Review

Advances in NMR Studies of Membrane Protein Structure and Function

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Abstract: As the functional core of biological membranes, membrane proteins mediate nearly all critical cellular processes, and their dysfunction is closely associated with the onset and progression of various major diseases. However, the inherent hydrophobicity of transmembrane domains, dependence on native lipid microenvironments, and prominent structural dynamics of membrane proteins pose substantial challenges to their structural and functional research. Although X-ray crystallography and cryo-electron microscopy (cryo-EM) are mainstream structural biology techniques, they each have limitations in membrane protein studies. Nuclear magnetic resonance (NMR) spectroscopy, as a powerful complementary approach, enables atomic-resolution structural analysis of membrane proteins in selected native-like membrane environments or *in situ* membrane systems, and can sensitively characterize conformational dynamics across multiple timescales as well as intermolecular interactions, offering unique advantages in membrane protein research. This review focuses on the core advances of NMR technology in membrane protein studies, systematically summarizes the key membrane-mimetic systems adapted for NMR analysis and the methodological developments of solution NMR, solid-state NMR (including magic-angle spinning, oriented-sample NMR, and dynamic nuclear polarization-enhanced NMR), as well as *in situ*/intracellular NMR. It further elaborates on the application achievements of NMR in membrane protein structure determination, molecular interaction analysis, and conformational dynamics research, aiming to provide a comprehensive reference for the in-depth investigation of membrane protein structure and function, and the development of membrane protein-targeted drugs.

Keywords: NMR; membrane proteins; MAS ssNMR; OS-ssNMR; DNP NMR

1. Introduction

As the functional core of biological membranes, membrane proteins are extensively involved in critical cellular processes such as cell signal transduction, transmembrane transport, and energy conversion [1]. Their functional abnormalities are closely associated with the onset and progression of various major diseases, making them the targets of approximately 70% of drugs in modern pharmaceutical research and development [2,3]. Despite their irreplaceable significance in the fields of life sciences and medicine, membrane proteins have long encountered technical bottlenecks in in vitro recombinant purification and homogeneous sample preparation. This is attributed to their inherent characteristics, including the strong hydrophobicity of transmembrane domains, the dependence of native conformations on specific lipid microenvironments, and prominent structural heterogeneity and dynamic properties. Consequently, progress in investigating the structure and function of membrane proteins lags behind that of soluble proteins, rendering them a pressing challenge awaiting resolution in the field of life sciences.

The main techniques currently used in membrane protein structure research fall into three categories: X-ray crystallography is a traditional core method for resolving protein structures at near-atomic or even atomic resolution [4]. To obtain clear and high-quality diffraction signals, crystals must possess a certain size and regularity to meet the technical requirements of X-ray diffraction measurements [5]. Although this technique has been applied for decades and continuously optimized, it still has obvious limitations: the crystallization process of membrane proteins is time-consuming and labor-intensive, and it is difficult to obtain large and well-ordered

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crystals; prolonged X-ray radiation damages the crystal structure, thereby compromising data quality; additionally, it cannot directly visualize hydrogen atoms—whose precise localization is crucial for accurately constructing atomic models and analyzing protein hydration shells [6].

Cryo-electron microscopy (Cryo-EM) can resolve biomolecular complexes of different molecular weight ranges (from small proteins to large viruses), retaining the native structure of membrane proteins and distinguishing heterogeneous complexes in samples [7,8]. In recent years, this technique has undergone a "resolution revolution" with significantly improved resolution [9], but it is still slightly inferior to X-ray crystallography; meanwhile, it has high requirements for sample purity and preparation technology, and subsequent computational analysis of three-dimensional structures also requires substantial computing power support [6].

