METHODS OF INTEGRAL MEMBRANE PROTEIN EXTRACTION AND THEIR LIMITATIONS

Anusha Kallakuri[a] and Harsha E. Rajapakse[b]*

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Membrane proteins makeup around one-third of all proteins in the body and play important roles in signal transduction, selective molecular transmission and membrane integrity. They also serve as important drug targets. Therefore, accurate structural and functional analysis of membrane proteins is critical. The extraction and study of membrane proteins is limited due to their amphipathic nature, making them difficult to solubilize in aqueous media. Integral membrane proteins can be extracted from bio-membranes with detergents and purified into detergent micelles, bicelles, bilayers, or other forms of amphipathic molecules. These platforms do not necessarily mimic bio-membranes and, therefore, may not reflect the integral membrane proteins' accurate biological structure and function. Recent studies have introduced better representations of the bio-membrane, such as membrane mimicking nanoparticles, lipid nanodiscs (LNDs) and their variations, and artificial polymers. Due to some limitations described below, there are continuous efforts to develop better platforms that correctly resemble plasma membranes, leading to the correct biological structure and function of membrane proteins for in vitro studies. This mini-review comprehensively summarizes the recent developments of membrane proteins extraction, their advantages and disadvantages.

*Corresponding Author
E-mail: hrajapakse@mec.cuny.edu
[a] Department of Chemistry and Environmental Science, Medgar Evers College, City University of New York, Brooklyn, NY, USA
[b] Department of Chemistry and Environmental Science, Medgar Evers College, City University of New York, Brooklyn, NY, USA

INTRODUCTION

Membrane proteins make up a significant percentage of the body's proteins, thus contributing to various biochemical processes in the human body. Consequently, they also play a critical role in disease, making them of great interest and the target of 50 % of modern drugs.[1] Common membrane protein drug targets range from enzymes, transporters, ion channels, and receptors.[2] Membrane proteins fold correctly and function naturally when embedded in the cell membranes. Biochemical analysis of these proteins requires careful separation, isolation and solubilization without destroying their native conformation. Due to their hydrophobic nature, these proteins are not water-soluble, and therefore, various solubilization methods have been discovered for their extraction from the membrane.[3] However, conventional methods remove lipid molecules that are in association with the proteins.[4,5] Removal of lipids from the protein background has previously shown to severely impact protein function due to the loss of protein–lipid interactions.[6, 7] Thus, there is a need to advance the current extraction technologies to preserve these lipid-protein interactions during membrane protein analysis.

Integral membrane proteins are a recognized target in biomedical and pharmaceutical applications. Therefore, it is highly important to understand the high-resolution structure, dynamics and functions of membrane proteins.[8] It is necessary to purify membrane proteins for in vitro analyses and structure determination. Traditionally, membrane proteins are extracted into detergent-based micelles. Other alternatives such as peptide-supplemented detergent solutions,[9] lipopeptide detergents,[10,11] amphipols,[12] amphibiopols,[13] and fluorinated surfactants[14] have been introduced. However, due to the minimal representation of the natural membrane and other limitations discussed in this review, these methods did not prove to be the utmost popular. In this review, the most recent methods that lead to a natural representation of integral protein structure and function are discussed.

1:1 Membrane proteins – structure and function

The cell's phospholipid bilayer has an amphipathic character, with hydrophilic head groups and hydrophobic fatty acid tails.[15] Approximately 30 % of the human genome encodes for membrane proteins[16] as they serve important functions, specifically in cellular transport and communication.[16] Membrane proteins are typically classified based on the nature of the proteins' interactions with the plasma membrane: integral (intrinsic) membrane proteins and peripheral (extrinsic) membrane proteins.

Integral membrane proteins are embedded within the lipid bilayer and contain hydrophilic and hydrophobic components. The hydrophobic side chains of these proteins interact with the uncharged fatty acyl chains within the plasma membrane, while the hydrophilic components interact with the polar head groups and face the cytosol/the extracellular fluid.[17] These proteins typically span the entire width of the membrane, hence their amphipathic nature. Integral membrane proteins that interact with more than one domain within the phospholipid bilayer are called transmembrane proteins.[17]

Peripheral membrane proteins do not interact with the hydrophobic interior of the plasma membrane but are instead anchored to the membrane via attachments to the integral membrane proteins or to the polar head groups of the phospholipid bilayer.[17]

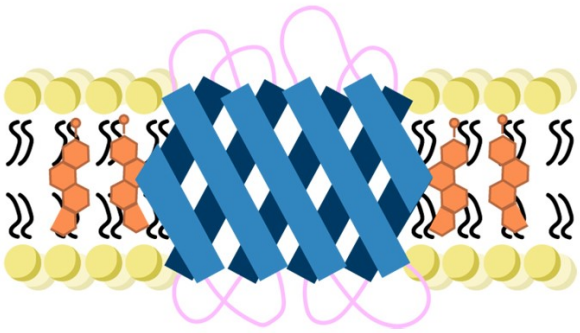

Figure 1. Graphic representation of an integral membrane protein embedded in the phospholipid bilayer. The membrane protein (blue) spans the width of the bilayer and displays an amphiphilic character. The hydrophilic portions of the protein face the cytosol/extracellular fluid and interact with the charged polar head groups (yellow) of the bilayer, while the hydrophobic portions interact with the uncharged fatty acyl tails (black) and other lipid components of the membrane, such as cholesterol (orange).

