

Solution NMR investigations of integral membrane protein structure and dynamics

Solution NMR investigations have contributed significantly to the development of NMR applications and to the understanding of the structure and dynamics of α -helical and β -barrel membrane proteins. In addition, the requirements of NMR solution biomolecular spectroscopy have pushed the development and engineering of robust expression systems and led to a greater understanding of membrane mimics, such as detergent and bicelles. These advances have enabled the solution NMR to identify, structurally map, and dynamically characterize membrane protein-protein interactions [1-4], membrane protein-ligand interactions [5-7], and membrane protein-lipid interactions [8-10].

Protein expression and isotope incorporation: advances and challenges

NMR requires protein concentrations in the range of hundreds of micromolar as well as expression systems that can incorporate NMR active nuclei. These limitations almost entirely exclude eukaryotic expression systems for complete membrane protein structure determination of membrane proteins [11,12]. However, specific side chain incorporation in has facilitated the applications of solution NMR to investigating membrane protein dynamics and conformational landscape of lower expressing proteins, including those from eukaryotic expression systems [13-17]. Membrane proteins can be isolated from the membrane or refolded from an insoluble fraction (e.g. inclusion bodies) [18,19]. Regardless of the approach, a robust assay of protein function or biological activity is essential as expression and purification conditions can drastically affect protein fold and function [20,21].

Directed evolution and optimization of *E. coli* expression of eukaryotic targets has facilitated breakthroughs in understanding membrane protein structure, function and dynamics [22-25]. However, membrane proteins from eukaryotic organisms may have post-translational modifications essential to form and function, which cannot be recapitulated in a bacterial expression system [26]. Eukaryotic expression systems are not as versatile for deuteration or complete labeling compared to bacterial expression systems, so alternative isotope incorporation strategies, such as single amino acid labeling, the use of isotopically labeled unnatural amino acids, or post-translational chemical modification with stable isotopes have been successfully used [13-17]. These labeling strategies are more suitable for studying protein dynamics or ligand binding as opposed to structure determination since the proteins are not uniformly labeled with ^{15}N and ^{13}C [27]. The power of this methodology is demonstrated by stunning investigations of G-protein

coupled receptors (GPCRs), such as the β 2-adrenergic receptor in which adopting ^{13}C labeling of lysine or methionine residues allowed elucidation of changes in dynamic behavior of the protein in response to ligand binding as well as the structural heterogeneity that exists even in bound states that is not captured well by other structural techniques [28,29].

In conjunction with these alternative labeling strategies, eukaryotic proteins may require extensive genetic manipulation to produce an optimally thermostable protein sample, with G-protein coupled receptors (GPCRs) being a prime example [30,31]. Optimization such as this is not universal and must be designed specifically within each experimental framework. In this context, NMR has also proved an incredible tool to assess functionality of membrane proteins genetically optimized for structure determination as elegantly demonstrated by Goba, et al. [32]. Codon harmonization, in which the codon usage in recombinant gene sequences is matched to those in the native sequences, has shown an increase in protein yield [33]. This specific codon optimization nearly doubles yield through the ability to mitigate protein misfolding. In addition to the expression system, the membrane mimic is an incredibly important factor for membrane protein integrity and physiological relevance [34-36].