Nuclear magnetic resonance (NMR) spectroscopy can resolve the three-dimensional structure of membrane proteins at atomic resolution in native or near-native lipid bilayer environments. Unlike techniques that only present static structures, it is an effective tool for analyzing intrinsically disordered proteins—it can sensitively capture conformational dynamic changes of complexes, and explore protein dynamic characteristics through nuclear spin relaxation analysis [10]. Its advantages are reflected in: obtaining high-resolution structures in native conformations, providing information on conformational dynamics and flexibility, and being suitable for analysis in both solution and solid-state environments [6].

2. Expression Systems for NMR Studies of Membrane Proteins

Efficient heterologous expression of membrane proteins is a core prerequisite for their NMR structural and functional investigations, as high-resolution NMR spectroscopy relies on milligram-scale sample production, precise isotope labeling, and native conformation preservation—key guarantees for valid spectrum acquisition and dynamic characterization. Membrane protein overexpression commonly triggers host toxicity and protein misfolding, and to date, no single system can balance yield, labeling purity and functional integrity at the same time. Thus, an ideal system must reconcile expression efficiency, isotope labeling feasibility and native protein activity, providing high-quality samples for subsequent NMR analyses. Mainstream platforms include prokaryotic, eukaryotic and cell-free systems, with distinct pros and cons in NMR-related indicators.

Prokaryotic systems represented by *Escherichia coli* are one of the most widely used for membrane protein expression [11], featuring simple culture, low cost, fast growth and high adaptability [12]. Notably, this system enables efficient large-scale production and accurate isotope labeling (^{15}N, ^{13}C, ^{2}H) via isotope-enriched media or amino acid precursors [13], which is critical for solution NMR. However, *E. coli* possesses a distinct lipid composition and lacks eukaryotic molecular chaperones and post-translational modification (PTM) machinery, which may lead to misfolding or inclusion-body formation and thus restricting its application to membrane proteins with relatively simple structures.

Eukaryotic expression systems, encompassing both yeast and insect cell systems, are capable of providing PTM and a more native-like folding environment for complex eukaryotic membrane proteins. Yeast systems support scalable and cost-effective membrane protein production, enable ^{13}C methyl labeling and perdeuteration that meet NMR dynamics assay requirements, while suffering from inefficient folding and long induction cycles. Insect cell systems enable native-like folding and processing of membrane proteins, and recent studies have achieved site-specific isotope labeling of target amino acids under deuterated conditions [14], but are limited by high culture cost, slow growth and prohibitively expensive large-scale isotope enrichment, hindering routine NMR sample preparation [12,14].

Cell-free expression systems emerge as a promising alternative for NMR-oriented membrane protein preparation, avoiding host cytotoxicity caused by membrane protein overexpression with an open and controllable reaction environment [15]. More importantly, this system minimizes isotope scrambling by reducing amino acid metabolic interconversion, enabling flexible selective and site-specific labeling to simplify complex NMR spectra [15,16], making it highly suitable for recalcitrant membrane proteins that are hard to express in traditional cellular systems. In addition to conventional ^{15}N, ^{13}C, and ^{2}H labeling, ^{19}F labeling serves as an important complementary strategy in NMR studies of membrane proteins [17]. Owing to its 100% natural abundance, high gyromagnetic ratio, and absence of background signals in biological systems, ^{19}F offers unique advantages for investigating conformational changes and ligand-binding interactions of membrane proteins [17,18]. Cell-free expression systems provide flexible control over amino acid composition and effectively circumvent the cellular toxicity associated with fluorinated amino acid analogs, making them an important platform for achieving efficient ^{19}F labeling [19,20].

In summary, each expression system exhibits distinct advantages and limitations. The selection of a suitable expression strategy should be flexibly tailored to the structural complexity, modification requirements, and NMR labeling needs of the target membrane protein.