Proteins fold in a way that renders the most energetically favorable interactions by creating a structure with the minimum amount of free energy.[18] This occurs between the hydrophobic residues of the membrane protein and the phospholipid bilayer's fatty acyl tails and minimizes exposure of hydrophobic residues to water.[1] The overall charge of the residues can cause misfolding and thus a loss of function, most notably if point mutations introduce polar or charged residues into the protein structure.[1]

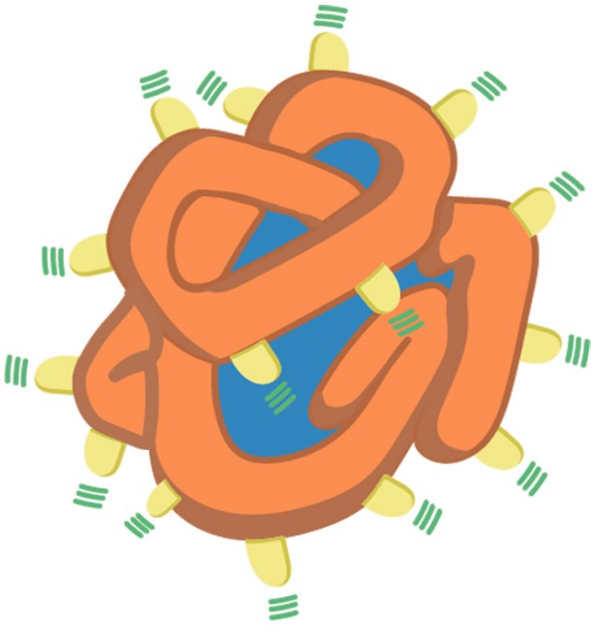

Figure 2. Graphic representation of a soluble protein. In an aqueous environment, soluble proteins fold in a way that renders the most energetically favorable interactions, similar to membrane proteins. The interactions between the hydrophobic residues dictate the structure by shielding these residues from the surrounding aqueous environment, creating a hydrophobic core (blue). This tertiary structure also puts the polar, charged residues (yellow) on the surface of the protein so that it can interact with the hydrophilic components in the environment. One of these interactions may include hydrogen bonds with the aqueous environment (green).

Transmembrane protein structure heavily relies on the folding of the secondary structure to achieve its native conformation.[18] Alpha helices are the most common secondary structure found within cell membranes, as they can satisfy the backbone hydrogen bond of the peptide while also folding to shield the hydrophilic residues from the surrounding hydrophobic environment.[18] This shielding is referred to as the hydrophobic effect and dictates the overall globular structure that the protein will have once it has formed its tertiary and/or quaternary structure.[18] According to Popot and Engelmen, individual helical structures are stable because the hydrophobic side chains make contact with the hydrophobic region of the lipid, which stabilizes a "transbilayer location" and forms hydrogen bonds that are strong in a "low dielectric environment".[19]

1:2 Why membrane protein extraction is challenging

The analysis of membrane proteins is significantly challenging compared to that of water-soluble proteins due to the following reasons, i) membrane proteins are expressed endogenously at low quantities; sufficient quantities of membrane proteins are usually unavailable, ii) there are many challenges on protein solubilization in aqueous media due to hydrophobic nature of the protein exterior such as possible exposure of hydrophobic regions; membrane proteins tend to aggregate, iv) extracted membrane proteins are often more susceptible to protease degradation v) due to large differences in the membrane structure from one cell to another, no singular protocol for membrane protein purification can be used; methods are often based on trial and error.

Except for the problem of low endogenous expression of membrane proteins, all other outstanding issues in membrane protein studies are due to the proteins' inverted polarity. Disregarding this inverted polarity, scientists use protocols that are developed for water-soluble proteins in the later steps of protein studies. Therefore, it is not surprising that multiple studies report inconsistency in their findings with integral membrane protein studies.

1:3 Traditional integral membrane protein solubilization

The first step of the traditional solubilization process of integral membrane proteins is to disrupt the membrane using either organic solvents or detergents. The organic solvent with hydrophobic property is the major component that dissolves the membrane, while detergents stabilize the membrane protein in the solution. Detergents solubilize proteins by binding to the hydrophobic surface of the protein on one side and interacting with the solvent on the other side. There are different types of detergents: ionic, nonionic, or zwitterionic.[20] The choice of detergent is based on trial and error. It is also necessary to optimize other conditions such as the concentration of the detergent use, buffer concentration, ionic strength, pH and temperature. Critical micelle concentration (CMC), which is unique to a given study, must also be considered. After the extraction, the pellet is washed and residual detergent is removed and resuspended in the appropriate buffer. Usually, high concentrations of the detergent are used at the initial extraction and the detergent is removed or exchanged to another detergent as needed by the subsequent steps.

