Solution NMR: A powerful tool for structural and functional studies of membrane proteins in reconstituted environments

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ABSTRACT

A third of genes in prokaryotic and eukaryotic genomes encode membrane proteins that are either essential for signal transduction, solute transport, or function as scaffold structures. Unlike many of their soluble counterparts, the overall structural and functional organization of membrane proteins are sparingly understood. Recent advances in X-ray crystallography, cryo-electron microscopy (CryoEM) and nuclear magnetic resonance (NMR) are closing this gap by enabling an in-depth view of these ever-elusive proteins at atomic resolution. Despite substantial technological advancements, however, the overall proportion of membrane protein entries in protein data bank (PDB) remains < 4%. This paucity is mainly attributed to difficulties associated with their expression and purification, propensity to form large multi-subunit complexes, and challenges pertinent to identification of an ideal detergent, lipid or detergent-lipid mixture that closely mimic their native environment. NMR is a powerful technique to obtain atomic-resolution and dynamic details of a protein in solution. This is accomplished through an assortment of isotopic labeling schemes designed to acquire multiple spectra that facilitate deduction of the final protein structure. In this review, we discuss current approaches and technological developments in the determination of membrane protein structures by solution NMR and highlight recent structural and mechanistic insights gained with this technique. We also discuss strategies for overcoming size limitations in NMR applications, and explore a plethora of membrane mimetics available for the structural and mechanistic understanding of these essential cellular proteins.
I) Introduction

Membrane proteins (MPs) constitute 30% of genomes in both prokaryotes and eukaryotes. Essential for cell homeostasis, they are specifically involved in signal transduction, solute transport, post-translational modification and as scaffold proteins maintaining the overall integrity of the outer envelope. MPs are classically divided into subclasses based on their location in the membrane, viz. (Fig. 1): a) integral (or intrinsic) MPs (IMPs), spanning one or both leaflets of a lipid bilayer, b) lipo-proteins, anchored to the membrane through a post-translationally modified lipid (myristoyl/palmityl/prenyl) or glycolipid (glycosyl-phosphatidylinositol); and c) peripheral (or extrinsic) MPs, interacting with IMPs at the aqueous/membrane interface. This review mostly focuses on IMPs (unless specified otherwise), a subclass notoriously recalcitrant to structural investigation. Typically, IMPs are classified into two subgroups, largely based on the secondary structure of their trans-membrane region: most eukaryotic IMPs are α-helical proteins, with one or more trans-membrane (TM) α-helices interconnected through flexible loops, while gram-negative bacilli and eukaryotic mitochondrial organelle predominantly house β-barrels porins in their outer membrane (OM). Albeit few, there are outliers to this classification: autotransporters, β-barrel porins with α-helical complexes partially inserted within their cores; secretion pores, for example Wza, responsible for the transport of capsular polysaccharide across the OM. A complete catalog of available MP structures can be found at the “Membrane Proteins of Known Structure” database curated by Dr. White’s lab at UC Irvine (http://blanco.biomol.uci.edu/mpstruc).

It is hard to undermine the complexity of basic principles that govern the structure and function of IMPs, especially when one factors in the diverse milieu of lipid molecules that surround them. Not surprisingly, IMPs constitute a meagre fraction of structures in PDB (less than 4%) though they constitute more than 50% of therapeutic drug targets [1]. And G-protein coupled receptors (GPCRs), with seven TM α-helices, represent the most elusive yet sought after subgroup in drug development [2]. For a complete list of GPCRs, validated as potential therapeutic targets, the database maintained by Dr. Gloriam’s group at the University of Copenhagen, in collaboration with the EU COST Action ‘GLISTEN’ (http://gpcrdb.org) serves as a good resource. In this review, we focus on structural data and functional studies carried out by solution NMR of IMPs in general.

In the current era, robust technological developments in the fields of X-ray crystallography, NMR and CryoEM has enabled an ever increasing addition of structures to the protein database. X-ray crystallography, with the caveat of obtaining a crystal, remains the gold standard for obtaining atomic resolutions structures. CryoEM, with the advantage of simpler sample preparations, is the current revolution, well on its way towards reaching higher resolutions. While structures provide a clear snapshot of a protein, dynamic properties govern its functional output. Solution NMR, a technique limited by a protein’s size, is potentially the best tool to study protein dynamics along with its structure. We envision a future where a synergy of aforementioned techniques will help quickly delineate the structure and dynamics of a protein to better understand its function within a cell.

II) Solution NMR - A Brief Overview

II.a NMR: Conceptual Summary

Nuclear Magnetic Resonance (NMR) is a powerful biophysical tool to ascertain atomic resolution details of a protein. It relies upon the
basic quantum mechanical property of nuclear spins. Atoms with non-zero spin numbers, when placed in a magnetic field, are at different energy levels. We can stimulate and follow transitions between these levels at specific resonance frequencies. The most prominent feature of NMR spectroscopy, is its ability to differentiate chemically identical atoms, experiencing small variations in their magnetic environment influenced by their neighboring atoms. This property, substantiated by half a century of scientific and technological developments, allows for the assignment of each peak in the spectrum to a specific atom within a molecule of interest.

Delineating a protein’s structure can be broadly split into identifying the atoms and their three-dimensional position with respect to each other. Nuclear Overhauser Effect (NOE) provides the main source of geometric information utilized for structure determination by NMR [3] and requires the assignment of each proton resonance in a spectrum of the target molecule. For small proteins of hundred residues or less, this could be accomplished through conventional homonuclear 1H two dimensional (2D) experiments developed in the late 1970s and early 1980s [4]. For larger proteins, overlapping chemical shifts and increases in linewidth (due to increased rotational correlation time, \( \tau_c \)), greatly complicates spectral analysis demanding an improvement of spectral resolution [5]. Multidimensional NMR using \(^{15}\)N and/or \(^{13}\)C chemical shifts through the introduction of triple-resonance heteronuclear experiments [6-8] allows for the determination of proteins structures in the 25 kDa [9] range, while incorporation of deuterium, replacing protons at sites not amenable to exchange, further pushed the limit to 35 kDa [10] by suppression different relaxation pathways.

Among the different applications of NMR, viz., solution, solid state and oriented sample, solution NMR has found wide usage for structural and function studies of IMPs under conditions that closely reflect their native environments. It also allows for real time mechanistic investigation [11] and makes possible “in-cellulo” applications [12]. In addition to structure determination, solution NMR provides information on protein dynamics [13]; folding [14]; enzymatic reaction rates [15, 16]; oligomeric states [17]; ligand binding, (quantitative in favorable cases of fast exchange [18]); and has been extensively used to facilitate drug discovery [19, 20]. The overall size of the molecule determines the success of NMR applications, which in the earlier 1980s was below 10 kDa which later progressed to 25-35 kDa around mid-1990s. Currently the size limit has been pushed even further, to upwards of 100 kDa [21].