3. Overview of Methodological Development in Membrane Protein NMR

3.1. Membrane Mimetic

Solution NMR spectroscopy is a cornerstone tool in membrane protein structural biology, enabling atomic-resolution structure determination and characterization of dynamic processes. However, membrane proteins exhibit high hydrophobicity and are prone to aggregation and inactivation in conventional aqueous solutions. This compromises rapid isotropic molecular tumbling—a critical prerequisite for solution NMR to attenuate transverse relaxation decay and acquire high-resolution spectra. Thus, the development of appropriate membrane-mimetic systems is indispensable: such platforms must integrate a native-like lipid microenvironment with solution dispersibility, preserving the native structure and function of membrane proteins while ensuring sufficient NMR signal sensitivity and resolution. Currently, mainstream membrane-mimetic systems encompass detergent micelles, isotropic bicelles, nanodiscs, liposomes, styrene-maleic acid (SMA) copolymer lipid particles (SMALPs), reverse micelles, each with distinct structural and functional characteristics tailored to specific membrane protein classes and NMR applications (Table 1, Figure 1).

Table 1. Comparison of membrane-mimetic systems for NMR studies of membrane proteins.

Membrane Mimetic	Compatible NMR Modalities	Key Advantages	Limitations	Suitable Protein Classes
Micelles	Solution NMR	Easy preparation, high stability, well-established protocols	High curvature, limited bilayer mimicry, may destabilize protein folding	Small single-pass membrane proteins, peptides
Bicelles	Solution NMR, oriented-sample ssNMR	Bilayer-like environment, size-tunable, isotropic tumbling	Limited loading capacity, detergent present	Small to medium membrane proteins, TM domains
Nanodiscs	Solution NMR (small discs), ssNMR	Detergent-free, monodisperse, tunable lipid composition, high stability	Complex preparation, limited to small discs for solution NMR	β-barrel proteins, GPCRs, transporters
SMALPs	Solution NMR	Detergent-free extraction, preserves native lipids	Narrow pH range (~6.8–7.5), divalent cation sensitivity, sample heterogeneity	Integral membrane proteins, native lipid environment studies
Reverse micelles	Solution NMR	Low-viscosity solvent, no deuteration required, tunable size	Non-native environment, limited to specific surfactant systems	Membrane proteins in organic solvents, hydrophobic peptides
Liposomes	ssNMR	High biomimetic fidelity, tunable lipid composition, supports native conformation	Large size (slow tumbling), incompatible with solution NMR, labor-intensive preparation	Integral membrane proteins, α-helical membrane proteins

