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REVIEW

# Membrane Proteins in Nanodiscs: Methods and Applications

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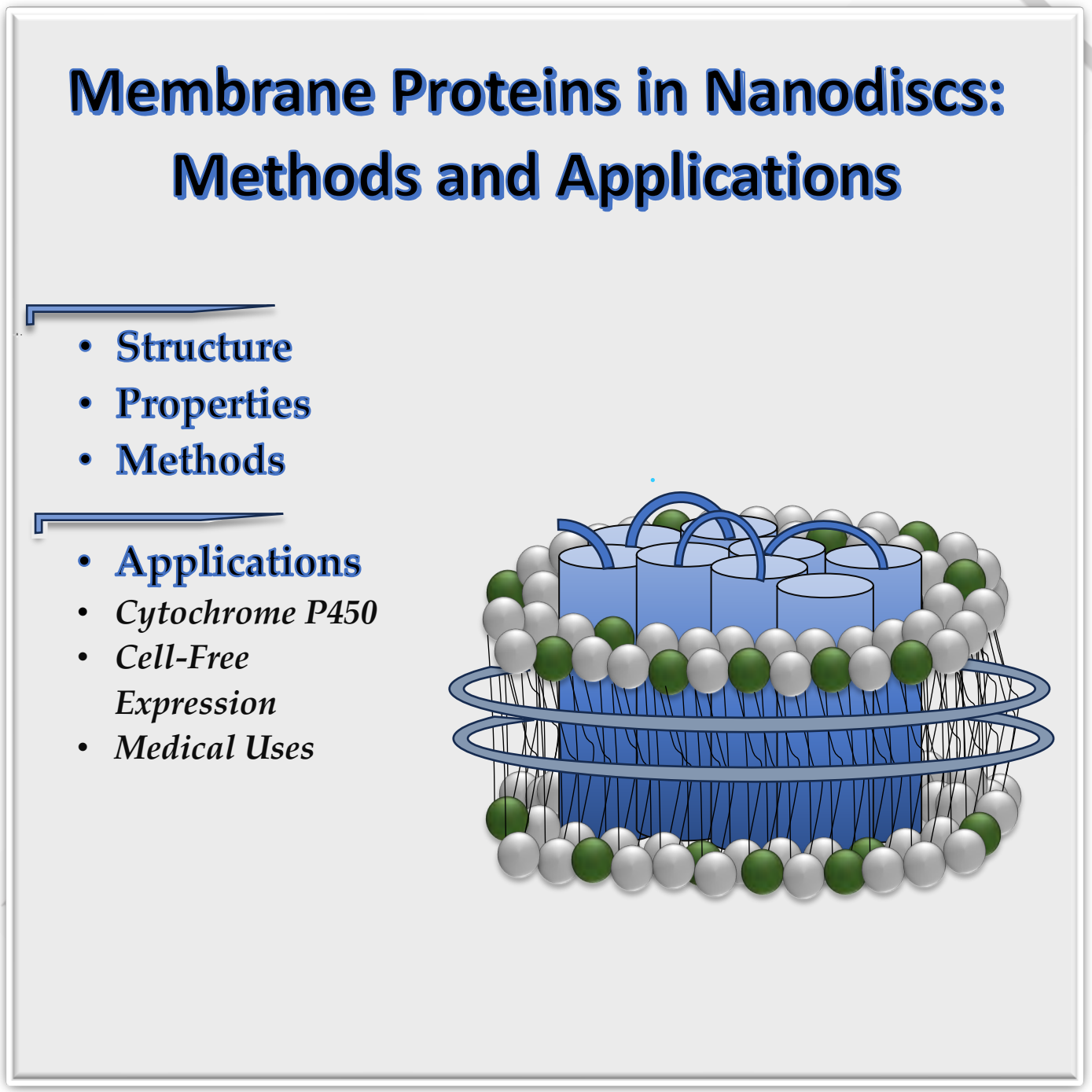

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REVIEW

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**Abstract:** Membrane proteins, a principal class of drug targets, play indispensable roles in various biological processes and are closely associated with essential life functions. Their study, however, is complicated by their low solubility in aqueous environments and distinctive structural characteristics, necessitating a suitable native-like environment for molecular analysis. Nanodisc technology has revolutionized this field, providing biochemists with a powerful tool to stabilize membrane proteins and significantly enhance their research possibilities. This review outlines the substantial advancements in nanodisc methodologies and applications from 2018 to 2024. We cover the development of various nanodisc models, as well as structural and functional studies of membrane proteins that utilize nanodiscs, highlighting their medical applications.

## 1. Introduction

Biological membranes play a crucial role in biological systems. They act as barriers, creating a stable environment that protects cells and constituting intracellular structures. All biological membranes are composed of various lipids, with each membrane having a specific composition. Due to the amphiphilic properties of lipids, biological membranes typically exist as bilayers in an aqueous environment.

Membranes form the boundary between the internal environment of cells and their external surroundings, while membrane proteins serve as gatekeepers, determining which molecules and signals can cross the membrane. Membrane proteins are indispensable in biological entities, functioning as receptors, transporters, and enzymes, and are involved in crucial cellular processes such as cell proliferation and differentiation, energy conversion, signal transduction, and material transport. Membrane proteins constitute approximately 30% of the human proteome and are the targets of over 60% of all drugs.[1][2] Therefore, the study of membrane proteins is a significant and expansive field in biochemistry and life sciences.

However, studying membrane proteins poses challenges because they do not lend themselves to the standard methods used for soluble proteins. Many existing research techniques are only applicable to soluble substances, and membrane proteins often form insoluble aggregates that are difficult to study. Moreover, membrane proteins can exhibit altered properties when removed from their native lipid bilayer, necessitating a suitable environment for their study.[3] In recent years, several methods have been developed to address these challenges.

Detergents were initially used to extract proteins from cell membranes. These molecules can insert their hydrophobic tails into the lipid bilayer, disrupting the membrane and extracting membrane-embedded proteins.[4]

When the critical micelle concentration (CMC) is reached, the the dehydration of the hydrophobic tails drive the detergent molecules to spontaneously form micelles, encapsulating the membrane proteins. This approach allows the use of conventional techniques to study membrane proteins and remains in use today. Another detergent-based model is the bilayer mixed micelle, or bicelle, which is a mixture of detergents and long-chain lipids. Although both micelles and bicelles are used in solution and solid-state NMR applications, micelles can impact the structural folding of proteins, making bicelles a preferable model membrane. However, detergents often reduce or abolish the stability and functionality of proteins and can also influence membrane protein dynamics.[5] Furthermore, some membrane protein systems require specific types of phospholipids to maintain activity, necessitating the use of a detergent-free model necessary for membrane protein research.