II.b Overcoming Size Limitations

Crucial breakthrough, which increased the susceptibility of the technique to proteins of higher molecular weight, resulted from combining the outcomes of the following factors (discussed briefly hereunder: i) wide spread availability of high field (above 700 MHz) superconducting magnets equipped with cryo-probes and simultaneous four channels decoupling; ii) application of TROSY-based acquisition protocols; and iii) universal adoption of novel isotopic labeling schemes.

The advantages of high magnetic fields, that drove the design and construction of bigger (often synonymous with “better”) magnets, are based on two general principles: i) direct proportionality of the spectral resolution to the field strength (\( \delta \sim B \)), and ii) signal-to-noise, proportional to the field strength in the power of \( 3/2 \) (\( S/N \sim B^{3/2} \)). An additional important advantage of high field magnets was
appreciated with the development of TROSY-based protocols. TROSY, Transverse Relaxation Optimized Spectroscopy [22], relies upon the notion that the two major relaxation pathways, chemical shift anisotropy and dipole-dipole coupling, are mutually cancelled out in one of the individual multiplet components of the single quantum spectrum, resulting in sharper NMR peaks (even for very large proteins). The efficiency of cancellation depends upon the strength of magnetic field. Optimal field strengths for TROSY was estimated to be around 900 MHz for the amide-detected-TROSY protocols. More recent studies of 15N-detected TROSYs, however, purport the achievement of maximal peak heights at even higher field strength of 1.15 GHz [23]. Low temperature (“cryo-“) probes, which detect signals at lower than ambient temperatures, additionally improved the sensitivity through white noise reduction (N ~ kT), reflected in a higher signal-to-noise ratio (S/N ~ 1/kT). Simultaneous decoupling through four channels (1H, 15N, 13C, 2H) is essential to take full advantage of data acquisition on highly deuterated 15N/13C labeled samples as it increases signal-to-noise ratio by consolidating all signal intensities of multiplets into single peaks.

Though 15N/13C isotopic labeling schemes and triple resonance spectroscopy provide an opportunity to study large macromolecular systems, proteins are still subject to 1H-1H dipolar spin relaxation, which adds to the heteronuclear 1H-X (X=15N or 13C) spin relaxation. Together, these relaxation pathways reduce the efficiency in magnetization transfer during heteronuclear multidimensional experiments, as reflected by the substantial loss of sensitivity and resolution of spectra for proteins above 25 kDa. In remediation, carbon-bonded proton substitutions with deuterium can take advantage of the 6.7-fold lower gyromagnetic ratio of 2H relative to 1H, effectively reducing the relaxation pathways as compared to fully protonated samples. Uniform random 2H incorporation at varying levels or selective methyl protonation on fully deuterated samples [24] are methodologies that incorporate such substitutions. One should exercise caution by not eliminating all protons in a sample as they constitute the main source of geometric information essential for structure calculation. Other, more exotic examples of novel labeling schemes include “reverse isotope” approaches, where specific protonation of 14N-labeled residues have been employed in a fully deuterated 15N-labeled background [25, 26], and segmental labeling, which allows the observance of signals of select amino (N-) or carboxyl (C-) terminal region along a peptide chain [27]. Employing alternatives to well-established (prokaryotic) E.coli expression systems, like Pichia pastoris [28] or Chinese hamster ovary (CHO) cells [29], may be deemed necessary when post-translational modifications are essential for proper protein folding. Cell-free (CF) expression systems [30] are particularly gaining traction as they can successfully produce recombinant IMPs which are otherwise difficult to overexpress due to their endogenous toxicity leading to cell death [31]. Dötsch* lab, for example, has introduced a strategy to generate small isotropic bicelles with IMPs that have been co-translationally embedded into nanodiscs during CF expression [32]. The advantage of this method (generating a proper fold while maintaining a small size) becomes clear when we discuss the pros and cons of different membrane mimetics in the next sections.

III) Membrane Mimetics

Stabilizing membrane proteins outside their native membrane environment remains the
achilles heel for proteins of this family. Liposomes, spherical vesicles composed of a lipid bilayer, was the earliest system developed to house membrane proteins. Alec Bangham and colleges successfully employed liposomes around mid-1960 [33, 34] to study different aspects of membrane biophysics. Though liposomes could routinely vary their lipid composition, net charge, shape, temperature and lyotrophic phases, they remain too large for solutions NMR studies. Even the smallest unilamellar vesicles are only best suited for binding experiments than structure determination. In the upcoming sections we follow the chronological development of different approaches, undertaken to increase the susceptibility of solution NMR to larger systems.

**III.a Organic Solvents**

Organic solvents or their mixtures can solubilize complex membrane proteins by mimicking a hydrophobic environment to accommodate the hydrophobic core of an IMP. An obvious advantage is the lack of additional molecular weight brought upon by the system itself with the downside of compromised protein stability. While organic solvents may preserve secondary structural elements, it could still perturb a protein’s tertiary structure, as was observed for DAGK (trimer of 13 kDa subunits, each containing three TM helices) [35]. When using organic solvents, it is recommended to obtain additional corroborating data to improve the overall confidence in the structural information on IMPs, as demonstrated by the following two case studies. Girvin and co-workers showed the subunit c of F1-F0 ATPase (two TM helices connected through a short loop) to adopt a stable, native-like fold in at least one organic solvent mixture [36, 37] and determined a high resolution structure of the dimer (pdb: 1A91/1COV). Based on their NMR and cross-linking data, they were able to generate a functional model of α1-ε12 complex (pdb: 1C17). Yang and co-workers defined the interface of major platelet integrin αIIbβ3 transmembrane-cytoplasmic heterodimer (pdb: 2KNC), as supported by numerous mutational and biochemical data, which allowed them to propose a mechanistic model for the activation of this key cell surface receptor [38].