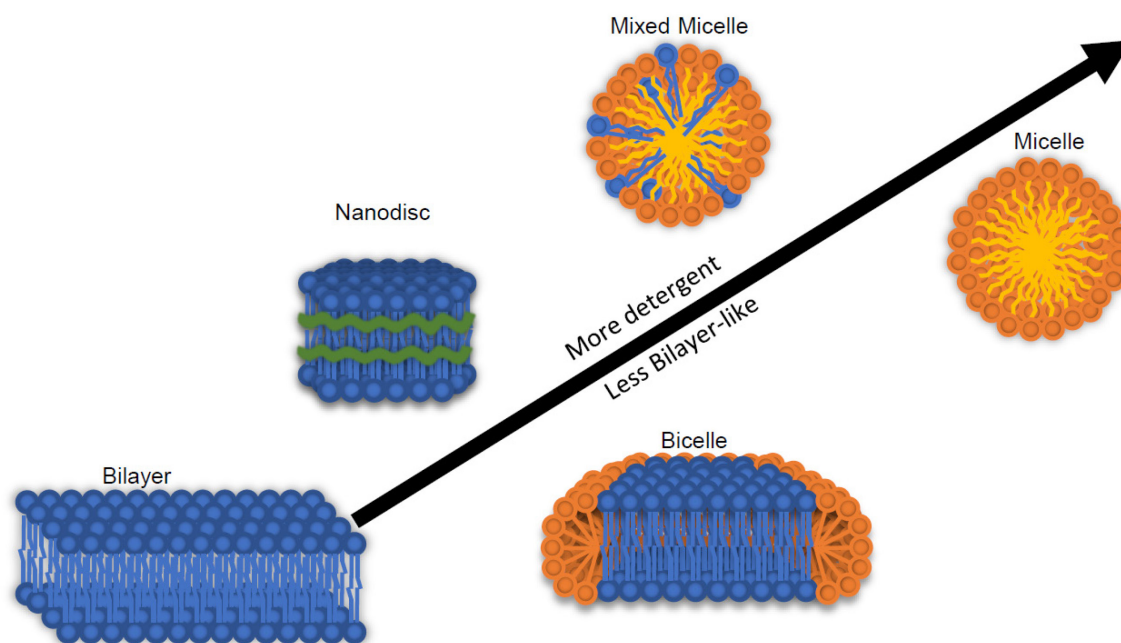


Figure 1. Representation of membrane mimics for membrane protein solution NMR arranged in order of least detergent/more bilayer-like to most detergent/least bilayer like.

Lipid molecules are represented in blue, while detergents are represented in orange.

Extraction and solubilization impacts NMR spectral quality

Micelles. For solution NMR investigations, membrane proteins need an apolar solvent around the hydrophobic transmembrane regions yet be soluble and freely tumbling in an aqueous solvent. In addition, if the membrane protein is expressed to the cell membrane, then the protein needs to be extracted from the membrane as well as stabilized in aqueous solution. Choosing the correct membrane mimic for membrane protein solubilization and stabilization can have an outsized impact on the success of a study. Choosing an incompatible membrane mimic can result in insoluble, destabilized, and/or not functional membrane protein [37-42]. Fortunately, a host of membrane mimics have been developed for use in membrane protein NMR, such as micelles, bicelles, and nanodiscs (Figure 1) [43,44]. Each mimic system has well-documented advantages and disadvantages and within each system, much work has been done to characterize the myriad combinations of the constituent component detergents, lipids, and membrane scaffolding proteins [45-48]. Despite the large body of literature surrounding membrane mimics, identifying the right membrane analogue for a given system is often a matter of empirical screening, which is often costly and time-consuming [49-51].

Traditionally, amphiphilic surfactants are used; however, additional systems have been developed and used. Membrane proteins are extracted from the bilayer with the addition of detergent through detergent-lipid interactions that destabilize the lipid-lipid interactions and yield solubilized mixed lipid-detergent assemblies [52]. The protein of interest is then purified from the rest of the solubilized membrane components and most weakly associated lipids are removed. The resulting protein-detergent micelle complexes are then empirically screened for solubility, stability (days for spectral acquisition), and quality NMR spectra (evaluated by chemical dispersion, uniform intensity of peaks, and a match of the number of expected and observed resonances). While detergent micelles are used in most extractions, the final mimic system may change for NMR analysis.