To provide a native-like environment for membrane proteins, various biological materials have been used to construct model systems. Although early systems like liposomes were groundbreaking, they exhibited significant limitations, which led to the development of more advanced mimetic systems. Recent innovations such as nanodiscs, SMALPs, and peptidiscs have proven to be superior alternatives, each featuring distinctive components that enhance their effectiveness in studying membrane proteins.[6]

This review will primarily focus on nanodiscs. Nanodiscs are discoidal lipid bilayers stabilized by two units of membrane scaffold protein (MSP), which are amphiphilic helical proteins that stabilize the lipid structure in aqueous media. This structure is derived from human high-density lipoprotein (HDL), a lipid and cholesterol carrier in the blood composed mainly of lipids and apolipoprotein A1. In addition to their advantages in stability, homogeneity, and compositional control, nanodiscs are also highly compatible with techniques such as NMR spectroscopy and chromatographic purification. Since their first report in 2002, these features have made them exceptionally suitable for a broad spectrum of membrane protein studies. As the number of applications has grown, the technology has also advanced. Previous studies have been systematically reviewed by Sligar et al. in 2017.[4] This article aims to provide an overview of the rapid developments in nanodisc methods and applications from 2018 to 2024.

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# REVIEW

**Table 1.** Difference between Nanodiscs and Bicelle.

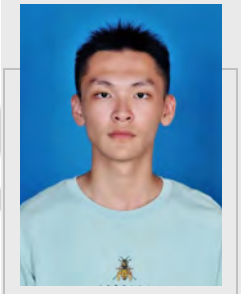
| Field | Nanodiscs | Bicelle |
|---|---|---|
| Component | Composed of phospholipids and membrane scaffold protein. [4] | Spontaneous assembly of long-chain phospholipids and short chain phospholipids. |
| Size | Diameters range from 6 to 20nm. [4] | Diameter depends on the ratio of long-chain phospholipids to short chain phospholipids. |
| Stability | Surround by membrane scaffold protein [4], without detergent. [5] | Dependent on specific temperature. |

Chan Cao is a professor and principal investigator at Nankai University. She holds Master and Ph.D. degrees from Nankai University, and has conducted joint Ph.D. research and postdoctoral studies at Harvard University (Medical School). Her research team is dedicated to elucidating the molecular mechanisms of biomolecular drug targets on cell membrane, with the aim of leveraging these insights for therapeutic innovation.

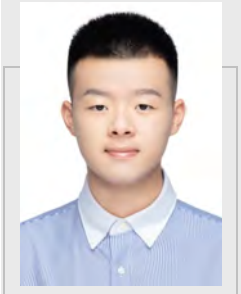
Jiaxu Guo is now pursuing his Bachelor's degree in Chemistry at Nankai University, Tianjin, China. His research focuses on organic chemistry and biochemistry including transition metal catalysis and membrane protein research.

Qinghan Hou received his Bachelor's degree in Chemistry from Nankai University, Tianjin, China. He is now pursuing PhD at California Institute of Technology, California, USA.

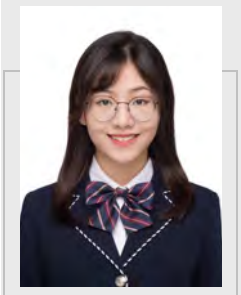
Yulin Tan is now pursuing his Bachelor's degree in Chemical Biology at Nankai University, Tianjin, China. He is currently conducting research related to membrane proteins.

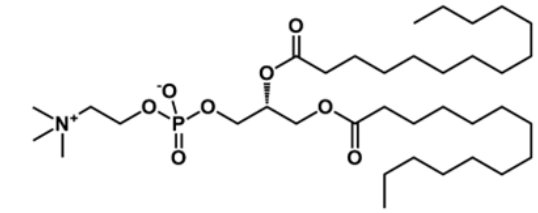


Ruoheng Fu is now pursuing his Bachelor's degree in Chemistry at Nankai University, Tianjin, China. He is currently conducting research related to membrane proteins.

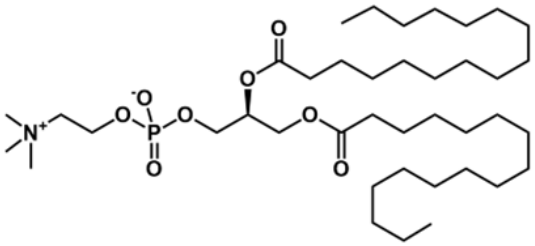


Xuanwei Huang is now pursuing her Bachelor's degree in Chemical Biology at Nankai University, Tianjin, China. Her research focuses on molecular mechanisms of molecular chaperone, membrane proteins and integrin.

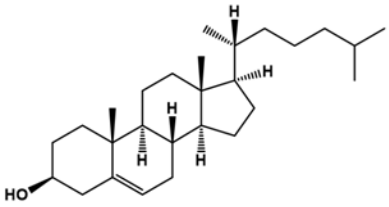




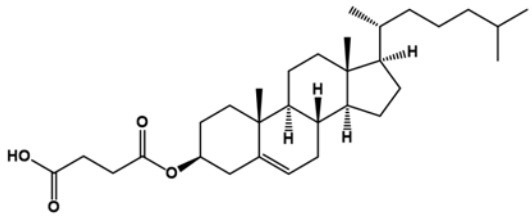
**1,2-dimyristoyl-*sn*-glycero-3-phosphocholine**
(DMPC)
Neutral



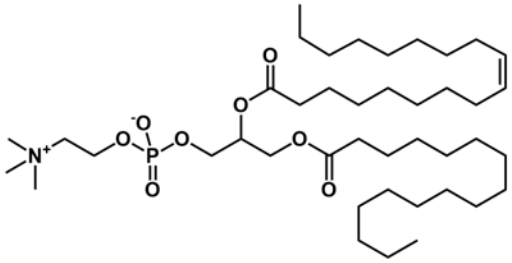
**1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol)**
(DMPG)
Negatively charged



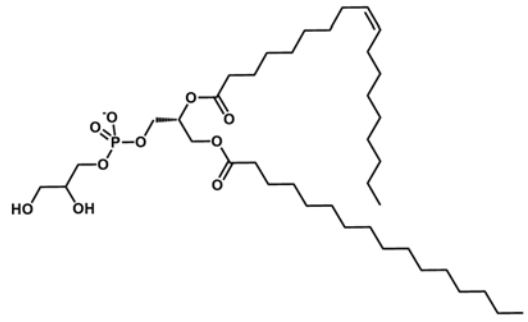
**Cholesterol** (CHL)
Neutral



**Cholesteryl hemisuccinate** (CHS)
Negatively charged



**1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine**
(POPC)
Neutral



**1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol**
(POPG)
Negatively charged

**Figure 1.** Common phospholipids for constructing nanodiscs and their chemical property.

## 2. Structure and Properties

Although nanodiscs have been widely used in the study of various membrane proteins and other biochemical problems, our understanding of their structure and detailed properties remains incomplete. The seemingly simple bilayer structure surrounded by amphiphilic helical proteins is, in fact, quite complex and was not systematically investigated until 2005.[7] In recent years, advancements in precise instrumental analysis and molecular dynamics simulation technologies have enabled scientists to obtain more accurate information about nanodiscs, contributing to the improvement of the biophysical tool itself and related studies.

### 2.1. Self-Assembly of Nanodiscs

Lipids and MSPs spontaneously integrate under appropriate conditions to form the stable macromolecular structure of nanodiscs. This self-assembly process is driven by the strong hydrophobic interactions between the amphiphilic helices of the proteins and the acyl chains of the lipids. In the 1980s, Jonas et al. first developed a method for reconstituting high-density lipoproteins by removing detergent from a mixture of lipids and scaffold proteins,[8] which significantly advanced subsequent applications of nanodiscs and laid the foundation for the construction of nanodiscs. A deep understanding of how nanodiscs form during detergent dilution is crucial in this area of study.