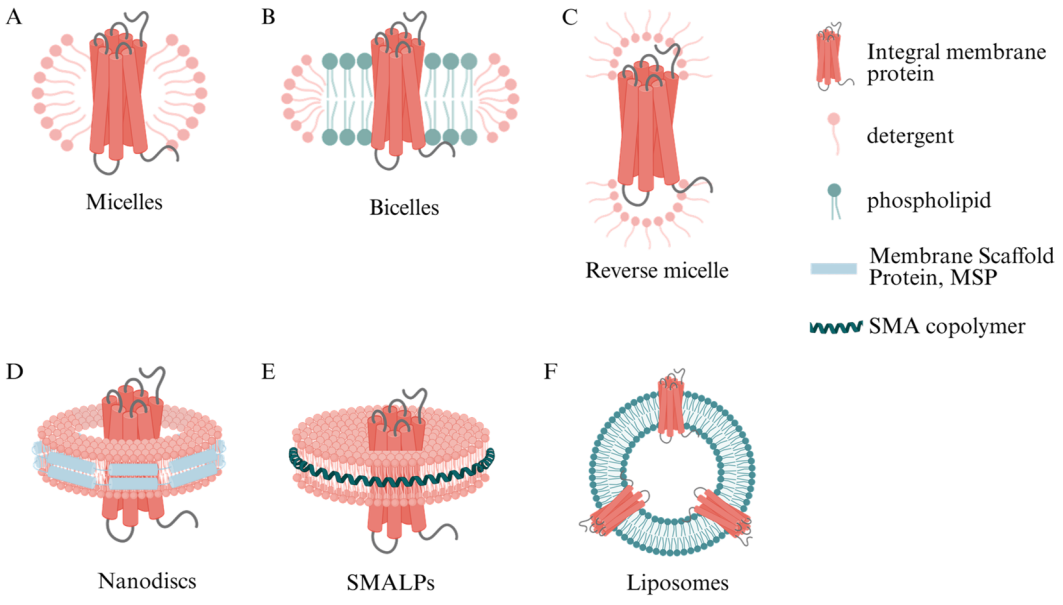


Figure 1. Membrane mimetic systems for NMR studies of membrane proteins. (**A**) Micelles: self-assembled monolayer of detergent molecules with hydrophobic tails inward and hydrophilic heads outward. (**B**) Bicelles: disc-like bilayer with a planar lipid region surrounded by detergent rim. (**C**) Reverse micelle: inverted structure with

hydrophilic heads inward. This system is commonly used in solution NMR to mimic the hydrophobic environment of membrane proteins but is less frequently applied in solid-state NMR. (**D**) Nanodiscs: lipid bilayer disc stabilized by two membrane scaffold proteins (light blue). (**E**) SMALPs (styrene-maleic acid lipid particles): lipid bilayer disc encircled by an SMA copolymer belt (dark green), which extracts native lipids together with the membrane protein. (**F**) Liposome: spherical closed vesicles composed of continuous phospholipid bilayers that encapsulate aqueous interior compartments. All systems aim to provide a native-like lipid environment for membrane protein structural determination by NMR.

3.1.1. Micelles

Micelles are single-layered aggregates self-assembled by amphiphilic molecules (detergents or short-chain lipids) when their concentration in aqueous solutions exceeds the critical micelle concentration (CMC). In this structure, the hydrophobic groups of the molecules aggregate inward to form a dense core, while the hydrophilic segments arrange outward to interact with the aqueous phase [21,22]. Common types include short-chain lipid micelles and detergent micelles. Although the hydrophobic core of lipid micelles is closer to the natural lipid environment, their size and stability are usually weaker than those of detergent micelles, so they are rarely used alone for membrane protein NMR. Detergent micelles are easy to prepare and have higher stability, making them the preferred membrane-mimetic system, which have been widely used for structural determination of small single-pass membrane proteins or peptides [14,23]. Researchers have verified the structural integrity of membrane proteins using various detergents (including *β*-D-octylpyranoglucoside (OG), *n*-dodecylphosphocholine (DPC), 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), etc.) [24]. However, detergent micelles are not ideal membrane-mimetic environments. In membrane protein research, the selection of detergents needs to be optimized according to the characteristics of the protein itself, and the specific scheme often needs to be determined through repeated experiments [25]. In addition, detergent micelles have limited supporting effect on membrane protein folding. Their monolayer structure and highly curved nature caused by small radius of curvature are significantly different from natural lipid bilayers, which may further induce misfolding or aggregation of proteins and peptides [26,27].

3.1.2. Bicelles

Distinct from micelles, bicelles represent a class of discoidal nanoscale bilayer assemblies that self-assemble in aqueous environments through the mixing of long-chain phospholipids capable of forming lipid bilayers with detergent molecules or short-chain phospholipids (e.g., DHPC) at a defined ratio [28,29]. The molar ratio of long-chain lipids to short-chain lipids is termed the q value, and a key advantage of bicelles lies in their size tunability via adjustment of this q parameter. Generally, when the q value is large, the bicelles are large and tumble slowly, which results in spontaneous orientation and anisotropic behavior in the magnetic field. At this time, the solution NMR spectrum peak is easy to split and widen, but it can be used for directional solid-state NMR (ssNMR) research [21]. When the q value is small (about 0.25–0.5), the bicelles are usually “lipid-poor and detergent-rich”, showing fast isotropic tumbling characteristics—that is, isotropic bicelles. They combine a bilayer-mimetic environment with solution-state NMR-friendly dynamic properties, making them very suitable for solution NMR research [23,30].