In detergent micelles, charged detergents have been identified to destabilize tertiary membrane protein structure, whereas nonionic and zwitterionic detergents micelles, such as *n*-dodecyl- β -D-maltoside (DDM) and dodecylphosphocholine (DPC), respectively, stabilize membrane proteins [53-56]. Historically, nonionic protein – detergent micelles are used to determine protein structures with X-ray crystallography [57,58] and zwitterionic detergents from NMR [56]. This could arise from the requirement that proteins not interact in NMR yet need to interact to form a crystal lattice.

82 Thus, detergent micelles that have electrostatic repulsive headgroups would be more favorable for
83 NMR to reduce exchange processes and higher molecular weight complexes.

84 For quality NMR spectra, the molecular weight of the molecular complex should be minimized
85 including the molecules that solubilize the membrane protein. With respect to detergents, long-
86 chain alkyl chains result in larger aggregates that may be unsuitable for high-resolution
87 spectroscopy [14,59]. Even in cases where a protein is successfully solubilized, the membrane
88 mimic can cause loss of function or destabilization of the overall structure and dynamics [60,61].
89 For instance, although quality NMR spectra for the ADP/ATP mitochondrial carrier transport
90 protein AAC3 were obtained, Kurauskas *et al.* demonstrated that carrier was not functional in DPC
91 micelle, but was functional when reconstituted into a DDM micelle [62]. Detergents can be mixed
92 to tune mixed micelles of different properties to match membrane [63,64] and membrane protein
93 properties. In addition, lipids with specific properties (e.g. charged head groups, alkyl chain length,
94 or known modulators of function) can be added and will partition into micelles; however, these
95 mixed micelle – lipid assemblies will have different properties [65].

96 *Bicelles*. Mixtures of lipids and detergents are referred to as bicelles (Figure 1) and have proven
97 extremely useful in solution NMR studies. An ideal bicelle has a segregated lipid core with a
98 detergent belt surrounding the core lipids. When the lipid component is present at molar ratios less
99 than 1:1, the lipids can mix with detergents to form mixed micelles [65,66]. The concentration for
100 which lipid segregation occurs is dependent on the lipid and detergent monomer properties and the
101 presence of protein. Membrane proteins can be purified in bicelles or reconstituted into liposomes
102 and solubilized with the addition of the detergent at the appropriate concentration to form the
103 desired bicelle. The quality of the NMR spectrum not only depends on the bicelle used but how
104 the bicelle is formed as was shown for EmrE [61,67].

105 There are many detergents, detergent mixtures, and bicelles to screen empirically for folded,
106 functional, membrane proteins with quality NMR spectra. The major challenge is line broadening
107 that could be due to (i) exchange processes due to interactions between solubilized complexes, (ii)
108 conformational heterogeneity which is very common in α -helical proteins, (iii) correlation time of
109 the complex, and (iv) partial unfolding or denaturation of the membrane protein. There is no magic
110 bullet as to what membrane mimic is the best for all proteins. However, several factors can be
111 considered to narrow the choice, such as aggregate size, hydrophobic thickness, and composition.
112 Hydrophobic mismatch between a membrane protein and the membrane mimic environment can

impact protein structure [68-70]. Inner and outer membrane proteins have a hydrophobic thickness of ~ 29 Å and ~ 24 Å, respectively [71,72]. A surfactant's ability to match the hydrophobic thickness is critical, but not necessarily sufficient. The maximum hydrophobic thickness of a micelle is determined by the amphiphile tail length in a pure micelle and can be titrated with detergent mixtures; however, as the detergents are mixed the aggregate shape and size change as well [73]. Electrostatics are important to both mimic the membrane environment and to reduce interactions between solubilized complexes. A mixture of surfactant and lipid that can provide the required charges and meet the hydrophobic thickness may be most desired.

Finally, this section would not be complete without mentioning the innovative use of reverse micelles to encapsulate membrane proteins for NMR solution studies [74-76]. These systems, although seemingly the least membrane-like, result in quality NMR spectra and stabilize membrane protein structures.