An initial strategy for examining nanodisc formation involved using coarse-grained (CG) simulation and small-angle X-ray scattering (SAXS). From 2000 to 2020, research on the self-assembly process of nanodiscs was continuously advanced. A detailed introduction to this work is provided below to enhance understanding of nanodiscs self-assembly. In 2007, Shin et al. investigated the formation of lipoprotein particles,[9] and later, the same group provided insights into the assembly of nanodiscs by studying the reverse process, adding cholate to preformed particles.[10] Their work revealed that during self-assembly, the components first formed a heterogeneous structure. As the concentration of cholate decreased, the scaffold proteins began to encircle the lipids and detergent molecules. A further reduction in cholate concentration led to the formation of lipid bilayers and the double-belt conformation of scaffold proteins. The discoidal structure of nanodiscs was ultimately formed when most of the cholate was removed from the mixture.

Camp et al. further examined the self-assembly process in 2020 using fluorescence and optical spectroscopy.[11] Their findings indicated that the process was both reversible and controllable. By adjusting the amount of detergent, the system could be maintained at thermodynamic equilibrium points. The study showed that as detergent was gradually removed, phospholipids began to form bilayers when the detergent concentration fell below the CMC. As the detergent concentration decreased further, the tyrosine residues in MSP became less hydrated, likely due to the accumulation of lipids near the hydrophobic regions of the protein. With a more significant reduction in detergent concentration, MSPs began binding lipids and forming larger complexes.

A systematic study by Skar-Gislinge et al.[12] demonstrated that the form and shape of the nanodisc assembly products are influenced by the preparation procedure. Underloaded nanodiscs were generated if lipids were insufficient, while other MSP-lipid particles appeared when lipids were in excess. The study concluded that a lipid-to-MSP ratio of 70:1 was optimal for reconstituting POPC-MSP1D1 nanodiscs.

Research into the self-assembly of nanodiscs is crucial for developing efficient protocols for related experiments and provides a more accurate framework for designing new nanodisc systems. Further studies are needed to understand how membrane proteins correctly assemble into lipid bilayers.

### 2.2. Properties of Lipids

The complexity of nanodiscs largely arises from the structure of the lipids they encircle. Although nanodiscs can be used as native-like membrane mimics in most conditions, the lipids within nanodiscs exhibit notable differences compared to those in their native membrane environment. Previous studies have shown that the phase transition temperatures of lipids in nanodiscs are higher than those in lamellar lipid systems.[13] Additionally, lipids in DMPC-MSP1Δ (1-22) nanodiscs have lower configurational entropies and higher order parameters than those in native bilayers.[14] These differences can be attributed to the inward pressure exerted by the scaffold proteins. It is also well-established that lipids at the rim of the nanodiscs exhibit altered characteristics due to direct contact with the MSP,[13] a phenomenon that has been further confirmed and studied recently.

The properties of lipids within nanodiscs are influenced by the surrounding scaffold and vary heterogeneously within the nanodisc, as well as between nanodiscs of different sizes. Molecular dynamics (MD) simulations [15] have shown that lipids near the scaffold protein exhibit perturbed properties, while those located more centrally behave more like lipids in an unconstrained bilayer. Schachter et al. conducted a comprehensive study on the elastic nature of nanodiscs in 2020,[16] finding variations in lipid properties from the center to the edge of the disc. In small nanodiscs, such as those encircled by MSP1 and MSP1E3D1, the bilayers were stiffer (with smaller area-per-lipid and higher tilt and bending moduli) in the center and softer at the rim. In contrast, larger nanodiscs, such as those encircled by MSP2N2, exhibited the opposite trend. Another study [17] indicated differences in lipid properties between nanodiscs of different sizes by examining hydrogen bonding at the lipid-water interface. The dynamics of hydrogen bonds at smaller nanodisc interfaces were faster, likely due to a greater proportion of MSP-perturbed lipids compared to larger nanodiscs. Stepien et al. identified three distinct populations of lipids in nanodiscs based on their structural and dynamic properties at different temperatures.[18] The first population, located adjacent to the MSP, remained in a liquid phase even below the melting temperature (Tm). Above Tm, the lipids were divided into an outer, less-ordered portion and an inner, more-ordered portion.

These findings help scientists to better understand the environment in which membrane proteins situate their transmembrane (TM) domains and aid in the future applications of nanodiscs. However, the lipid environment within nanodiscs containing membrane proteins is likely different from that of an empty nanodisc, and further studies are needed to explore these differences.

## 3. Methods

To accommodate various applications in biochemistry and physiology, the classic nanodisc structure, originally built with apolipoprotein A1 (ApoA1), has undergone several evolutions. By

altering the length and helical sequences of membrane scaffold proteins (MSPs), researchers have developed nanodiscs with a range of sizes and shapes. Further efforts such as using different compounds as membrane scaffolds have been made to improve nanodiscs, making them more stable, monodisperse, or larger.This section discusses the nanodisc methods and techniques developed since 2018, including innovations in traditional MSP nanodiscs and nanodiscs using scaffolds other than MSPs.

## 3.1. Self-Assembly of Nanodiscs

The most common method for reconstituting nanodiscs is through the removal of detergent from the mixture, and this remains the primary approach for almost all nanodisc preparations. However, new methods, such as incubation techniques,[19]-[25] are also being utilized. In recent years, developing new types of nanodiscs to make them more native-like and stable has become a hot topic, and several advancements have been made in this area.

A recent approach to enhancing nanodisc performance involves covalently linking the N- and C-termini of MSPs to form circularized nanodiscs. These circularized nanodiscs have demonstrated significantly improved homogeneity, stability, and defined shapes.[26] The enhanced stability allows for the creation of larger particles and expands their applicability to a broader range of studies, such as virus research. Several studies have further explored this topic in recent years. Miehling et al. introduced circularized nanodiscs based on split intein ligation,[27] and Yusuf et al. reported an optimized strategy for sortase-mediated ligation, which achieves circularization with fewer byproducts and higher yields using a detergent-assisted method. These circularized nanodiscs have been proven to be sufficiently stable under conditions typically used for biophysical measurements (including various lipid compositions, buffers, temperatures, and concentrations) and have been successfully applied in thermodynamic studies and structural determinations.[28] Another form of circularized nanodiscs was developed using DNA origami,[29] which will be discussed later.

Another innovative approach,[30] developed by McLean et al., focuses on generating nanodiscs better suited for analytical purposes. They modified the amount of tryptophan and tyrosine residues on the MSPs to achieve either reduced or enhanced fluorescent responses. These nanodiscs are expected to be highly useful in future studies.

## 3.2 Other Methods

In addition to MSPs, researchers have explored alternative scaffolds for nanodiscs, leading to significant advancements. The first report of nanodiscs using polymer scaffolds was by Knowles et al.,[31] who utilized styrene maleic acid (SMA) copolymer. Compared to MSPs, SMA copolymer-lipid nanoparticles (SMALPs) are stable, can be produced without detergent, and are capable of extracting proteins directly from their native membranes,[32] offering advantages over MSP nanodiscs. However, SMA is prone to instability in the presence of divalent cations and acidic conditions, and controlling the size of SMALPs can be challenging. Despite these limitations, research on SMA continues to advance. For instance, Andrew et al.[33] found that SMALPs can extract large quantities of proteins from cells and tissues without causing cell damage, which is promising for future research.