**III.b Micelles**

By far the most successful method to solubilize IMPs are detergents that spontaneously form a micelle. Their overall smaller size [39], relative to planar bilayers, makes them the best choice of a membrane mimic. A variety of detergents have been used (SDS, DDM, OG, DPC, LDAO, LMPC, LMPG, DHPC, etc.) to access the structural integrity of membrane proteins [40, 41], compare their compatibilities with enzymatic functions [39] and determine the quality of corresponding NMR spectra [42]. A subset of detergents with polar or zwitterionic head-group attached to a medium-chained aliphatic tail has found widespread usage (Fig. 2). From an NMR perspective, DPC, dodecyl-phosphatidyl-choline (Foscholine 12), is the most widely used detergent, followed by LDAO, lauryl-dimethyl-amine N-oxide, and LMPC, lyso-myristoyl-phosphatidyl-choline or, in some specific case, like potassium channels, lyso-myristoyl-phosphatidyl-glycerol (LMPG). Additional lipid supplementation, in small amount, had shown drastic improvement in the stability and/or functional activity of IMP [43] without a perceptible increase in the overall size [44]. The following consideration should be taken into account while planning experiments involving detergents: i) although popular, SDS - sodium dodecyl-sulfate with negatively charged head group - should be sparingly used as it can destabilize a protein’s conformation; ii) the actual size of mixed micelle depends less on the
type of detergent and more on the properties of the membrane protein itself, where the extent of molecular size is governed by detergent coverage of the transmembrane region; iii) the presence of free detergent and a strong hydrophobic environment could substantially impede interaction to ligands and could lead to progressive denaturation of the soluble binding targets; and iv) loose packing of detergent head-groups, micellar curvature, and tighter acyl chain interactions at the core may destabilize an IMP conformation and alter dynamics and oligomerization states leading to noticeable differences with respect to these properties in a flat lipid bilayer system [45-48]. The above observed caveats have warranted the need to develop alternative, nanoscale-phospholipid bilayer systems, as discussed in the following sections.

Ill.c Bicelles

Prestegard’s research group first introduced bicelles, flat phospholipid bilayer discs decorated by detergents at their edges (Fig. 3). A bicelle’s conception was derived from X-ray studies of a mixture of bile salt (CHAPSO, for example) and phosphatidylcholine (DMPC) that formed discoidal bilayers of uniform size. They further demonstrated spontaneous orientation in a magnetic field [49, 50], thus instituting a novel medium for oriented protein samples. Sanders and co-workers further extended the system by substituting bile salts with DHPC, a short chain phosphatidylcholine with two six carbon acyl chains [51]. These bicelles, or “binary bi-layered mixed micelles”, quickly replaced DMPC/CHAPSO mixture since phosphatidylcholine is a more abundant natural constituents of biological membranes. Other bicellar compositions of longer chain phospholipids, forming wider bilayer, or lipid mixtures mimicking eukaryotic membranes, providing a “close to” natural environments have also been used for NMR studies [52-55].

In addition to their width and charge, the diameter of a bicelle can also be regulated, offering magnetic properties suitable for alignment. Only systems with magnetic susceptibility anisotropy sufficiently large to overcome Brownian motion, which correlates to a diameter beyond 20 nm, can spontaneously align in a magnetic field. Bicellar diameter (D) depends on the lipid to detergent ratio, often referred to as the “q” number [56]. It can be calculated according to the equation originally proposed by Mazer and coworkers [57] for ideal bicelles and later modified by Vold’s research group [58] to take into account differences between headgroup areas of DHPC and DMPC (k defines this ratio, which was experimentally found to be 0.6; r is the radius of the rim, which equals 2 nm for DHPC):

\[ D = k r q \left( \pi \left( \frac{r^2 + \frac{8k}{q} \frac{1}{q}}{2} \right) \right) \]

The q parameter was later modified to accommodate free detergent concentration of a low amphiphilic sample, especially where the total lipid and detergent concentration is below 5% w/v [59]. For DMPC/DHPC bicelles with lipid concentrations of 3-40% w/v, in aqueous solutions, and \( q > 2.3 \), spontaneously align above the DMPC gel-to-liquid transition temperature (typically within 30° to 50°C) [60]. Bicelles are oriented with their bilayer normals perpendicular to the direction of the applied magnetic field. This orientation can be flipped parallel by doping bicelles with paramagnetic ions, such as lanthanides Eu³⁺, Er³⁺, Tm³⁺, and Yb³⁺ [61], aromatic molecules [62] and even by incorporating membrane proteins, such as gramicidin A [63]. This flip could often result in an improved spectrum quality for bilayer constituents even in the absence of its rapid rotation around bilayer normal, and therefore is
particularly useful in determining $^{15}$N-labeled protein amide bond orientations by solid-state NMR. At smaller $q$ ratios of ~ 0.25 - 0.5, bicelles are typically “lipid-poor” and “detergent-rich”, exhibiting rapid isotropic tumbling ideal for solution NMR. However, their hydrophobic cores are quite similar to that of a lipid-detergent mixed micelle. At $q > 0.6$, assemblies are most often too large to yield high resolution spectrum, though they do possess fully segregated lipid cores [64]. A number of well-defined oligomers, composed of TM helices, has been determined in isotropic bicelles with $q$ ranging between 0.5 and 0.6. They include structures with pdb: 5JYN [65], 6E8W for HIV gp41 TM trimers [66], 2NA7 (human), 2NA6 (mouse) for TM trimers of Fas/CD95 death receptor [67], and 6NHY (mutant) or 6NHW (wild type) for trimer or dimer of trimers, respectively, of DR5 [68]. A detailed protocol for samples preparation at these conditions and suggested set of experiments to access oligomerization and TM partitioning has been published recently by Chou’s research group [69]. The authors particularly emphasize the utilization of PRE (Paramagnetic Relaxation Enhancement) experiments and OG-label method, they introduced, for these studies [70]. This approach was used to confirm the unusual hexameric structure of HCV p7 channel originally determined in DPC micelles (pdbid: 2M6X) [71] and recently challenged by Oestringer et. al [72]. At $q < 0.25$, ideally mixed micelles are formed with no observable lipid segregation.

The ability of diluted (< 5% w/v) bicelles to orient in magnetic field was later proven useful by Tjandra and Bax [73] to generate an alignment medium for measurement of residual dipolar coupling (RDC), an additional resource for distance restraints in determining NMR structures.

### III.d Amphipols

Detergents remain the current gold standard for solution NMR studies, but as indicated in the micelle section, they render IMP-ligand interaction studies untenable. Amphiphilic polymers, with a backbone of alternating hydrophilic and hydrophobic chains, represent an alternative solution to micellar system. Pioneered by Popot’s research group [74, 75], amphipols (APols) have shown the ability to extract and stabilize IMPs in the absence of conventional detergents or denaturants. Amphipol’s applicability to solution NMR remains challenging, with no available representation in the PDB database, thus far. A potential explanation may reside in a heterogeneous local environment produced by the interaction of amphipols with residues from the transmembrane region, as it’s difficult to control the exact sequence of alternating chains within the polymer. The resonance frequencies may reflect this diversity, due to the extreme sensitivity of NMR to local environment, and might be averaged for the same atoms from different molecules, resulting in peak broadenings and reduced spectrum quality. Moreover, polyacrylate based amphipols, like A8-35, are sensitive to pH <7 and multivalent cations [76]. PMAL polymers [77] containing a sequential pair of alternating hydrophobic and hydrophilic groups might ameliorate the shortcomings of APols. This was clearly demonstrated through higher thermal stability for NavM sodium channels [78]. IMPs extracted by amphipols, however, can be transferred to lipidic mesophase, as shown for bacteriorhodopsin (pdb: 4OVO), resolved at 2.0 Å by X-ray crystallography [79]. Amphipols do seem suitable for single particle electron microscopy. A 19 Å 3D map of the 1.7-MDa amphipol-solubilized super-complex I(1)II(2)IV(1) (2YBB) from bovine heart, obtained by cryo-EM, revealed an amphipol belt replacing the membrane lipid bilayer [80]. More recently, a number of amphipol stabilized TRP
channels (pdb: 6BO9, 5YDZ, 5YE1) and PMAL stabilized polycystin 2-l1 (6DU8) have been characterized by cryo-EM at high resolutions.