Nanodiscs. Assemblies of lipids surrounded by an amphipathic scaffold that can be a protein, synthetic polymer, or DNA are referred to as nanodiscs (Figure 1) [77,78]. The most common nanodisc used in solution NMR are scaffolds formed by membrane scaffolding proteins (MSP) [79,80]. MSP nanodiscs take advantage of the α helical, amphipathic proteins from apolipoprotein A-1, first reported by Sligar and coworkers. Within these systems, the radius of the disc is modulated between 8-16 nm with different scaffolding proteins [79] and the lipid composition can be manipulated [81]. Applications of MSP for membrane protein solution NMR experiments were reviewed recently [82] and a more recent demonstration of the benefits of circularized nanodiscs for solution NMR studies of membrane proteins was published [83]. Although nanodiscs require membrane protein isolation and purification in detergent, the advantage is that they are a discrete size and the molecular understanding of their structure and stabilization of the bilayer is well understood. Alternative to MSP nanodiscs, synthetic nanodiscs, such as styrene maleic acid copolymers (SMAs), diisobutylene-maleic acid (DIBMA), and poly(acrylic acid-co-styrene) (AASTY) have gained recent popularity. While all three synthetic polymers provide nanodisc structures for stabilization, the attractive feature of these polymers is they eliminate the need for detergents for extraction and solubilization [85-88]. Where MSP nanodiscs typically contain artificial lipids, synthetic nanodiscs have the ability to use the native cell phospholipids. Each polymer has its own unique drawbacks, but one that is ubiquitous to all the synthetic polymers nanodiscs is their variable size, due to the different lengths of polymer

chain. SMA, the longest synthetic polymer in use, has an extensive database for successful solubilizations of membrane proteins. Additionally, SMAs have been shown to have a slight increase in yield when compared to DIBMA [89]. However, like AASTY, SMAs contain an aromatic ring that absorbs light at wavelengths of 280 nm, which can make quantification of proteins troublesome [90]. Due to their relatively new use, membrane protein structure determination has been limited in these systems, but they have been successful in characterization studies of membrane proteins. SMAs used in solution NMR, was shown to maintain the physiological activity, and allow for the dissociation measurement of the Smoothed (SMO) protein of the GPCR family to its known ligands[5]. One drawback to many synthetic nanodiscs, is a sensitivity to divalent cations. Because of this, development of electroneutral SMA and DIBMA derivatives, containing a sulfobetaine group, were developed to advert cation incompatibility [91].Recent reports indicate the lipid properties are different in nanodiscs than bilayers. Lipid and protein dynamics are reportedly different in membrane scaffolding protein nanodiscs [92] and copolymer assemblies [93] compared to lipid bilayers and more investigation is required to determine if these packing properties impact membrane protein function.

Solution NMR Investigations of Membrane Proteins: Beyond Structure Determination

Solution NMR has determined the structure of numerous membrane proteins. However, solution NMR approaches can offer more than structure determination and are now facilitating the investigation of catalysis, ligand binding, conformational dynamics, and lipid -protein interactions. Saturation transfer difference (STD) NMR is a powerful technique used to investigate protein-ligand interactions [52]. The STD-NMR experiment relies on the weak-binding ligand to exchange between the bound and free ligand state and can be useful in understanding the protein-lipid interactions. In protein-lipid interactions investigations with STD, the protein resonances are selectively saturated, and magnetization is transferred to a first layer of lipids surrounding the protein. While the saturation energy is continually applied, lipids are allowed to exchange, increasing the rf-saturated lipid population. Gawrisch *et al.* measured, by using STD-NMR, the individual strength as well as the statistics of the lipid-protein interactions in membranes with incorporated bovine rhodopsin [94]. STD combined with high throughput screening also allowed researchers to map the binding epitopes of epinephrine and A-61603 to both adrenoreceptors, revealing the selectivity of the α_{1A} -AR-selective agonist for one adrenoreceptor over the other [25]. ^{19}F -transfer NMR has been used to report on conformation