Recent years have seen modifications to polymer scaffolds to address these limitations. For example, Ravula et al. introduced a functionalized SMA polymer, SMA-EA, with a lower molecular weight for nanodisc scaffolds.[34] This modification allowed for the formation of uncoiled polymer conformations and improved size regulation. Further functionalization led to the creation of SMA-QA,[35] which enhanced stability in the presence of divalent metal ions and under low pH conditions. Hall et al. introduced a styrene maleimide copolymer (SMI) that self-assembles with lipids similarly to SMA, forming SMI-lipid particles [36]. Additionally, Ravula et al.[37] reported several non-ionic amphiphilic polymers that resulted in nanodiscs resistant to divalent metal ions and acidic conditions.

SMA synthesized using reversible addition-fragmentation chain transfer (RAFT) reactions has also been employed to form nanoparticles with controlled sizes.[38] However, RAFT-synthesized copolymers were initially found to be poorly homogeneous. Cunningham et al.[39] addressed this issue with an iterative RAFT method, and Smith et al.[40] used another RAFT-generated copolymer, AASTY, to form nanodiscs. Both approaches yielded homogeneous copolymer scaffolds and controlled nanoparticle sizes.

Gavin et al. investigated the interaction between SMALPs and lipid monolayers and found that SMALPs can perform lipid exchange at two different interfaces.[41] Despite their convenient

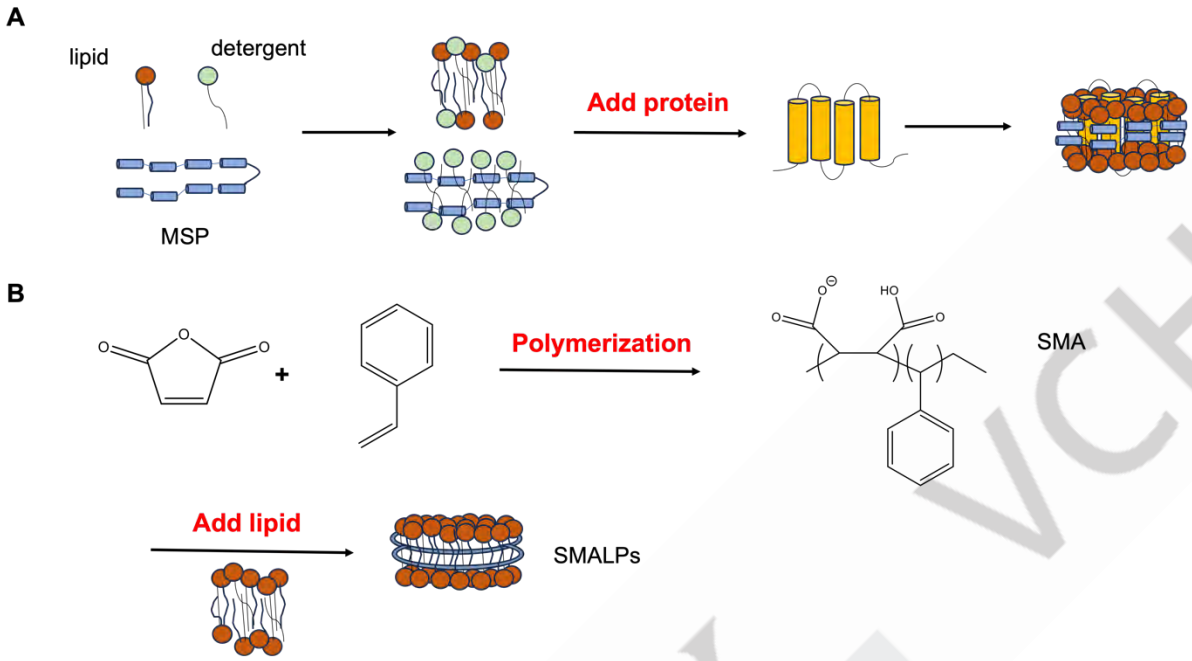



**Figure 2.** Illustration of nanodisc self-assembly process. (A) The process begins with MSP, lipids, and detergent all together in micelles. (B) Synthesis of SMA and formation of SMALPs.

assembly process and applications in membrane protein studies, the lipid dynamics in SMA nanodiscs differ significantly from those in bulk lipid bilayers due to strong interactions between lipids and SMA. This has been confirmed by EPR spectroscopy ,[42] which revealed differences in the order parameters of lipid acyl chains between SMALPs and vesicles. These considerations are important for using SMALPs in protein structural studies.

In 2023, further research explored the limitations of SMALPs, revealing a variety of structures,[43] including polymer-lipid hybrid microemulsions, polymer-encapsulated nanovesicles, polymer-lipid hybrid microcapsules, and lipid-doped polymer particles. These different forms of SMALPs exhibit varying degrees of self-assembly and functional properties, challenging the traditional view of SMALPs as simple nanovesicles and highlighting their diverse nature. Further research on SMALPs may foucus on overcoming these limitations, and contributing to the development of nanodiscs with enhanced performance.

Research has also focused on constructing nanodiscs with DNA scaffolds. In 2018, Zhao et al. introduced circularized nanodiscs made from DNA origami, which allowed for the creation of larger discs for studying viral entry processes. Small non-circularized MSP nanodiscs were attached to a DNA barrel and recombined into a large lipid bilayer with additional lipids, resulting in nanodiscs with diameters of ~45 and ~70 nm, suitable for binding proteins and viruses. Another study by Iric et al. reported nanodiscs scaffolded by a single DNA strand.[44] This DNA helix was alkylated to impart hydrophobicity on the inner side, producing smaller nanodiscs with diameters below 40 nm, but at a lower cost. According to theoretical studies by Maingi et al.[45] , DNA-scaffolded nanodiscs have lipid properties more similar to native bilayers compared to MSP nanodiscs. However, this method is still in the early stages of development and requires further investigation.

Additionally, saponins have been utilized to form nonionic lipid nanodiscs, advancing protein structural studies. A heterogeneous mixture of naturally extracted saponin molecules can create size-tunable nanodiscs, facilitating the development of a new class of membrane systems. These systems feature desirable properties such as tunable sizes, magnetic alignment capabilities, specific surface characteristics, varied lipid compositions, enhanced protection against structural denaturation, and resilience to pH fluctuations and divalent cations. Saponin nanodiscs have the potential to significantly enhance NMR, X-ray crystallography, and Cryo-EM studies. Furthermore, they provide nontoxic and biodegradable nanoparticle platforms for drug delivery. [46]

## 4. Applications

Nanodiscs simulate biological membranes and provide a near-native environment for membrane proteins. The use of nanodiscs in structural analysis has significantly advanced our understanding of these essential biomolecules. Beyond membrane protein studies, nanodiscs have been applied to various biochemical and medical challenges. This section highlights recent research to illustrate the diverse applications of nanodiscs.