**II. e Nanodiscs**

Nanodiscs, a detergent-free flat discoidal system, developed by Sligar’s group, contains a phospholipid bilayer encased within two anti-parallel amphipathic helical Membrane Scaffold Protein (MSP) (Fig. 4) [81-84]. Nanodiscs are derived from Apolipoprotein A-1 (apoA1), originally involved in cholesterol transport. In the absence of cholesterol, apoA1 forms nascent discoidal particles containing phospholipids. Upon cholesterol ingestion, the proteolipid transforms to a spherical shape, eventually ending up in the liver [85]. Full length apoA1 is a helical protein with an N-terminal four helix bundle and two C-terminal helices (2A01). A 43 amino acid truncation at the N-terminus results in a circular belt like structure made of amphipathic helices (1AV1) [86]. This deletion, containing ten alpha helical repeats is referred to as MSP1. A larger construct, designed with two MSP1 molecules connected through a stable linker, is termed MSP2. MSP1, MSP2 [82] and other variants are broadly classified as MSPs. The diameter of the disc is determined by the number of helical repeats in the annular MSP. A bilayer of desirable thickness can be achieved by using multifarious lipids like DMPC (dimyristoyl-phosphocholine), DMPG (dimyristoyl-phosphorylglycerol), POPC (palmitoyl-oleoyl-phosphocholine), DPPC (dipalmitoyl-phosphocholine), or lipid mixture like E. coli lipid extracts, etc. [87]. Lipid molecules varying in their acyl chain length provide bilayers with altered thickness (Fig. 4) which may be paramount to IMPs that either diverge in the expanse of the bilayer or incur transmembrane reorientations as a result of activation. This nascent promiscuousness of the system for incorporated lipids offers a wide diversity of customizable discs gratifying stability requirements for different membrane proteins. Altogether, nanodiscs provide a competitive edge over other systems for studying IMPs due to their soluble nature, ease of concentration, monodispersity, temperature stability, and compatibility with cell free expression system.

The prototypical MSP construct, MSP1D1 [88], with ten amphipathic helical repeats, forms discs of ~10 nm diameter with an overall MW ~150 kDa. Variation in the number of helical repeats within the MSP1D1 construct led to the development of nanodiscs with different diameters. Solution NMR applications are amenable to an upper limit of ~100 kDa. Consequently, redesigning nanodisc to obtain smaller sizes was an obvious alternative for application of nanodiscs in solution NMR. Wagner’s group removed three intermediate helices (helices 4-6), either individually or together, based on the rationale of helical insertion for larger MSP constructs like (MSP1E3D1) while trying to maintain terminal interactions that may potentially stabilize the discs. The smallest disc (ΔH4-H6,) ~ 6.4 nm in diameter was kinetically unstable, forming larger aggregates of ~11 nm over time. The next smaller disc (ΔH4-H5 construct), was stable overtime with a diameter of 7.3 nm with an overall MW of ~70 kDa (Fig. 4) [89]. Concurrently, we also independently developed smaller discs by sequential removal of three terminal helices, ΔH8-H10. Our approach additionally confirmed the irrelevance of terminal interactions as inconsequential for nanodisc formation. Our smallest disc, called D7 containing the first seven helices (Fig. 4), is 7 nm in diameter and ~62 kDa [90]. Both ours and Wagner’s efforts independently demonstrated the applicability of small discs for NMR studies. Additionally, we also developed an alternative “on-column” method for nanodisc reconstitution on chromatographic resins. From
a structural perspective, it is important to note that though Wagner’s $\Delta H_{4H5}$ yielded the smallest stable disc, the $\Delta H_{5}$ construct, with a slightly larger diameter of about 8.4 nm (Fig. 4), provided the best NMR spectrum and was selected for deducing the structure of OmpX (pdb: 2M06). We find that identifying the oligomeric state of a $\beta$-barrel porin in a nanodisc may be important before pursual of NMR studies. Image averaging of negatively stained TEM images serve as a good visualization tool for identifying oligomeric states [91].

Larger discs, necessary for incorporation of larger integral membrane complexes, have been investigated as well. Sligar’s lab fused two MSP1 molecules together into a single construct to yield ~17nm discs [92]. A separate approach for generating larger nanodisks was demonstrated by Opella’s group [93]. Multiple copies of an amphipathic 14-residue peptide, mixed with phospholipids at a predefined ratio, produced MACRODISC of ~ 30 nm diameter. The increased diameter generated sufficient magnetic susceptibility anisotropy to overcome tumbling averaging motions allowing them to align in an external magnetic field. Similar to anisotropic bicelles, macrodiscs can be used as an alignment media for RDC measurements of soluble macro-molecules [94].

A notable modification of the nanodisc system has recently come from Wagner’s group [95]. Discs of controlled diameters (9, 11, 30 or 50 nm) were produced using sortase A based system that recognizes a consensus LPGTG sequence near the C-terminus and a single Gly residue at the N-terminus of MSP. These covalently circularized nanodiscs (cNDs) exhibit enhanced stability, defined diameter sizes and tunable shapes. Overall the improvements in cNDs are manifested through better NMR spectral quality for two tested IMPs, VDAC-1, a $\beta$-barrel membrane protein, and GPCR, NTR1.

A crucial parameter to consider in lipidic systems is the bilayer thickness. An optimum lipid alkyl chain length should be selected to accommodate the entire span of an IMP’s hydrophobic region. Mismatches, potentially occurring when the protein’s hydrophobic thickness is less or greater than that of the lipid bilayer, may cause curvature and/or disorder of the bilayer near the protein’s core surface [96]. In few certain cases, this condition may seem favorable, for example folding study on $\beta$-barrel proteins [97]. But most often, the functional states of channels or receptors, as well as enzymes activity, will be altered due to conformational distortions associated with membrane mismatch which can be determined by functional assays [98]. The anticipated thickness for a protein of interest should be experimentally explored by testing various lipid compositions. A complex lipid composition is often essential to mirror natural bilayer properties and maintain the functional activity of a particular IMP [99]. Nanodisc’s resilience towards the inclusion of a cornucopia of lipids, divergent in their alkyl chain lengths, degree of saturation, polarity of head group, etc., is a prominent advantage of this system.