INTEGRAL PROTEIN EXTRACTION METHODS AND THEIR PITFALLS

2:1 Detergent effects on isolated integral membrane proteins

Detergents simulate the native lipid bilayer environment of the isolated protein being studied,[21] and therefore, they are commonly used in membrane protein isolation and solubilization. They are amphipathic molecules with hydrophilic head groups and hydrophobic tails that extract the membrane protein from its lipid bilayer while spontaneously forming micelles around the proteins.

There are a variety of detergents, classified into different groups based on their structure. The most commonly used detergent in protein analysis is dodecyl maltoside (DDM), which works by forming either a micellar structure or a “monolayer” around the protein, thus preventing any “nonspecific aggregation" in an aqueous solution.[21]

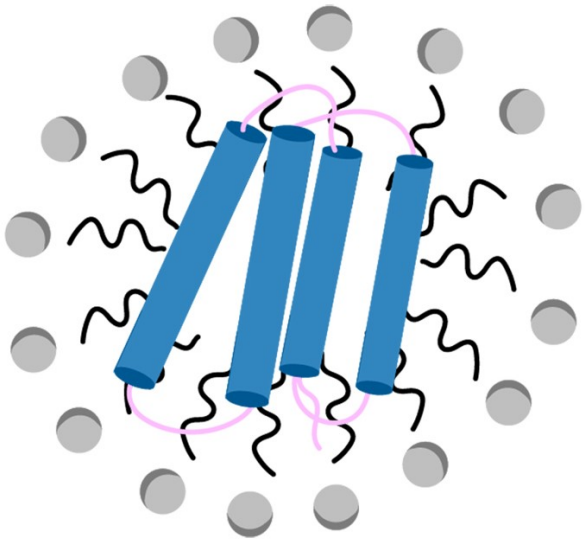

Figure 3. Graphic representation of a micelle. Micelles contain both hydrophilic polar heads (grey) and hydrophobic fatty acyl tails (black), which interact with the hydrophobic residues on the membrane protein (blue). Micelles are formed when a monolayer of detergent surrounds the protein, with the detergent's hydrophilic component facing the aqueous solution.

The micelles are soluble in an aqueous solution, which dissolves the proteins despite their hydrophobic character. The aqueous spaces on the sides of the membrane differ in chemical composition, pH, and electrical potential. These factors are believed to contribute to the stability and folding of membrane protein regions exposed to the aqueous environment, as they also do in the folding of soluble proteins.[22]

A good detergent will simultaneously allow the transmembrane α-helices of a membrane protein to pack correctly and interact with the helix bundle's outer surface. This will prevent exposure of the hydrophobic helices to water and aggregation.[23] An ideal detergent will also have little structure of its own, allowing protein intramolecular interactions to determine the helices' packing.[23] Due to its importance in the overall structure and orientation of a membrane protein, removing the lipid bilayer via detergent could lead to a large change in the structure of the protein.[23] The overall structures of membrane proteins determined from crystals grown from detergent micelles give a preview of the structure in the native membrane. However, they are not always accurate.

As previously described, detergents simulate the native lipid bilayer environment of the isolated protein being studied.[21] Additionally, according to Henry and Sykes, the use of organic solvents and micelles of strong detergents (i.e., SDS) are useful in determining structure for amphiphilic peptides and small membrane proteins.[24] However, the results with larger proteins are inconclusive compared to those of water-soluble proteins and smaller peptides. They require a “milder treatment" to preserve the tertiary structure, suggesting that detergents have a strong effect on protein structure.[24]

The authors Bayburt and Sligart acknowledged that in detergent, there is a chance that the protein can aggregate in the process of detergent solubilization.[25] Additionally, with the varying results associated with the type of detergent and speed of its removal, the protein structure may not always resemble its native conformation in vivo.[25] The fact that the appropriate use of detergent is essential is further demonstrated by Lee and Bååth. The use of detergents affects the native fold of the membrane proteins and their interactions with other proteins, including antibodies.[26] Lee and Bååth used cadherin-11 (Cad11) and investigated the impact of detergents on membrane protein complex isolation. They found out that different detergents gave different results with immunoprecipitation.[26] For instance, dodecyl maltoside (DDM) was able to immunoprecipitate Cad11–mAb 1A5 as a single complex whereas, acetyl glucoside could not, suggesting interference from the detergent with the interaction between mAb, 1A5, and Cad11. Additionally, Triton X-100, cholate, CHAPSO, Zwittergent 3-12, Deoxy BIG CHAP, and digitonin were able to solubilize Cad 11 while Brij-35 was unable to, suggesting differences in their behavior and interactions with the proteins and lipids.[26] They also report differences in the association of p120 catenin with Cad11 identified based on the detergent used.[26] These findings suggest molecular-level differences in the attractions between detergents and membrane proteins. The detergent’s interactions with the proteins also have an effect on protein-protein interactions.[26]

Guan and Smirnova describe how the content of phospholipids extracted with detergent affect the crystallization of membrane proteins. In the process of crystallization of lactose permease (LacY), using the same protocol, they ended up with three different types of crystals: hexagonal crystals with low diffraction, orthorhombic crystals with better diffraction and crystal structure, and a tetragonal form.[27] They reported that the crystals all appeared at different time periods and that the tetragonal form was the most difficult to reproduce. Most importantly, they reported that the crystals' purification by gel filtration yielded only hexagonal crystals, suggesting the need of phospholipids as an impurity with the protein extracted to maintain its structure.[27] In several membrane protein crystallization projects, the formation of the inverted dimer was observed.[27, 28]. This could be due to nonspecific hydrophobic interactions between the two monomers. By these means, protein-protein interactions replace detergent - phospholipid interactions.