Through such small-sized isotropic bicelles, the solution structure and dynamic characteristics of a variety of membrane proteins have been successfully characterized, including HIV gp41 transmembrane trimers [31], the Fas/CD95 death receptor transmembrane trimer [32], the curved α-helical structure of the KCNE3 transmembrane domain [33], and EmrE [34]. The interaction between the SARS-CoV-2 E protein transmembrane domain and glycyrrhizic acid [35], as well as the conformational transition of EnHD upon membrane binding [36], have also been characterized. However, the diameter of isotropic bicelles is usually only 5–20 nm, and their spatial capacity is limited, making them unsuitable for NMR analysis of large-sized membrane protein complexes.

3.1.3. Nanodisc

Nanodiscs are a detergent-free, flat disc-shaped system assembled from phospholipids (e.g., DMPC) and an amphipathic α-helical membrane scaffold protein (MSP) [37], and owing to their excellent monodispersity, high stability, and precise tunability of lipid bilayer size and composition [38,39], they have become a widely used and high-quality membrane-mimetic system for solution NMR studies of membrane proteins in recent years [26]. Because conventional nanodiscs are too large to be directly used for solution NMR structural analysis of integral membrane proteins, researchers adjusted the molecular weight of the MSP and successfully constructed a small MSP nanodisc with a diameter of approximately 8.4 nm [40]. This nanodisc is large enough to accommodate a single membrane protein molecule and exhibits sufficiently rapid tumbling to meet the demands of high-resolution

solution NMR. Subsequently, the smallest nanodisc, named D7 (diameter: 7 nm; molecular weight: ~62 kDa), was developed and experimentally confirmed to be suitable for NMR studies [23]. Using nanodisc technology, the Wagner group achieved high-quality NMR spectra of the *E. coli* outer membrane β-barrel protein OmpX and further determined its atomic-resolution structure using nuclear Overhauser effect (NOE) restraints, obtaining the first high-resolution structure of a membrane protein within a nanodisc [40].

Although nanodiscs have significant advantages, they still have limitations: on the one hand, due to the minimum requirements for scaffold protein stability and lipid loading, it is extremely difficult to prepare smaller nanodiscs by further truncating MSP; on the other hand, conventional larger nanodiscs are difficult to use directly for solution NMR because of their slow tumbling. Therefore, the development of nanodiscs that provide an appropriate membrane environment while meeting the rapid tumbling requirements of solution NMR (whether through size optimization or other strategies) still requires further research, which depends on advanced sample preparation methods and NMR techniques.

3.1.4. Co-polymer Discs

SMALPs are disc-shaped aggregates composed of a lipid bilayer encapsulated by a styrene-maleic acid (SMA) copolymer (at ratios of 2:1 or 3:1). In aqueous solution, they spontaneously assemble into particles of ~10 nm in diameter containing about 140 lipid molecules, and their size can be controlled by adjusting the lipid-to-SMA ratio [26,41]. Although SMALPs are structurally similar to nanodiscs, they are not based on a protein-coating strategy; instead, they utilize SMA copolymers to encapsulate the lipid bilayer. [14]. Compared with traditional nanodiscs, SMALPs have a lighter polymer belt and lower overall molecular weight, which may provide better relaxation properties for NMR detection. These aggregates can extract integral membrane proteins from cells without detergent and retain some of the native membrane environment, thereby maintaining the structural and dynamic integrity of the membrane proteins [42].

However, the pKa of SMA is 6.5, and experiments need to be carried out within a narrow pH range (approximately 6.8–7.5), which is unfavorable for solution NMR applications [43]. At the same time, divalent cations destabilize SMA copolymers and cause their disassembly. TEM observations reveal heterogeneity in SMALPs samples, which can reduce NMR spectral quality. In addition, the strong ultraviolet absorbance of the styrene group can interfere with analysis [23,44]. To this end, the researchers used acetic anhydride, triethylamine, and sodium acetate to prepare maleimide via dehydration, and then synthesized styrene-maleimide quaternary ammonium salt (SMA-QA). This compound features a quaternary ammonium group as the hydrophilic moiety, which greatly improves its tolerance to pH variations and metal ions. NMR results show that small-sized SMA-QA nanodiscs exhibit fast tumbling characteristics, while large-sized ones can be magnetically aligned. Such improved polymer-lipid discs are expected to be useful for solution or ssNMR studies [45].