III.f Saposin A discs

Two Saposin A proteins coalesce together around lipid molecules forming a nanoscale saposin A (SapA) disc named “Salipro®”. SapA disc differ from nanodisc in two general ways: (a) SapA proteins form a discontinuous belt around the lipid bilayer, and (b) the diameter of SapA disc is dynamic and unlike the need for MSP of varying length, multiple SapA proteins accommodate IMPs of varying sizes. The apo (lipid/detergent free) form of SapA has a closed architecture that becomes extended upon binding to LDAO molecules [100]. Individual detergent bound SapA proteins are brought together through the hydrophobic interaction of core acyl chains (Fig. 5A). LDAO
containing SapA disc is ~3.2 nm and contains 40 detergent molecules in total, arranged asymmetrically with 24 molecules in the upper and 16 in the lower leaflet of the bilayer. Empty SapA discs have a significantly smaller MW of 43 kDa, with respect to nanodiscs. Similar to nanodisc, SapA discs are also compatible with a variety of lipid molecules and is flexible enough to accommodate large membrane protein complexes with variable molecular weights, as demonstrated by cryo-EM structures of archaeal mechanosensitive channel T2 (32.9 kDa), with four predicted transmembrane helices forming as a putative homo pentamer, and bacterial peptide transporter PepT\(_{502}\) (56 kDa), with 14 transmembrane helices forming a homo tetramer [101].

Theoretically, there are certain advantages off employing SapA discs in solution NMR. SapA discs are compatible over a wide range of pH [100]. It has exceptional stability, withstanding several freeze-thaw cycles, and demonstrate high thermostability (0-95°C), allowing for NMR experiments, to be conducted at higher temperatures as is desirable for larger systems rendering better spectral quality. Lastly, protein incorporated discs are homogeneous as confirmed by negatively stained TEM images. However, the system does bring along a few caveats: (a) the propensity of the reconstituted IMP for non-native spontaneous oligomerization; and (b) a potential for spurious interaction between the IMP and SapA, especially since the lipid content around the reconstituted IMP is “very tight”. Though the system is not without its challenges, SapA discs do offer a viable alternative to nanodiscs. As of 2018, there are no protein structures available in SapA discs. The \(^{15}\)N-HSQC spectrum of OmpX, incorporated in SapA disc [102], was found similar to the spectrum of OmpX in \(\Delta 5\)H-nanodisc (for which the structure has been determined by solution NMR [89]). The spectrum of SapA incorporated phototaxis receptor sensory rhodopsin II (pSRII, 24.6 kDa, potentially a homodimer in this setting), was compared to the one in c7-DHPC micelles and appeared to be more heterogeneous and less resolved. Additionally, the functionality of SapA incorporated \(\beta_1\)-adrenergic receptor (\(\beta_1\)AR, 36 kDa, a GPCR) was also tested and confirmed by NMR [102].

### III.g Co-polymer discs

SMALPs, branded Lipodisq®, are discoidal lipid-polymer aggregates, where the outer annulus of the lipid bilayer is formed by Styrene-Maleic acid (SMA) copolymer composed of Styrene and Maleic acid in ratios of 2:1 or 3:1 [103]. These discoidal particles assemble spontaneously in aqueous solution and require very low concentration of SMA, as reflected by extremely low molar ratios of lipid to polymer, forming a disc with 140 lipids molecules and a median diameter of ~ 9 nm (Fig. 5B). SMALP has been used to form bilayer discs with POPC [104] and DMPC [105]. Interestingly, SMA copolymer can directly solubilize membrane proteins from its native membrane, isolating patches of flat bilayers with endogenous lipids surrounding the IMP. The mitochondrial respiratory Complex IV was successfully isolated from its native membrane using SMA which retained its enzymatic activity [106]. The presence of a lighter copolymer belt, in comparison to amphipathic protein in nanodiscs, results in an overall lower MW assembly and might offer favorable relaxation advantages for detection using NMR. However, as of writing this review, no structures of IMPs in SMALPs are available. Since SMA has a pKa of 6.5, NMR experiments involving SMALPs need to be performed within a narrow pH range of 6.8-7.5. Additionally, divalent cations destabilize SMA copolymer, causing their dissociation, and hence should be avoided. Also, the major hydrophobic moiety of the polymer, styrene, is purported to
nonspecifically interact with the aromatic side-chains of proteins and has a propensity to absorb in the UV range which could impede accurate analysis by various biophysical techniques. We believe, a major drawback of SMALPs, akin to amphipols, is its sample heterogeneity as observed by TEM [105], which should translate into an NMR spectrum with compromised quality.

However, there have been successful attempts recently to enhance the stability and homogeneity of lipid-polymer discs by the modification of functional units and/or by exploring other types of styrene based or styrene free polymers. Styrene Maleimide Quarternary Ammonium (SMA-QA), derivatization resulted in an improved stability at low pH and discs with controlled size [107]. One could envision the use of such polymer-based lipid-discs in solution NMR or as macro-nanodiscs in solid-state NMR applications. In addition to their use as an alignment media, these discs are tunable in their size and resistant to presence of divalent metal ions [107, 108]. Styrene free polymer disc with a polymethacrylate frame work [107] has also been designed as a cheaper alternative to SMA. These are amphiphilic and can easily be derivatized with a variety of side chains. The charge on the polymer plays a vital role in the functional reconstitution of membrane proteins: a high charge density around disc’s could produce unexpected and undesirable interactions [109]. Polyacrylic acid (PAA), with systematically varied hydrophobic groups through a robust functionalization method, was utilized to probe how alkyl-PAA affects the formation, stability, and other discs properties [110]. It was indeed confirmed that the choice of hydrophobic group can have a noticeable effect on the polymer solubilization properties. Another aliphatic copolymer with alternating diisobutylene/maleic acid, DIBMA, was recently shown to form nano scale discs [111]. DIBMALPs, are compatible with both long and short chain phospholipids, unfazed by the presence of cations and do not absorb at 280nm. Though the lack of potentially interfering hydrophobic groups make them an attractive alternative, DIBMALPs form slightly larger, heterogeneous discs than SMALPs. Further rigorous experimentation will determine the merit of the application of copolymer systems with NMR.