### 4.1 Studies of Membrane Proteins

As a model mimicking biological membranes, nanodiscs are primarily used to study membrane proteins. They allow us to observe and analyze membrane proteins' structures and functions in vitro, providing insights into their roles in cellular processes. Here, we discuss recent advancements in structural studies of membrane proteins.

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### 4.1.1 Structural Studies

To obtain high-resolution structural data, a variety of techniques are employed (Figure 3). Nuclear Magnetic Resonance (NMR) spectroscopy is a key method for studying membrane proteins. Recent advances in different NMR techniques include:

- **NMR and HDX-MS:** These techniques were combined to reveal the structure of the Bak transmembrane helix in MSP1D1DH5 nanodisc.[47]
- **NMR Spectroscopy:** This was applied to determine the solution structure of the TREM2 transmembrane helix (TMH) in MSP1D1DH5 nanodisc.[48]
- **$^{17}$O NMR:** Samuel D. McCalpin et al. found that magnetically aligned nanodiscs can be used as an alignment medium for measurement of $^{17}$O RQC in an $^{17}$O-labelled small molecule in the aqueous phase, making it possible for further structural studies with $^{17}$O NMR.[49]
- **NMR Assignment Procedure:** The application to helical membrane proteins were developed by Laurens Kooijman et al. in 2020, Combining amide- and methyl-derived NMR data from double- and triple-resonance-based NMR experiments with 3D/4D NOESY data, water/lipid accessibility data.[50]

Additional techniques such as computational methods, isothermal titration calorimetry (ITC), and Paramagnetic NMR relaxation enhancement (PREs) have significantly advanced our understanding of protein-membrane interactions.[51-56] Notably, PREs was used to study the KRAS [57], and the cytb5-CYP2B4 complex was studied by $^{19}$F-NMR.[22] What's more, heteronuclear NMR such as $^{31}$P and $^{14}$N are also used in saponin nanodiscs.[46]

**Table 2.** Overview of Functional Studies on Membrane Proteins Utilizing Nanodiscs

| Year | Proteins Mentioned | Investigated Problems | Nanodisc Scaffold | Reconstitution Methods | Significance | Reference |
|---|---|---|---|---|---|---|
| 2018 | β $_2$AR,$\beta_2$AR-β- arrestin 1 (PDB: 3SN6) | Mechanism of the formation of the phosphorylated GPCR- arrestin complex. | MSP1 | Incubation. | This study revealed the conformational changes of $\beta_2$AR upon ligand binding, enhancing the understanding of GPCR signaling through a native-like environment provided by nanodiscs. | [19] |
| 2021 | α - Syn (PDB: 1XQ8) | Probed the interactions and fibrillation behaviour ofα- Syn using styrene- maleic acid nanodises, containing zwitterionic and anionic lipid model systems with and without cholesterol. | SMA copolymers | Incubation. | The research found that α-Syn aggregation in a membrane environment is related to its biological function, with nanodiscs helping to confirm key interactions in the aggregation process, advancing the understanding of Parkinson's disease mechanisms. | [23] |
| 2019 | Arfl (PDB: 1J2J) | Established a function al environment for biophysical studies of Arfl effectors and interactions at the membrane. | MSP1D1,MSP△H5, MSP△H4,5 | Detergent removal. | It analyzed the effects of Arf1 by reconstructing a membrane environment with nanodiscs, revealing its important role in membrane protein interactions and providing new insights into intracellular signaling. | [80] |
| 2020 | mouse TRPV3 (PDB:6MHO 6MHS 6DVZ) | Determined the ap o state structure of mouse TRPV3 in a lipid nanodise, revealing the lipid- protein interaction in TRPV3, and proposed the lipid-associated gating mechanism of TRPV3. | MSP2N2 | Detergent removal. | The study elucidated the lipid-protein interaction mechanisms of TRPV3, with the application of nanodiscs enabling high-resolution structure determination under near-native conditions, providing important structural foundations for drug design. | [69] |
| 2020 | Syt1-SNARE (PDB: 5KJ7 1KIL) | Investigated the interactions between the $Ca^{2+}$ sensor synaptotagmin-1 and the SNARE complex on PIP2- containing nanodiscs. | MSP1E3D1 | Detergent removal. | Using nanodisc technology, this study confirmed the regulatory role of $Ca^{2+}$ in SNARE complex formation, further elucidating the molecular mechanisms of neurotransmitter release. | [82] |
| 2020 | DRD2 (PDB: 6CM4) | Elucidated the structure of an agonist-bound activated DRD2- Gi complex reconstituted into a nanodisc. | MSP1D1 | Detergent removal. | This research characterized the structural dynamics of dopamine receptors in a membrane-like environment, demonstrating how nanodiscs preserve receptor functionality, which is crucial for understanding their role in neuropharmacology | [83] |

Cryo-Electron Microscopy (cryo-EM) is another powerful technique for obtaining high-resolution structural data. As an example, Yifan Cheng's research group at UCSF recently published a study in *Cell* using nanodiscs to investigate the structure of TGF-β signaling-related complexes under cryo-electron microscopy.[58] Specifically, the nanodisc serves as a membrane model that stabilizes membrane proteins and their complexes, overcoming the instability of membrane proteins in solution encountered with traditional methods. In this study, the authors reconstituted full-length αvβ8 (αvβ8fl) into lipid nanodiscs