In this well-equipped biotechnological era, scientists still rely on a trial-and-error approach to choose the correct detergent for membrane protein extraction, solubilization and study.[29] Theoretically, the same detergent should be able to stabilize the membrane protein at all the stages of a membrane protein study. Yet, the best detergent for protein solubilization is not usually the most suitable detergent for the purification and crystallization of a given protein.[29] This evidence suggests that the detergents' role is not stabilizing the protein but interacting with the environment. On the other hand, plasma membranes make interactions with the surface of the membrane proteins to stabilize the protein itself.

Additionally, several studies discuss that the proteins extracted using detergents are unstable due to the differences in physical characteristics, such as thickness and lateral pressure of the detergent micelles compared to that of the membrane structure.[30-32] Moreover, evidence from previous works reveals destruction of membrane proteins' structure and function due to extraction into detergents.[33-36]

2:2 De-lipidation due to detergents

Lipidation is a post-translational modification that targets proteins to membranes in organelles, vesicles, and the plasma membrane. Examples of lipidation include myristoylation, palmitoylation, and prenylation.[1] All types of lipidation increase the affinity of proteins for the membrane by increasing their hydrophobicity.[1] However, each type of modification gives the protein a distinct membrane affinity.[1] Lipidation serves to regulate and control interactions between the membrane and protein, such as enzymatic actions, protein conformation, and structural stability.

The modification of proteins by these lipid components is essential in determining their function. When membrane proteins are solubilized, lipids in the micelles play an important role in stabilizing the protein. Therefore, the detergent-lipid-protein ratio is an important factor for the successful solubilization of membrane proteins. When the detergent concentration is too low, it will not be able to extract the integral proteins into solution. However, when the detergent concentration is gradually increased, the plasma membrane "dissolves" due to disruption of hydrophobic interactions that hold the lipid molecules together and micelles containing detergent-lipid-protein are formed.

Excessive amounts of detergent then start to bind lipids in the detergent-lipid-protein complex and remove the micelles' lipid component. This de-lipidation process leaves detergent-protein complexes. There are many reports discussing that the high concentrations of the detergent concede the protein's activity. [20, 37]

Yang and Wang studied the interactions between the first nucleotide-binding domain (NBD1) of the cystic fibrosis transmembrane conductance regulator (CFTR)NBD1 and three classes of detergents. Cationic, zwitterionic, and nonionic detergents were unable to solubilize the protein without denaturing the protein.[38]

More importantly, they reported an irreversible denaturation of the protein due to detergents and the possible formation of the non-native helical structure.

The authors also reported a significant thermal destabilization. As indicated by the circular dichroism (CD) signal, secondary structures were intact.[38] Therefore, their observation of a significant decrease of calorimetric enthalpy when unfolding the protein is most likely due to the disruption of its tertiary/quaternary structures. Their findings also suggest that the detergent monomers destabilize NBD1 by binding to the unfolded state.[38]

2:3 Other platforms

Amphiphilic polymers called amphipols were discovered to overcome many of the limitations of detergents. Nevertheless, amphipols did not completely resemble bio-membranes and were also reported to denature delicate membrane proteins when extracted. Liposomes and bicelles are lipid-based bio-membrane imitations and are considered better choices than detergents. The reconstitution of membrane proteins into liposomes typically begins with the isolation of cellular membranes[39] followed by disruption of the bilayer using either organic solvents or detergents. The use of organic solvents often denatures the proteins and therefore makes them lose their functionality. Thus, the most widely used strategy for membrane protein reconstitutions is detergent-mediated reconstruction.

Detergent extracted membrane proteins are mixed with detergent-solubilized lipids in a process that creates lipid-detergent-protein and lipid-detergent micelles. Liposomes are then formed in the solution upon removal of the detergent. Because they contain membrane proteins, these liposomes are called proteoliposomes. How and whether these membrane proteins correctly get anchored into the liposomes is not yet fully understood.