**III.h Peptidiscs:**

Amphipathic peptides have been recently used for “lipid free” IMP reconstitution forming “peptidisc” [112]. Watson and colleagues have shown that bi-helical peptides wrap around the hydrophobic parts of detergent purified IMPs, eventually displacing detergent molecules (Fig. 5C). Since being a lipid-free reconstitution, we are inclined to believe that the replacement of detergents by peptides should result in their close association to IMP and thereby become an obvious concern for a direct interaction between the two components. Future studies comparing NMR spectra of a known protein in detergent micelle and peptidisc should address this query. Peptidisc can be reconstituted in solution using a mixture of peptides and IMPs or directly on a chromatographic resin bound to target IMP. Reconstitution on resin provides a single step enrichment of the reconstituted protein similar to our on-column method, developed for nanodiscs [90]. Being a fairly new system, it remains to be seen whether their stability and homogeneity will allow for high resolution structure determination of reconstituted IMPs.

**IV) Structural Gallery**

Although there is an increase in the overall number of IMP structures determined by solution NMR, it remains significantly lower than X-ray crystallography. Here we present a
comprehensive overview of the structures in PDB as of mid-2019 (Fig. 6). These are predominantly smaller IMPs with one to two trans-membrane α-helices, although several porins composed of membrane-spanning β-barrels have been defined as well. Additional strategies for resonance assignments, which include trypsinization and use of synthetic peptides, have shown promising potential towards structural characterization of β-barrels such as Opa60 [113] with long flexible extracellular loops.

The majority of IMP structures have been determined in micelles, followed by isotropic bicelles. Nanodiscs, in our opinion, are yet to become a true choice of a mimetic with only structures of two outer membrane porins and a monomeric BclxL TMD helix (pdb: 6F46) available thus far. The first structure of a β-barrel protein, E. coli OmpX (pdb: 2M06), solved in nanodiscs, was obtained using small discs formed by the ΔH5 MSP deletion construct [89]. A later study, using RDC measurements of OmpX in nanodiscs with Pf1 phage as an alignment medium (pdb: 2MNH), helped refine the structure of the classical eight-stranded β-barrel fold with better defined orientation of N-H bonds [114]. A second structure of the Yersinia pestis outer membrane protein Ail (pdb: 5VJ8) in the ΔH5 discs composed of DMPC/DMPG mixture, was achieved by solid-state NMR data acquired with membranes containing lipopolysaccharide (LPS) [115]. The membrane composition had a marked effect on protein dynamics, with LPS enhancing conformational order and slowing down the 15N transverse relaxation rate.

Two dimeric conformations, one of F0-F1 ATPase subunit C (pdb: 1A91/1COV) and major platelet integrin αIIbβ3 transmembrane-cytoplasmic heterodimer (pdb: 2KNC), may be considered as reliable representatives of IMPs in organic solvent mixtures. Other membrane mimetics (amphipols, Salipro, and SMALPs), discussed in this review, are yet to prove their applicability towards structural studies using solution NMR.

V) Mechanistic Studies

HSQC chemical shift perturbations (CSP), observed upon addition of potential ligands, is generally the core experiment to study binding. The utility of nanodisc for monitoring resonance frequencies (or chemical shifts) of two membrane proteins was demonstrated early on through: (i) CD4 mutant, containing a single transmembrane and cytoplasmic tail, where the aliphatic 1H-13C HSQC chemical shifts were compared to ones in DPC micelles [116]; and (ii) VDAC-1, human anion channel protein, where 1H-15N TROSY HSQC spectra in LDAO isotropic bicelles and MSP1D1 nanodiscs were compared in the presence and absence of its native ligand NADH [117], later followed by a similar investigation of VDAC-2 [118]. Quite expectedly, spectra in nanodiscs show broader peaks than in micelles. Most peaks overlap, though some demonstrate differences in chemical shifts reflecting the variations arising from a micellar to a bilayer packing. Other examples of HSQC-based IMP studies in nanodiscs include: (i) the voltage sensing domain (4TM) of KvAP channel, which was characterized and shown to maintain a proper conformation only in a zwitterionic environment [119]; (ii) effect of different membrane mimetics on the structure of bacteriorhodopsin (7TM) [120]; and (iii) importance of lipid deuteration shown for YgaP (2TM and a cytoplasmic Rhodanese domain) in d54-DMPC containing MSP1D1 nanodiscs [121].

A 22 residue long amphipathic peptide nanodisc was used to study protein-protein interactions within the 70 kDa cytochrome P450 complex (CYP2B4-Cyto b5), defining the binding
surface of Cytb₅ [122]. NMR data-driven model of GTPase KRas-GDP, tethered to a lipid-bilayer nanodisc (pdb: 2MSC/2MSD/2MSE), was published in 2015 [123] and later refined in 2018 (pdb: 6CC9/6CCX/6CCH). In this combined computational approach, support was derived from experimental distance restraints obtained from paramagnetic relaxation enhancement (PRE) and additional NOE experiments in the refined version. HADDOCK [124] simulations allowed to distinguish the reorientation of the effector binding site on K-RAS4B GTPase anchored to (MSP1D1) nanodisc previously occluded by the anionic membrane.

Lipid bilayer interactions of the substrate-binding hemopexin-like (HPX) domain of membrane type-1 matrix metalloproteinase (MT1-MMP) were investigated in MSP1D1 nanodiscs by PRE experiments, fluorescence, and mutagenesis, followed by docking and MD simulations [125]. They helped define the insertion mode of HPX, which appears to happen through the blades II (pdb: 6CM1) and IV (pdb: 6CLZ) from the opposite sites of its β-propeller fold.

It’s our contention that real-time in-vitro monitoring of functional activity of IMPs by solution NMR methods should be further explored in nanodiscs. The accessibility of either sides of a receptor, surrounded by a lipid membrane could enhance the interaction of a potential ligand(s). Phosphorylation, a classic example for cell surface receptor activation, can be monitored by NMR. A proof of concept demonstration of phosphorylation has been reported in nanodiscs under tightly controlled conditions for neurotensin receptor 1, a GPCR class member, through the addition of G protein-coupled receptor kinases [126]. We also provided the first demonstration (Fig. 7) of in-vitro phosphorylation by mixing SRC-kinase and nanodiscs containing the transmembrane and cytoplasmic domains (TMCD) of recombinant integrin β₃ subunit in an NMR tube [127].

Attempts to incorporate individually purified IMPs into a nanodisc while forming a heteromeric complex bring to light problems associated with proper stoichiometry and relative mutual orientation. Wagner and colleges demonstrate a generalized approach for co-reconstitution of a membrane protein-DNA adduct where complementary DNA strands attached to different protein initiate oligomerization [128]. Fluorescently labeled VDAC was used to demonstrate the formation of heterodimeric vs heterotrimeric complexes.