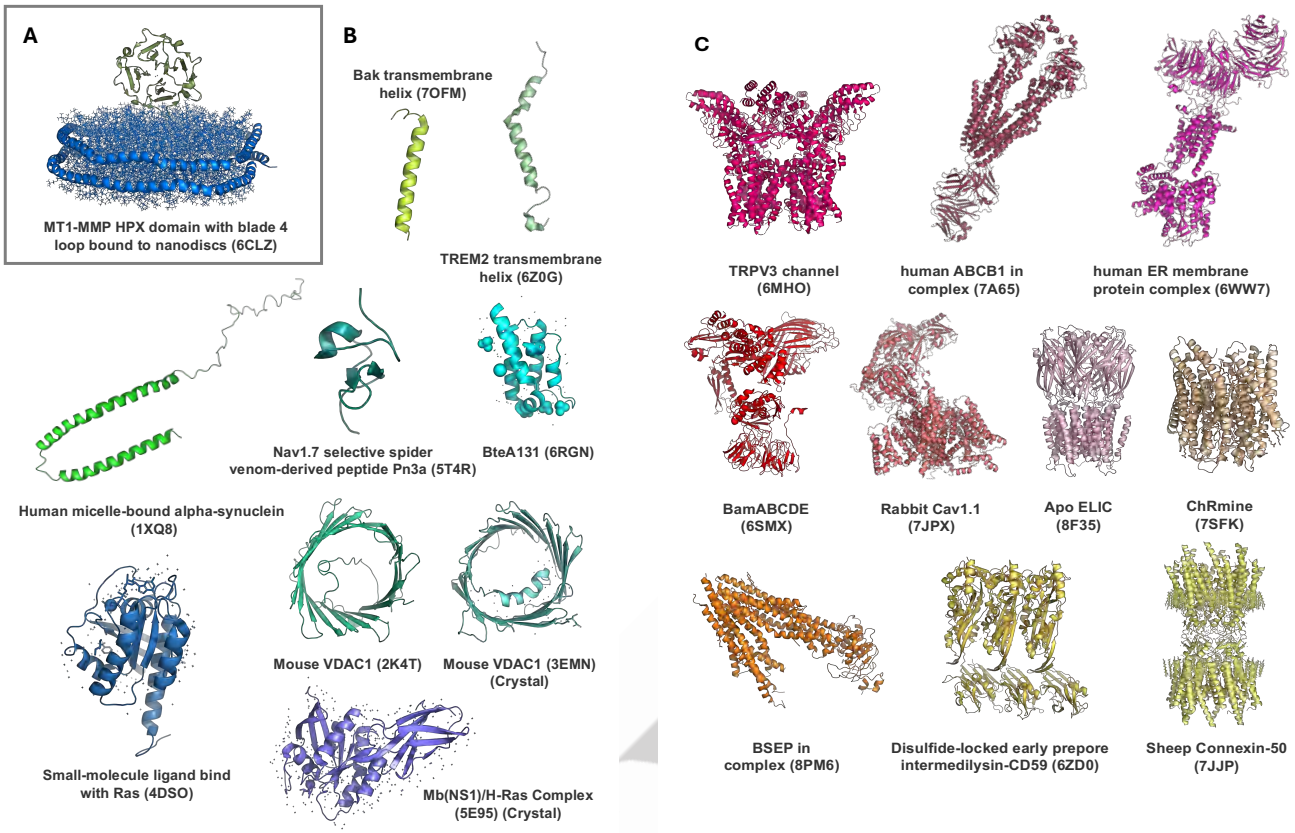


**Figure 3. Schematic Representation of Protein Structure Analysis Using Nanodiscs, 2018-2023.** (A) Illustration of a protein embedded in a nanodisc. (B) NMR solution structure and crystal structure of membrane protein in nanodisc (C) Cryo-EM structure of membrane protein in nanodisc. Structures were visualized using PyMol. Corresponding PDB numbers are cited next to each protein name.

(αvβ8fl-nd), which restricted its originally flexible legs, thereby stabilizing the integrin structure and improving the resolution of cryo-electron microscopy imaging.

The nanodisc provided a biocompatible environment that allowed the TGF-β complexes (such as L-TGF-β1/GARP and avβ8/L-TGF-β1/GARP) to assemble and be observed under near-physiological conditions. This approach not only enhanced the imaging quality of the complexes in cryo-electron microscopy but also enabled researchers to obtain high-resolution structural information, revealing the dynamic changes and conformational flexibility in TGF-β signaling. By using nanodiscs, the authors were able to effectively observe the interactions between the integrin and TGF-β complexes, thereby gaining insights into their roles in autocrine and paracrine signaling.

Here are several examples of recent advancements of membrane protein structures within nanodiscs:

- **NaChBac and E. coli BAM Complex:** Analyzed by cryo-EM to reveal structural details.[59]
- **ABCB1-Cryo-EM Structures:** Investigated by Kamil Nosol et al., showing the complex with antibodies and substrates.[60]
- **Human EMC-Cryo-EM:** Demonstrated substrate insertion mechanisms in nanodiscs.[61]
- **Cav1.1-Cryo-EM:** Reported in 2021, detailing structures in the presence of various antagonists and agonists.[62]
- **Human BSEP-Cryo-EM:** Revealed the basis of small-molecule inhibition by glibenclamide.[63]
- **CDC Studies:** Cryo-EM was used to understand structural transitions and cholesterol requirements for cell lysis.[64]
- **Connexin-46/50-Cryo-EM:** Provided a 3D reconstruction and insights into gap junction stabilization and protein-membrane interactions.[65]
- **pLGIC and ELIC-Cryo-EM:** Vikram Dalal et al. discovered that different nanosheets produce distinct structures.[66]
- **Single-particle cryo-EM :** Doreen Matthies et al. used Single-particle cryo-EM detected the structure of the Kv1.2–2.1 paddle chimera channel reconstituted.[67]
- **Electron cryomicroscopy:** Yuan Gao et al. combined electron cryomicroscopy with lipid nanodisc technology to ascertain the structure of the rat TRPV1 ion channel.[68] Hiroto Shimada et al. determined the apo state structure of mouse TRPV3.[69] Kyle Tucker et al. presented cryo-EM structures of ChRmine.[70]

Further advancements in nanodisc research including methods to determine the number of membrane proteins inserted into lipid nanodiscs were developed in 2020[71] and used in 2024 to count membrane proteins.[72] This innovation opens new possibilities for nanodisc applications.

Overall, structural studies of membrane proteins often require the integration of multiple analytical techniques. These advancements help biochemists build more accurate molecular models and lay the groundwork for understanding protein functions and mechanisms.

### 4.1.2 Analytical and Functional Studies

Biological membranes are central to numerous biochemical processes, making the study of reactions and binding interactions crucial. Nanodiscs provide a valuable platform for these studies, particularly in analyzing protein-protein interactions and dynamic processes.

#### Protein-Protein Interactions

Nanodiscs have played a crucial role in the study of protein-protein interactions. A method combining NMR and SAXS [73] has been developed for this purpose. For example, it was discovered that nanodiscs containing DHA chains and other acyl chains can redistribute protein conformations and influence interactions between A2AAR and G proteins,[74] revealing an extended network of protein-protein interactions at the GPCR–G protein interface compared to structures obtained in detergent micelles.[75]

#### Dynamic Studies

Nanodiscs are also used to investigate dynamic processes within membranes:

- **Single-Molecule FRET Assays**: These have been used to study the conformational dynamics of SNARE/Munc18-1 complexes as they progress towards the SNARE complex.[76]
- **Fluorescence-Based Kinetic Analysis**: Applied to amyloid fibrillation studies, this method has provided insights into the inhibition and mechanism of K-RAS4B, aiding the development of inhibitors for membrane-associated proteins.[77]
- **Lipid Dynamics**: Studies on E. coli OmpX have shown how membrane lipids modulate protein function through dynamics on the picosecond to millisecond timescales.[78]

Recent studies have reported significant advancements in understanding membrane proteins through nanodiscs, as detailed in Ttable 2 [19][21][23][69][79]-[85]. These findings demonstrate the rapid development and substantial impact of this field, greatly enriching our knowledge of membrane proteins and making notable contributions to biochemistry.

## 4.2 Cytochrome P450

Here we use Cytochrome P450 (P450) as an example to demonstrate how nanodisc technology has propelled advancements in the field. P450 enzymes are a widespread family of monooxygenases involved in various physiological and biotechnological processes. In eukaryotes, P450s are membrane proteins typically associated with mitochondrial or endoplasmic reticulum (ER) membranes.[86] Studying P450s requires mimicking their membrane environment accurately.

### 4.2.1 Obtaining Stable P450

Lipid membranes are crucial for the catalytic activity of P450s, but their complexity can challenge the dynamic molecular characterization of P450-lipid interactions.[87] Traditional detergent-based methods can alter membrane protein structures, deviating from their physiological states. The charge consistency between nanodiscs and membrane proteins also impacts stability.