Wang and Tonggu report that the binary lipid-detergent system behaves differently from that of the pure components and having membrane proteins as another component complicates the interactions.[39] The unknown effects of unknown lipid-lipid, protein-lipid, protein-protein and various other interactions on solubilization, characterization, proteins' structural stability and protein function could be a reason to obtain different results with different membrane protein analytical methods.[39]

They also emphasize that the efficacy of reconstitution depends on many factors, including the membrane protein of interest and the previous protocols and reagents used. The detergent removal rate is also important, as it decides the proportion of protein-lipid in the vesicles. Another problem associated with this is that the integral proteins can anchor the liposomes in the incorrect orientation, leading to no function. Young and Rigaud reported two orientations of Ca^{2+}-ATPase in the bilayer after reconstitution.[40] Similarly, Wang and Sigworth reported similar orientation problems for the human large-conductance calcium and voltage-activated potassium channel (BK).[41]

RECENT DEVELOPMENTS

3:1 Bicelles

Bicelles are long-chain phospholipid aggregates that are arranged in disk-shapes with flanking rims made of either detergent or short-chain phospholipids. The lipid-detergent ratio is important, as this ratio determines the size of the bicelles. The choice of the specific type of phospholipid and its charge controls the surface charge of the bicelle's flat region.

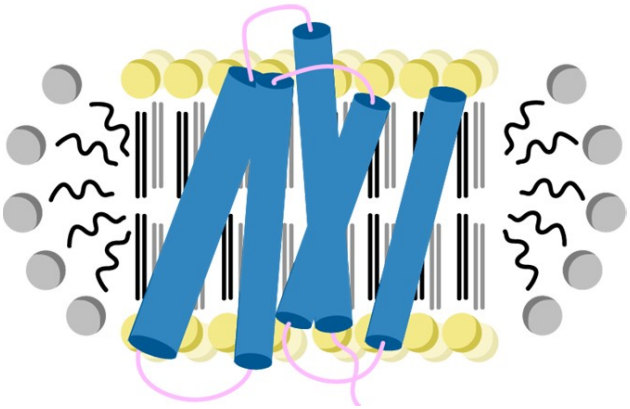

Figure 4. Graphic representation of a bicelle. Bicelles are made of long phospholipid chains (yellow) surrounding the membrane protein (blue), with the flanking rims consisting of detergent molecules (grey). These molecules more closely resemble a lipid bilayer with the phospholipid component. The size of the bicelle is determined by the ratio of lipid to protein to detergent.

Bicelles resemble bio-membranes better than the above methods and therefore have been used extensively for structural studies of proteins. The enzyme diacylglycerol kinase (DAGK) was found active in bicelles while there was no activity reported in micelles.[42] The protein staphylococcal multidrug resistance pump (Smr) did not show significant activity in multiple detergent systems but was still active and interacting with its substrate in the bicelle system, indicating the preservation of the native structure.[43] Bicelles also have the ability to align in a magnetic field spontaneously. This allows solid-state NMR studies to characterize G-Protein Coupled Receptors (GPCRs). As a result, bicelles have been successfully used in many NMR studies [44] and crystal structure determinations.[45]

3:2 Nanodiscs

Nanodiscs are membrane mimics that provide membrane proteins with a hydrophobic environment while keeping them in a soluble form for a variety of downstream applications. Nanodiscs are disc-shaped, nanoscale phospholipid bilayers made of a single or multiple phospholipids. To maintain its size, two amphipathic alpha-helical protein molecules run around its edge. Nanodiscs provide a more membrane-like surrounding for the membrane proteins compared to that of detergent micelles or liposomes. Therefore, nanodiscs are believed to preserve better isolated integral membrane proteins' structural and functional integrity than the above methods. Nanodiscs were originally inspired by high-density lipoproteins (HDLs), specifically human apolipoprotein A-1, a scaffolding protein in HDLs. HDL's used to scaffold nanodiscs are either extracted, synthetic or a homologous version from zebrafish. The choice of nanodisc and its lipid composition is generally decided based on the protein of interest.

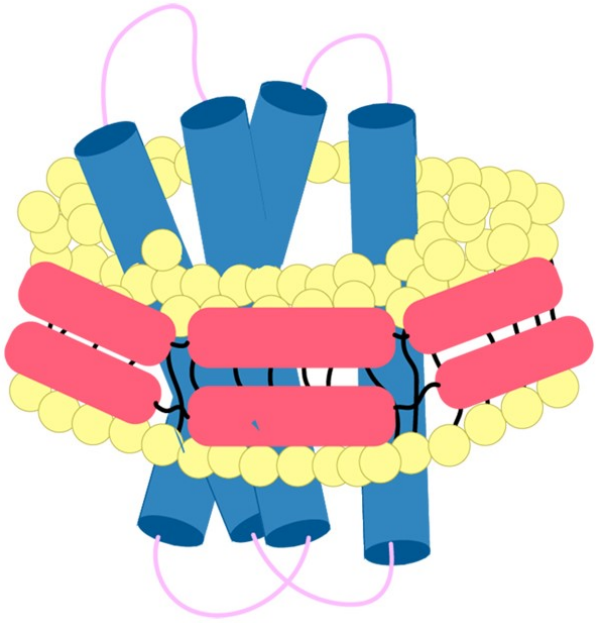

Figure 5. Graphic representation of a nanodisc. Nanodiscs are disc-shaped molecules that resemble a nanoscale phospholipid bilayer. The membrane protein (blue) is surrounded by phospholipids (yellow) and is held by membrane scaffold proteins (red) and maintains the size of the molecule.