dynamics on the binding groove of the neurokinin 1 receptor (NK1R) [95]. Pan *et al.* demonstrated that the orthosteric binding groove on NK1R fluctuates with amplitudes of 6 to 8 Å, highlighting a multistep selection of orthosteric ligands not captured in current static structures. Dynamic nuclear polarization (DNP) has gained significant attention recently, where polarization is transferred from an electron spin ensemble to a nuclear spin ensemble. The large difference in relaxation rates between electron spins and nuclear spins results in hyperpolarization of the nuclear spin ensemble [96]. Overcoming the inherent low sensitivity of NMR spectroscopy has allowed researchers to selectively enhance signals in ligand-binding sites [97]. Additionally, DNP provides an opportunity for a high-throughput, quantitative analysis of ligand-protein interactions using solution NMR [98]. This provides enhanced signal, and atomic-level structural detail in a single scan allowing for rapid analysis.

In studying large macromolecular complexes, increasing the isotopic labeling diversity is often important. Obtaining structural restraints using paramagnetic relaxation enhancement of ^{13}C labeled methyls is well established in beta-barrel [99,100] and alpha-helical proteins [101,102]. Recently, Huang *et al.* used paramagnetic relaxation of a ^{19}F label to elucidate information of the conformational dynamics of the membrane transporter, GltPh [103]. This approach not only allows for the identification of the multiple states of functional proteins, but also allows for the measurement of the exchange rates [104].

Fragment based drug discovery (FBDD) has become a powerful complementary approach to high throughput screening to identify drug-like candidates in the early stages of drug discovery [105]. FBDD utilizes libraries of small molecules called “fragments” that binding is evaluated against a target protein of interest. Using small molecules allows for a more efficient exploration of the ensemble of all possible molecular conformations presenting drug-like properties [106]. FBDD’s ability to screen potential therapeutics is demonstrated in the screening of several Glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC) inhibitors using an enzymatic assay and a WaterLOGSY NMR experiment [107]. The authors identified 10 fragment hits that directly bind and inhibit the GPI-PLC, an enzyme active in the pathogenesis of trypanosomiasis caused by *Trypanosoma brucei*.

Solution NMR is also able to estimate dissociation constants of specific lipid-protein interactions. The Tamm laboratory was able to investigate lipopolysaccharide (LPS) binding to the membrane protein OprH. Through a series of ^{15}N - ^1H TROSY and HMQC experiments the

authors determined which specific residues interact with LPS based on chemical shifts upon LPS addition. Additionally, to quantitatively determine the binding of LPS to OprH, the authors generated chemical shifts from TROSY cross-peaks that followed Langmuir binding isotherms, from which dissociation constants could be derived [109].

Most NMR experiments performed to date are at physiological pressure, however high-pressure NMR is emerging as a unique and complementary approach to chemical and temperature perturbation [110]. Pressure is a fundamental thermodynamic variable that can report on protein stability, function, and dynamics [111] and, with NMR, can be monitored at a site-specific atomic level [112]. Specific to membrane protein applications, high-pressure NMR can also be used to modulate the main gel-fluid phase transition and gelation of lipids [113]. Pozza *et al.* used high-pressure NMR to show the fluidity modulation of phospholipids in the interplay between lipids and the membrane proteins OmpX and BLT2 [114]. The conformational equilibria of the apo β 1-adrenergic receptor was investigated with high-pressure solution NMR with the fully active conformation (only reached in the ternary agonist·G protein effector complex) accessed with moderate pressure [115]. More recently, high-pressure NMR was used to demonstrate that the observed allosteric effects of cholesterol on adenosine A_{2A} receptor originate from changes in membrane properties not through direct interactions [9].

As demonstrated with these examples, the applications of solution NMR to integral membrane proteins are emerging beyond structure determination and with a focus on the strengths of spectroscopy in detection and dynamics.

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