Since GPCRs constitute an essential subclass of cell surface receptors and serve as potential drug targets for intervention in various diseases it is important to understand its structure-activity relationships in native environments. It is also well recognized that GPCRs are flexible and highly dynamic receptors that can adopt various shapes and oligomeric states. From the perspective of drug development, an investigation on how ligand binding affects GPCR’s overall motility and the dynamics of its side-chains under the conditions mostly closely mimicking native membrane environment is warranted. Solution NMR is uniquely capable of providing this type of information even for very large macromolecular complexes with the extensive use of ¹³C-Methyl-TROSY-based spectroscopy on perdeuterated samples [129]. However, specific ¹³C labeling of aliphatic methyl groups in deuterated proteins has been primarily limited to recombinant proteins overexpressed in E. coli. Many of the most interesting targets, including several GPCRs, require expression and purification of the receptors from mammalian cells to ensure proper folding and post-translational modifications. And this calls for novel schemes of effective isotopic labeling in eukaryotes. Clark and co-workers from Dr. Rosenbaum and
Dr. Gardner labs have demonstrated the feasibility of efficient $^{13}$C isoleucine δ1-methyl labeling in a deuterated background in *Pichia pastoris*. This was first demonstrated for maltose binding protein (MBP), as a proof of concept, and compared with the recombinant protein overexpressed in *E.coli*. The authors have also show that this method can be used to label eukaryotic proteins, such as actin. [130]. They later extended their approach to wild-type human A$_{2a}$R GPCR, resolving 20 out of 29 expected peaks in the Ile δ1 region of $^{13}$C-TROSY spectrum [131]. Their data further indicate that low Na$^+$ concentration is necessary to allow large agonist-induced structural changes, and how the pattern of side-chain dynamics is quite different between agonist and inverse agonist bound receptors, with the inverse agonist suppressing fast ps-ns timescale motions at the G proteins binding site. On a separate note, with the help of a thermostabilized mutant of the turkey β$_1$-adrenergic receptor (β$_1$AR), $^{15}$N-labelled at valine residues, Grzesiek’s research group has shown that GPCR motions can be followed at virtually any backbone site as well [132]. They also provide a thorough protocol for cost-effective isotopic labeling of proteins expressed in insect cells, using β$_1$AR and CCR5 as examples [133]. Altogether these impressive studies illuminate the unique capabilities of solution NMR for studying dynamics that enable a better understanding of GPCR’s functionality.

VI) Hybrid Techniques:

A combination of different experimental techniques, coupling their individual advantages, has proved beneficial for structural studied of IMPs. Park and colleges have explored membrane-bound form of the major coat protein of Pf1 bacteriophage (pdb: 2KSJ) through orientation restraints derived both from solid-state and solution NMR experiments [134]. The spectra were obtained in glass-aligned planar lipid bilayers, magnetically aligned bicelles, and isotropic bicelles. Isolated resonances only from the mobile N-terminal helix were observed in the solution NMR spectra, whereas resonances exclusive to the immobile transmembrane helix were observed in solid-state $^1$H/$^{15}$N-separated spectra in magnetically aligned bicelles. Thus using a combination of techniques the dynamic properties of Pf1 were addressed, allowing for a mechanistic view of the protein’s rearrangement during bacteriophage assembly. Veglia’s research group also used a similar approach to study phospholamban, in monomeric (pdb: 2KB7) [135], pentameric non-phosphorylated (pdb: 2KYV) [136], and serine-phosphorylated (pdb: 2M3B) [137] forms, outlining a detailed protocol to determine structure, topology, and depth of insertion of membrane proteins using a hybrid, solution and solid-state NMR restraints [138].

VII) Structural Discrepancies

Application of different biophysical techniques to structure determination of an IMP may result in inconsistencies, especially when different membrane mimetics are used for the study [139]. One should exercise extreme caution analyzing functional consequences of these structural deviations, taking into account known limitations for each method. Validating structural data in question under native lipid environment becomes imperative, even though high resolution structure determination under such condition might be impractical. In Figure 8 we present examples of few such cases.

Diacyl Glycerol Kinase (DAGK), has been studied by solution NMR in DPC micelles [140] (pdbid: 2KDC), X-ray crystallography in lipidic cubic phase [141] (pdbid: 3ZE3, 3ZE4, 3ZE5), oriented sample solid state NMR in liquid...
crystalline lipid bilayer [45] and magic angle spinning (MAS) solid-state NMR in native *E. coli* lipid membrane [142]. While all the methods agree on a trimeric architecture composed of three TM (plus one amphipathic) helices from each monomer, their arrangements, secondary elements and conformations are quite different. Two major differences between NMR and X-ray structures (Figure 8A) that stand out are: i) in solution NMR model helices 1 and 3 are domain-swapped, such that these helices primarily interact with helices from different monomers, and ii) all TM helices have an outward curvature, producing a barrel shaped structure. This significantly deviates from strictly cylindrical arrangement obtained from X-ray and might illustrate a potential artifact arising from detergent micelles. The unusual domains swapping, on the other hand, might reflect problems with structure calculation due to the dynamic state of the system and lack of multiple alignments followed by over-interpretation of PRE and RDC data. Low resolution solid-state NMR data does not support either model, but is more consistent with X-ray structure in terms of secondary elements and topology.

Phospholamban (PNL), a homopentamer expressed in sarcoplasmic reticulum that controls intracellular Ca$^{2+}$ levels through its phosphorylation state, is another example where solution NMR structure determined in DPC micelles [143] (pdbid: 1ZLL) differs significantly from the one obtained in lipid bilayer by a combination of solution and solid state NMR methods [136] (pdbid: 2KYV). The “bellflower” model originally proposed, (Figure 8B) with amphipathic helices of cytoplasmic domain sticking out into the cytoplasm, could reflect the influence of dynamic micellar environment combined with the effect of artificially introduced surface curvature. This conformation was not observed in a flat lipid bilayer where amphipathic helices were found interacting with the surface, consistent with an overall L-shaped architecture. Importantly, the ion channel activity of PNL, conceptualized on the idea of a bellflower pore, has not been substantiated in later experiments and theoretical calculations under more natural lipid surrounding (for details see review [139]).

Another case is a model of p7 (Figure 8C), a hepatitis C virus (HCV) small membrane protein (pdbid: 2M6X), presented by Chou’s research group in DPC micelles [71]. This model suggests an unusual mode of hexameric assembly, where the individual p7 monomers interact not only with their immediate neighbors but also with farther ones, forming a sophisticated, funnel-like architecture. Zitzmann’s and Schell’s research groups challenged this model with the evidence from their own solution NMR and SEC-MALS (size exclusion chromatography - multi-angle light scattering) studies claiming that p7 is monomeric in DPC micelles [72, 144]. It is worthwhile to note that DPC to protein ratios are very different between the two studies, with the latter using 40 times higher excess ([DPC/p7] = 250 vs 10,000). It has been known that high detergent to protein ratios may cause small membrane proteins denaturation and miss-folding [145], hence proper caution should be taken during reconstitution. Chou’s lab recently confirmed the original hexameric arrangement of p7 in isotropic bicelles [70].