Nanodiscs are believed to provide membrane-like surroundings to the integral membrane proteins. Many reports describe that the nanodiscs provide membrane proteins with a biologically relevant lipid bilayer environment. However, the phospholipids that are used in the nanodisc fabrication may have specific interactions with the protein of interest. Previous reports explain that adjusting the lipid to protein ratio is important but is tedious to determine due to the system complexity of two proteins interplaying with lipids.[46] Therefore, there are studies in which they modified the traditional nanodiscs with no lipids. Salvador and Glavier used the scaffold protein without added lipids as a minimal system to stabilize membrane proteins. They successfully used the bacterial transporter MexB in cryo-electron microscopy structural study.[47]

Nanodisc technology has been successfully used in many applications in various fields such as NMR, [48, 49] X-ray crystallography,[50] Cryo-EM,[51] electron microscopy, [52, 53] surfaces plasmon resonance,[52] single-molecule studies,[54] X-ray scattering,[55, 56] and charge- sensitive optical detection.[57]

One recent modification is the formation of libraries of membrane proteins in nanodiscs, where the membrane proteins are directly captured from the natural environment

of the protein of interest. This method is designed to improve the nanodisc in terms of how well it represents the biological membrane.[58-60] Furthermore, Wilcox and Marunde introduced a nanodisc based system to screen small molecule inhibitors in a high throughput assay. They encapsulated the solubilized synaptic membrane proteome into nanodiscs and demonstrated the system's ability to serve as a cell-free system to screen drugs to a synaptosome membrane protein library.[61] The ability to incorporate and regulate multiple protein molecules is one of the advantages of nanodiscs. Ligand binding and G-protein interactions of GPCRs have been successfully studied using nanodisc platforms. Information such as the effect of dimerization on GPCR activity has been determined as a result of nanodiscs. It has also been demonstrated that the monomer is enough for G-protein activation.[62-64]

3:2:1 DEVELOPMENTS TO NANODISCS

Native nanodiscs (SMALPs)

A recent development in membrane protein studies is that the native nanodisc with a section of the biological membrane can be fabricated for structural and functional studies without solely isolating the protein. These native nanodiscs are 5-50 nm in diameter and made by a linear polymer named poly(styrene co-maleic anhydride) (SMA) derivatives. SMA is an amphipathic molecule due to the presence of both hydrophobic styrene and charged, hydrophilic maleic acid moieties. Thus, the discs are water-soluble while they hold an intact piece of a membrane. These allow for the study of membrane proteins collectively as a complex (memteins) and their interactions with each component of the complex. Native nanodiscs can be used in studies of memteins by cryo-electron microscopy (cryoEM), nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR), mass spectrometry (MS), spectroscopy, small-angle x-ray scattering (SAXS), and x-ray diffraction (XRD).[65-71] However, SMA introduces a few challenges in the use of these SMALPs. SMA strongly absorbs in the UV region. Therefore, these nanodiscs are not the best application for fluorescence techniques.[72] These nanodiscs did not show a critical control of their size during the fabrication.[73] Low pH and divalent metal ions were also found to destabilize these nanodiscs. According to Lee and Knowles, SMA is also unstable at low pH and in the presence of divalent metal ions due to the presence of carboxylic acids as the hydrophilic portion of the polymer.[74] This problem arises because once these polymers dissolve in water, carboxylic acid groups dissociate into carboxylate form, which can chelate other metal ions.[75] This limits their application in certain proteins.

To overcome these limitations, other low molecular weight polymers can be used to form stable nanodiscs. Simple modifications to the monomer functional groups have been shown to change the properties of the resultant nanodisc.

Poly(methacrylate) (PMA) polymers

These polymers were originally proposed due to their amphiphilic nature. PMA-stabilized nanodiscs have been applied to the stabilization of helical intermediates of amyloid proteins. These are relatively easy and inexpensive to synthesize by a radical reaction, as well as change the functionality by altering the side chain.[76]

Poly(diisobutylene-co-maleic acid) (DIBMA)

DIBMA is another copolymer alternative to SMA-based nanodiscs. Due to the aliphatic nature of DIBMA, it does not interfere with the absorption or emission in optical microscopy. It is also insensitive to divalent metals at low millimolar concentrations.[72]

Styrene maleic acid – amine (SMAd-A)

SMAd-A is made by modification of SMA with a primary amine. These were invented to address the low solubility of SMA in acidic media. SMAd-A can tolerate low pH and is capable of solubilization of phospholipids into nanodiscs.[77]

Styrene maleic acid – ethanolamine (SMA-EA)

Ethanolamine functionalization improves size control. Thus, these nanodiscs can be formed in a smaller size for solution NMR and large size for solid-state NMR. The nanodiscs are negatively charged and preferably bind to negatively charged proteins.

Styrene maleimide quaternary ammonium (SMA-QA)

Sensitivity to pH can be effectively controlled by the introduction of a quaternary ammonium functional group.[78] The positive charge of the quaternary ammonium favors negatively charged proteins.