To address these challenges, several innovative methods have been developed:

- Histidine-Tagged Nanodiscs: Lan Zhao et al. created a method using membrane scaffold proteins with histidine tags to prepare charged nanodiscs. By adjusting the lipid-to-protein ratio, they controlled the particle size, distribution, and Zeta potential. At an optimal ratio of 60:1, uniform nanodiscs with an average size of 10 nm and a Zeta potential of -19 mV were obtained. These nanodiscs were successfully used for chromatographic studies, showing specific binding to cytochrome P450 in rat liver microsomes.[88]
- Apolipoprotein A-1 and Peptide-Based Nanodiscs: Shen Cheng and Zhiyuan Bo et al. replaced apolipoprotein A-1 with amphipols (Apols) to integrate CYP2B4 and P450-oxidoreductase (POR) into membrane mimetics. This method involved incubating the proteins with Apols overnight at 4°C, leading to spontaneous binding without detergents.[89]
- Non-Ionic Inulin-Based Polymer Nanodiscs: Bankala Krishnarjuna and Sang Choul Im et al. developed non-ionic inulin-based polymer nanodiscs to study membrane protein complexes with varying charges. Pentyl-inulin nanodiscs were used for high-resolution cryo-EM and solid-state NMR studies to characterize the structure and dynamics of transmembrane domains.[90]
- Positively Charged Nanodiscs: Thirupathi Ravula et al. found that positively charged nanodiscs enhanced the stability of reconstructed Cytochrome P450. Polymer-protein charge interactions were shown to be critical for protein stability and functionality in synthesized nanodiscs.[91]

These advancements facilitate the study of P450 enzymes and other membrane proteins in conditions that more closely mimic their native environments, providing valuable insights into their functions and mechanisms.

### 4.2.2 Research on the Structure and Function of P450

Recent studies have provided significant insights into the structure and function of Cytochrome P450 (P450) enzymes, highlighting the impact of various factors on their activity and stability.

#### Effects of Mutations

Ghulam Mustafa et al. investigated the effects of mutations in the N-terminal transmembrane (TM) helix residues of human steroidogenic enzymes CYP 17A1 and CYP 19A1 using (MD simulations. Their study revealed that mutations in the N-terminal TM-helix significantly affect the interaction and orientation of the TM-helix region and the spherical domains of the enzymes.

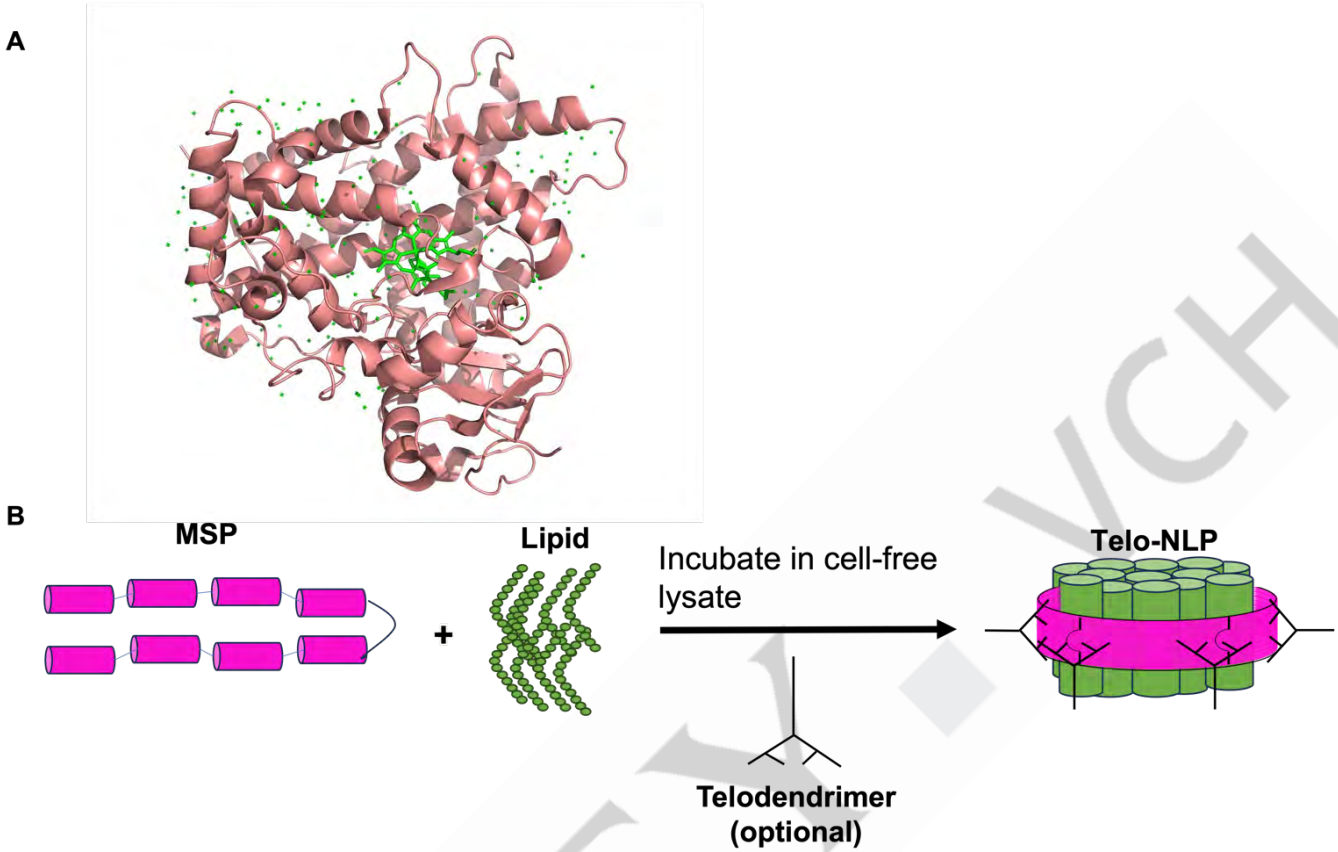


**Figure 4.** (A) 3D structure of cytochrome P450 (CYP2B4, PDB code is 1SUO); (B) Cell-free assembly of NLP.

Specifically, the orientation of the CYP 17A1 spherical domain is highly sensitive to changes in the N-terminal sequence. Removal of the anchor point at the end of the TM-helix led to unstable interactions of the TM-helix within the membrane, affecting enzyme stability and function.[92]

### Lipid Raft Formation

Carlo Barnaba, Bikash Ranjan Sahoo, and colleagues used nanodiscs to capture the lipid boundaries of Cytochrome P450 2B4 (CYP2B4). Their research provided evidence that CYP2B4 can induce raft domain formation in biomimetic compounds. Combining biophysical experiments with molecular dynamics simulations, they identified a sphingomyelin binding region in CYP2B4. The formation of lipid rafts induced by the protein increased the thermal stability of P450, demonstrating that P450 can dynamically interact with and alter its surrounding lipid environment, enhancing its function and stability.[87]

### Regulation by Lipid Composition

Hannah C. Huff, Demetri Maroutsos, and Aditi Das explored how the surrounding lipid environment regulates the enzyme function of CYP2J2-CPR, a membrane-bound cytochrome P450 involved in fatty acid and exogenous metabolism in the heart. They found that the composition of membrane lipids is crucial for CYP2J2-CPR interactions and ebastine metabolism. Anionic lipids increased the metabolic rate and coupling efficiency. The addition of sphingomyelin and 20% POPS to nanodiscs resulted in the formation of carebastine (CAR),[93] suggesting that these lipid components act as conformational regulators, influencing the release of hydroxybastine (HYD) and promoting the metabolism of escitaine into carebastine and hydroxybastine.