Bax’s research group investigated the HIV-1 gp41 viral coat protein in bicelles [146] (Figure 8D). They highlighted a deviation in gp41’s oligomeric state from the classical trimeric state as determined by multiple methods like, cryoEM (of full length gp160), X-ray crystallography and solution NMR (in isotropic bicelles) (pdbid: 5JYN, 6E8W) [65, 66]. Using a combination of various biophysical techniques, including measurements made under different alignment conditions for RDC.
restraints, PRE, AUC (analytical ultracentrifugation) and DEER (double electron-electron resonance) experiments, Chiliveri and co-workers found that gp41 TM domain is monomeric (pdbid: 6B3U), highly ordered and uninterrupted for a total length of 32 residues, extending well into the membrane proximal region. This contradicts the trimeric architecture of gp41 construct, containing membrane-proximal external region folded into three-folded cluster, from Chou’s lab. It is possible that the observed monomeric form represents a conformation that preludes trimerization. Nevertheless, it remains to be determined how constructs selection, reconstitution methods and/or lipid conditions factor into such observed differences.

Out of B-barrel membrane proteins, OmpX (Figure 8E) probably represents the best example, as it was extensively studied by solution NMR in DHPC [147, 148] (pdbid: 1Q9F) and DPC (pdbid: 2M07) [89] micelles, in nanodiscs (pdbid: 2M06, 2MNH), and by X-ray crystallography [149] (pdbid: 1QJ8). The most significant differences between micellar and lipid conditions were found at the B-strands edges, with nanodiscs structure having a few additional residues at the ends. It is best seen at the top of strands 1, 3, and 8 as well as at the bottom of strands 1, 2, 4, 5, and 8. Thus, as one may easily imagine, the stability at the edges of the barrel depends strongly upon the reconstitution environment. Varying length of lipids might accommodate residues of TM region differently. Functional assays in different mimetics should levy credence towards selecting the best mimetic for structural studies.

VIII) Conclusions and Future Directions:

In this review, we discuss technological developments that allowed for the investigation of membrane proteins using solution NMR techniques. We also analyze the advantage and challenges associated with different membrane mimetic systems utilized for studying IMPs. Of all the different newly emerging mimetics, it is interesting to note that there isn’t a single preferred mimetic for all IMPs. This strongly reflects on the complex architecture and dynamics of different IMPs. Though X-ray crystallography and, more recently, cryoEM demonstrate higher efficiency in determining structures of large macromolecular complexes, solution NMR remains the most compelling technique to investigate binding kinetics, conformational diversity and dynamic properties of membrane proteins, especially for small proteins recalcitrant to crystallization. In addition to attempts at delineating protein structure, understanding dynamic modalities of a protein that regulate its function could become the core strength of future NMR applications. Selective labeling and a combination of different experimental techniques will lead the future into answering specific questions regarding a protein’s modus operandi, taking advantage of both in-vitro and in-vivo applications of solution NMR. Altogether, we should be able to glean enough information to expedite novel strategies for therapeutic interventions of numerous diseases associated with IMP malfunctions.

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Footnotes:

MPs - membrane proteins, IMPs - integral (or intrinsic) MPs, TM - transmembrane, APols - amphipols, cNds - covalently circularized nanodiscs, SapA - saposin A discs, SMA - styrene-maleic acid, SMALPs - SMA lipid-polymer discs.
Figure 1. Classification of membrane proteins (MPs):
integral MP with a single TM helix (1), multi-pass TM helices (2), and β-barrel porin (3) that span both leaflets; monotopic amphipathic helix (4) that span a single leaflet; lipo-proteins (5, 6); and peripheral extrinsic proteins (7).
Figure 2. Micelles:

A) Spontaneous formation of a micelle from detergents or a mixed micelle with lipids/cholesterol. Also shown are IMPs (α-helical or β-barrel proteins) incorporated in micelles.

B) List of popular detergents used for IMPs in solution NMR.
Figure 3. Bicelles:

A) Diameters of spontaneously formed bicelles depend on q values, with higher numbers correlated with wider discs.

B) Most commonly used lipid, DMPC, which forms the bilayer and detergents, DHPC and CHAPSO that line the edges.

C) Spontaneously oriented bicelles with their bilayer normal perpendicular to the direction of the applied magnetic field; this orientation can be flipped parallel by doping bicelles with paramagnetic ions, including lanthanides.
Figure 4. Nanodiscs:

A) Visual representation of a nanodisc that is either empty or contains IMP. The outer belt protein MSP (green) while lipid molecules are colored by atom type with carbon (grey), oxygen (red), phosphorus (orange) and nitrogen (blue). Also, the approximate hydrocarbon thickness of nanodisc bilayers is shown for various phosphatidylcholines.

B) Schematic representation of the overall length and disc diameters for several MSP variants. Nanodiscs with smaller diameters (below 8.5 nm) are preferable for solution NMR studies. The largest “MACRODISC”, obtained from a 14 amino acid peptide, produces a disc of 30nm diameter and serves as an alignment medium.
Figure 5. Other potentially useful membrane mimetics:

A) Saposin-A in its detergent free form adopts a closed conformation that becomes extended when bound to detergent molecules (PDB ID: 4DDJ). Saposin-A lipoprotein disc, with a diameter of 3.2 nm, contains two Saposin-A proteins brought together by a lipid core.

B) Styrene-Maleic Acid Lipid Particle (SMALP) is shown where the synthetic Styrene-Maleic Acid copolymer forms discs by encapsulating lipid within its central cavity. SMA lipid discs or Lipodisq® have a diameter of 10nm. Other polymers shown to form lipid particles include methacrylate and diisobutylene-maleic acid (DIBMA).

C) Amphipathic peptides have been recently used for “lipid free” IMP reconstitution forming “peptidisc”: helical peptides wrap around the hydrophobic parts of detergent purified IMPs eventually displacing detergent molecules.
Figure 6. A comprehensive overview of solution NMR IMP structures in PDB as of June 2019.
Figure 7. Applicability of nanodisc systems for studying signaling pathways. The cytoplasmic tail of integrin β3 subunit is phosphorylated by Src kinase (in vitro) in an NMR tube: 15N labeled β3 incorporated discs were mixed with the kinase domain of Src kinase in the presence of ATP. Phosphorylation is manifested through chemical shift perturbations observed in the overlay of the 1H-15N-TROSY HSQC spectra obtained from the unphosphorylated (black) and bi-phosphorylated (red) β3 were collected on 600 MHz magnet. Also shown is the β3 tail sequence indicating phosphorylation sites at the two tyrosine residues.
Figure 8. An illustration with few examples of structural discrepancies.
Solution NMR: A powerful tool for structural and functional studies of membrane proteins in reconstituted environments
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