Poly(styrene-co-maleimide) nanodiscs (SMILPs)

As mentioned above, SMALPs are only soluble at higher pH values due to their carboxylic acid groups. To overcome that limitation, SMILPs have been employed. These are comparable to SMALPs by all the other properties.[79]

Covalently circularized nanodiscs (cNDs)

Despite challenges such as increased heterogeneity and low yield of production in E.coli when making large nanodiscs, nanodiscs of a size of 50 nm have been produced to accommodate large protein complexes. Padmanabha and Shih used DNA origami barrels as scaffolding corrals to recruit small non-circularized nanodiscs to the inner wall. The small nanodiscs fuse with each other upon the addition of excess fluid to result in a single large nanodisc. Accommodating large protein complexes is important because it enables the study of proteins and their interactions with other components in a complex.

Additionally, there are many potential applications of large nanodiscs. They can be used to study virus entry into cells and be applied to vesicular fusion studies.

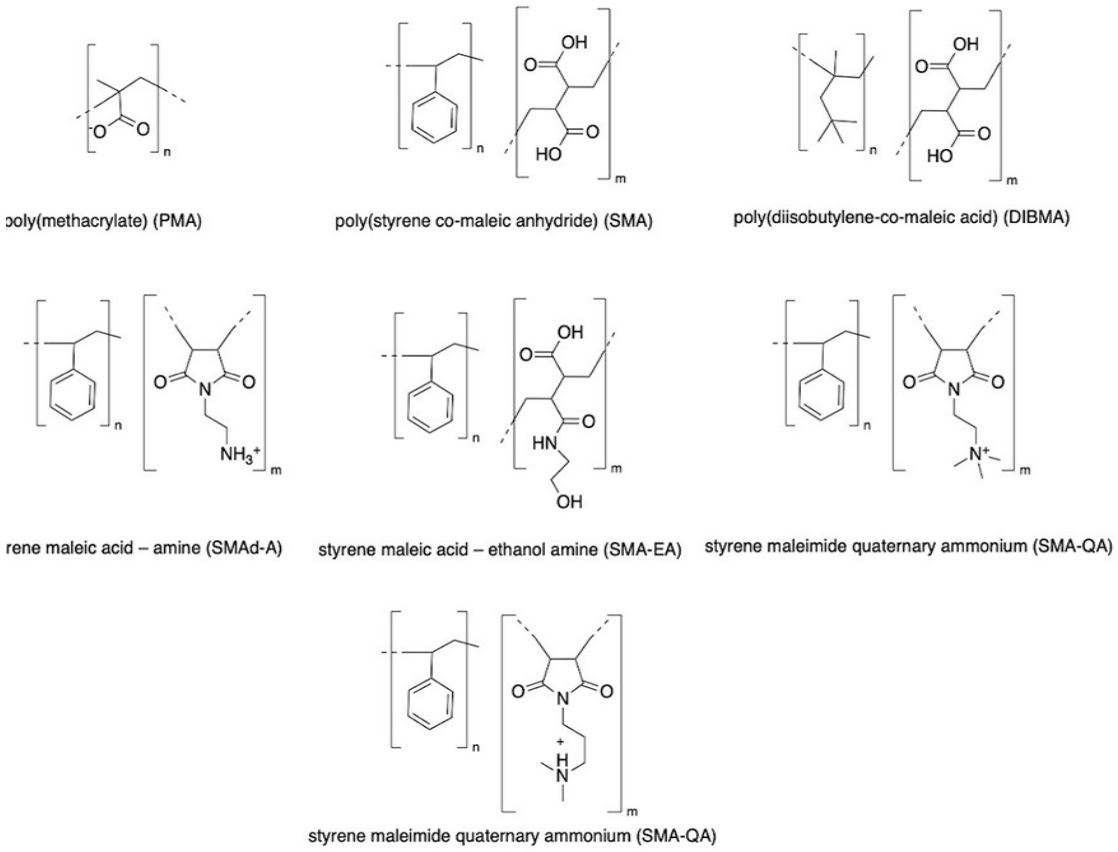

Figure 6. Visual representation of low molecular weight polymers used to stabilize nanodiscs. The addition of different functional groups to the base monomer alters the properties of the resultant nanodisc.

These can be further developed to help better represent the natural membrane by making them asymmetric. However, the development of larger nanodiscs is difficult because as the nanodiscs' size increases, they tend to aggregate.[80]

CONSIDERATIONS WHEN USING NANODISCS

Nanodiscs provide an isolated, controlled [64] system that mimics the membrane without external interference to study proteins. However, without neighboring lipid or protein interacting partners from the natural bio-membrane and a possible loss of critical protein-protein and protein-lipid interactions, the protein may not function optimally.