### Impact of Cholesterol

Cholesterol's role in P450 enzyme interactions was also studied. Nirupama Sumangala et al. examined the effect of cholesterol on the interaction between P450 19A1 and P450 reductase (POR). They found that cholesterol, concentrated near the membrane immersion area of P450, reduced the enzyme's flexibility and altered its position and orientation within the membrane. Nanodiscs containing 20% cholesterol and 80% phospholipids showed similar androstenedione binding rates and product formation compared to phospholipid-only nanodiscs. However, in cholesterol-containing nanodiscs, the electron transfer from POR to P450 19A1 occurred three times faster than in phospholipid-only nanodiscs. This indicates that cholesterol affects specific aspects of the POR-P450 19A1 interaction, potentially modulating the enzyme's catalytic activity.[94]

## 4.3 Cell-Free Expression with Nanodiscs

Cell-free expression systems offer a simplified and efficient approach for protein synthesis, avoiding issues related to protein toxicity and aggregation that often occur in cellular systems. This method is particularly useful for the production of membrane proteins. Nanodiscs are integrated into these systems to enhance protein stability and solubility. There are two primary strategies for combining nanodiscs with cell-free expression:

1. **Direct Addition of Nanodiscs:** In this method, pre-formed nanodiscs are added directly to the cell-free expression system, where they incorporate proteins expressed from plasmids. This approach ensures that the nanodiscs are pre-formed and ready to integrate membrane proteins.
2. **In-System Formation:** This strategy involves the addition of MSPs or plasmids encoding MSPs to the cell-free expression system, allowing nanodiscs to form within the system alongside membrane proteins. Theoretically, this "single-pot" reaction approach eliminates the need for pre-formed nanodiscs and can potentially increase protein expression levels as the amount of input nanodiscs is not a limiting factor.

The formation of nanodiscs in a cell-free expression system has been demonstrated as feasible[95]. For instance, human UGT2B7, an unstable enzyme, was reconstituted into nanodiscs using the TnT T7 Insect Cell Extract Protein Expression System with MSP1D1, POPC, and sodium cholate, resulting in nanodiscs containing a single protein monomer [96]. However, many studies favor using fully formed nanodiscs. For example, Do et al. used cell-free expression to investigate the influence of lipid composition on the proton-coupled folate transporter,[97] and G-protein-coupled receptors have also been studied using this method.[98][99]

A recent comparative study by Adi Yahalom et al. evaluated three strategies: 1) **Pre-assembly**: Integrating membrane proteins (PR) into existing nanodiscs; 2) **Co-assembly**: Assembling PR and nanodiscs together during nanodisc formation; 3) **Co-expression**: Adding nanodiscs or SAPA during protein expression. The study found that pre-assembled nanodiscs were the most effective for preparing high-quality membrane protein samples, while co-assembly and co-expression methods required longer and more complex procedures. Variability in template proportions during co-expression could affect the final product quality.[100]

In the past year, pre-assembly in cell-free expression has been employed to explore intermolecular interactions. For example, Eyitayo et al. discovered that the anti-apoptotic protein Bcl-xL could spontaneously insert into pre-assembled nanodiscs, promoting the insertion of Bax in a manner similar to mitochondrial insertion. This model system is suitable for studying the molecular aspects of Bcl-xL and Bax interactions during membrane insertion.[101]

## 4.4 Medical Uses of Nanodiscs

Nanodiscs, derived from human HDL particles, retain structural and functional characteristics in plasma and minimize immunogenic responses. This makes them valuable for various medical applications, including imaging and drug delivery.

1. **In Vivo Imaging:** Nanodiscs have been employed as contrast agents in magnetic resonance imaging (MRI) by combining them with $Gd^{3+}$ and fluorescent dyes.[102] They have also been used for tumor targeting in positron emission tomography (PET) imaging by modifying PEGylated nanodiscs with DOTA and antibodies.[103] In 2019, cell-free expression enabled the NMR-based structural studies, suggesting potential for future in vivo imaging.[104]
2. **Drug Delivery:** Nanodiscs are increasingly used in drug delivery, particularly in cancer therapy. Research has demonstrated their ability to enhance immune checkpoint blockade in cancer models,[105] overcome the blood-brain barrier to target brain tumors,[106] and deliver chemotherapeutic agents effectively.[107]
3. **Pathological and Pharmacological Studies:** Nanodiscs have also been utilized in studying pathological mechanisms and pharmacological responses. For example, they were used to investigate the membrane-targeting structure of Bordetella-mediated effector BteA, which could lead to new treatments for Bordetella colonization.[55] Another study revealed structural transitions required for oligomerization by observing CDC hemolysin binding to the human immune receptor CD59, which could guide the design of pore-forming proteins.[64] Recently, Benedicto et al. incorporated luteolin into a recombinant HDL complex consisting of apolipoprotein E3 (apoE3) and phosphocholine. This work demonstrated the potential for efficient delivery of luteolin to perinuclear sites.[108] Another remarkable application is for Alzheimer's disease (AD). The deposition of amyloid-β (Aβ) peptides has remained as the fundamental hallmark for pathogenesis, and nanodiscs paly an essential role in the study of Aβ peptides.[109]-[114]

## 5. Summary and Outlook

This article highlights recent advancements in nanodisc technology, which have significantly enhanced our understanding of nanodisc assembly, structure, and properties. The development of new methods has not only increased efficiency but also broadened the scope of research applications. Nanodiscs have proven to be highly effective tools in membrane protein studies and are demonstrating significant potential in medical imaging and therapy.

Despite their success in mimicking biological membranes, nanodiscs still face limitations. They do not fully replicate certain aspects of native bilayers, such as the asymmetry in lipid composition between the inner and outer leaflets, which is crucial for some transmembrane proteins. Additionally, the use of exogenous lipids in nanodiscs, rather than native lipids, may impact the performance of embedded proteins. Addressing these limitations and incorporating native lipids could enhance the functionality of nanodiscs. Future research should also focus on developing cost-effective methods for nanodisc production to further advance their applicability.

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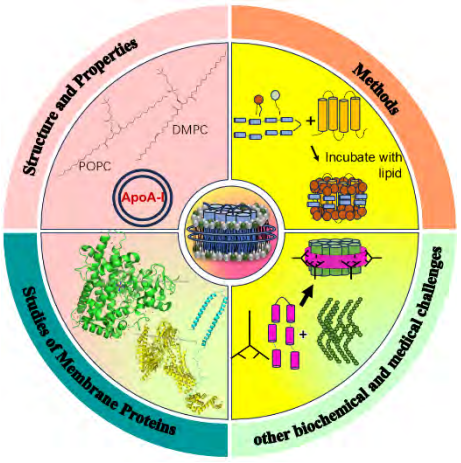


Our paper offers a thorough review of the advancements in nanodisc technology over recent years. We discuss the development of various nanodisc methods and explore their structural and functional applications in membrane protein research, emphasizing their potential impact on medical science.