies: the preservation of protein activity through binding or enzymatic assays; the assessment of proper protein orientation through proteolytic digestion or labeling techniques; and the confirmation of structural integrity through techniques such as electron microscopy or circular dichroism [113]. Furthermore, size-exclusion chromatography or dynamic light scattering are used to assess homogeneity and stability, and lipid content is adjusted and verified to replicate native circumstances. These thorough analyses guarantee that reconstituted systems perform as trustworthy platforms, providing important new information about the composition and role of membrane proteins.

9. Conclusion

The quest to find a better membrane model for studying membrane proteins in structural biology is constantly progressing. The ultimate objective is to choose a membrane system that offers a native-like environment, essential for extracting structural, dynamic features, functional properties and facilitating the study of complexes with interacting proteins. Furthermore, it should have better homogeneity and the final size of the particles must be improved for high-resolution solution-state NMR, considering functional aspects that necessitate characterization through accompanying biochemical experiments. Liposomes, which fall far right on the scale depicting near native membrane mimetics, despite having huge advantages like being detergent free among others, lacks several factors that they are big in size, expensive compared to traditional systems and often the orientation of the membrane of interest is not native. Therefore, the rapid advancement of lipid membrane mimics and the significant diversification of these compounds hold tremendous promise for the fruitful future investigation of the mechanisms underlying a variety of membrane proteins that have proven challenging to stable and analyze thus far.

CRediT authorship contribution statement

Rahul Yadav: Writing – original draft. **Debarghya Pratim Gupta:** Writing – original draft. **Chandan Singh:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors report no conflict of interest.

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Data availability

Data will be made available on request.

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