Most biochemical and biophysical techniques will not be influenced by the size of the nanodisc as long as it is fairly homogeneous. But in NMR, the size of the nanodisc matters. For example, solution NMR applications will strongly favor smaller nanodiscs since large nanodiscs take longer rotational correlation time and thus decrease the spectral resolution and sensitivity.[33, 81] On the other hand, for solid-state NMR applications, larger discs provide better magnetic alignment with the applied field, resulting in a better signal.[82, 83] The choice of lipids used to fabricate the nanodisc is important, as these lipids may interact with the protein of interest and may affect its native conformation. The lipid profiles of biological membranes are dynamic and vary among different membranes. Therefore, it is often difficult to define the exact native lipid composition for a given membrane protein and mimic it. Although a broad range of lipids are, in general, compatible with nanodisc platforms and allow manipulations as needed, nanodiscs are not necessarily formed with the expected ratios of the lipids while using a lipid mixture. Some lipids have a preference for other lipids and membrane scaffolding proteins can produce unexpected major products. Therefore, it is important to choose compatible lipids and lipids' desired ratios and membrane scaffolding proteins during the reaction. It is also important to analyze the composition of the nanodiscs produced after the assembly.[35]

Testing the stability of the nanodiscs during the full experiment time is crucial. For certain studies such as NMR, longer periods of data collection at higher temperatures may be applied. When nanodiscs are used in applications such as protein interaction studies, it is important to run controls for nonspecific interactions. The molecules may behave differently when they are in an aqueous phase and in a fabricated nanodisc. When the nanodiscs are produced, the functional groups or hydrophilic/ hydrophobic surfaces that are exposed may be different.[35] Therefore, in choosing the lipids for nanodisc formation, hydrophobic mismatch should also be considered as a factor.

Table 1. Summary of recent work on membrane protein extraction methods.

Extraction Technique	Description
Detergent	The solubilization of a membrane protein, Cad11, and other components in the membrane protein complex, was found to vary with the type of detergent used.[26] There is a critical micelle concentration (CMC) that must be met to form micelles. When extracting proteins, the detergent concentration should exceed the CMC.[29] Extra membranous soluble domains, such as nucleotide-binding domains, can be destabilized by detergents and, therefore, compromise membrane proteins' structural stability.[38]
Bicelles	Several membrane proteins have been successfully crystallized for further analysis with the use of bicelles, as the proteins can reconstitute in an environment that closely resembles the bio-membrane.[45] Bicelles formed from a combination of detergent and sphingomyelin and cholesterol-rich (SCOR) lipid mixtures have proven to be compatible with many techniques used to study protein structure due to their stability in varying conditions.[91]
Nanodiscs	Nanodiscs were found to control phosphatidylserine aggregation, a phospholipid that is normally recruited during the blood coagulation cascade.[25] A variety of specific tags are used on scaffolding proteins to assist them with protein targeting and isolation in vivo.[34] A bacterial transport protein, MexB, was stabilized by two membrane scaffolding proteins without lipids, potentially introducing a new, minimal form of the nanodisc.[47]
Amphipols	Amphipols' ability to refold makes them a useful tool to prepare membrane proteins for solution NMR analysis.[31] Lipid-detergent complexes face complications when interacting with membrane proteins, but the addition of amphipols may reduce this issue by removing the need for detergents.[39]

Maintaining the desired surface curvature is essential. This curvature and smoothness of the nanodisc's surface changes based on the lateral pressure provided to the nanodisc by the membrane scaffolding protein belt. [84]

SUMMARY AND FUTURE DIRECTION

Lipid membranes that act as a selective barrier for cells or for specific organelles perform functions essential for cell physiology and disease progression. Lipid membranes with the embedded proteins together coordinate these functions.[85-88] Therefore, it is essential to understand membrane protein function in the presence of its natural lipid environment. This is extremely challenging, as the lipid and protein composition and dynamics continue to change from one cell to another, as well as within the same membrane over time. It is essential to have a platform that can be easily adapted to represent a given membrane structure of interest and correctly mimic the plasma membrane. As described in this review, there have been many advances to the membrane structure memetics to study membrane proteins. Nevertheless, the possible effects of interactions between the protein of interest and the lipid components in the membrane mimetic platform on the protein structure, dynamics, and function will remain unknown.

Many integral proteins interact with the lipids in the internal regions and further anchor to the membrane cytoplasmic surface by lipids, primarily myristyl, palmityl, or prenyl groups. In addition, some proteins are anchored through the acyl chains of glycosylphosphatidylinositol from the outer surface. Most membrane surface proteins also interact with carbohydrates through glycosylation. These interactions may be an important part of their mechanisms but are not accounted for in current methods of analysis. According to the genome analysis studies, approximately 30 % of all proteins are membrane proteins.[89] Most importantly, membrane proteins are targeted by an estimated 50 % of all drugs.[90] Any drug that has a target inside the cell should be able to pass the plasma membrane. Therefore, understanding membrane proteins will also improve the effectiveness of the delivery of other drugs. Recent developments on covalently circularized nanodiscs (cNDs) have opened up another complete area of research by allowing the study of protein complexes.[80] Incorporation of plasma membrane fragments into these large nanodiscs will allow investigations of protein-protein, protein-lipid, protein-DNA and protein-carbohydrate interactions.

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