3.1.5. Reverse Micelles

Reverse micelles are a unique type of membrane mimetic system, which are water-in-oil molecular aggregates self-assembled from surfactant molecules. It consists of a nanoscale aqueous core encapsulated by a surfactant monolayer and dispersed in nonpolar hydrophobic solvents. The aqueous core can encapsulate membrane proteins and other biomolecules [46]. When dispersed in low-viscosity solvents, reverse micelles allow direct solution NMR investigation of membrane proteins without the need for protein deuteration or TROSY experiments, making them a unique and valuable tool in this research field. At the same time, reverse micelle systems are highly adjustable. Their particle size can be regulated by adjusting the hydration ratio W0, and the optimal composition can be identified by screening different surfactant types and ratios. Among them, the anionic surfactant bis(2-ethylhexyl)sulfosuccinate (AOT) is the most extensively studied reverse micelle system to date. It can form micelles without the addition of auxiliary surfactants or co-surfactants, and the procedures for sample preparation and pH pre-adjustment are both very straightforward [47].

The recently developed 10MAG (1-decanoyl-*rac*-glycerol)/LDAO (lauryldimethylamine-*N*-oxide) zwitterionic mixed reverse micellar system is prepared at a specific molar ratio with a small amount of *n*-hexanol. Slight adjustments to the formulation suffice for the encapsulation of the vast majority of proteins. This system allows precise control over the pH of the water core, achieves high structural fidelity of the encapsulated protein, and exhibits short molecular tumbling times, thereby effectively improving the applicability of reverse micelle technology [48].

A new type of membrane-mimetic reverse micelle (mmRM) system has recently been successfully developed and applied to protein NMR research. This system is formulated with a mixture of 1,2-dilauroyl-*sn*-glycero-3-phosphocholine and *n*-dodecylphosphocholine (DLPC:DPC), which stabilizes peripheral membrane proteins in

their native membrane-bound conformation. The prepared samples can remain stable for several months, providing a new technical strategy for long-term structural observation of membrane proteins [49].

3.1.6. Liposomes

Liposomes are spherical vesicular structures formed by the self-assembly of hydrated phospholipids, consisting of single or multiple lipid bilayers that enclose aqueous compartments. They serve as a classic biomimetic system for recapitulating native biological membranes [26]. Compared with membrane-mimetic systems such as micelles and bicelles, liposomes exhibit higher biomimetic fidelity in reproducing native membrane environments. Their membrane complexity can be further tailored by incorporating native lipids, including sterols and sphingomyelins, thereby supporting the proper folding and functional studies of membrane proteins. Liposomes are therefore a preferred carrier for studying membrane proteins by ssNMR spectroscopy [26].

In liposome systems, solid-state NMR has been widely employed to elucidate the structural features and dynamic properties of membrane proteins. For instance, high-resolution magic-angle spinning (HR-MAS) NMR studies on the KcsA potassium channel have revealed that its C-terminal domain undergoes a conformational transition from a rigid state to a highly dynamic state under acidic conditions. This work also achieved the first chemical shift assignment of the channel's C-terminal residues in a lipid-embedded state, providing structural insights into the mechanism underlying the pH-dependent tetramer stability of the channel [50]. Ong et al. reconstituted the multidrug efflux pump EmrE into DMPC liposomes. By means of DNP-enhanced solid-state NMR, which yielded a 19-fold signal enhancement, they directly confirmed the specific interaction between the substrate TPP^+ and the key residue E14 on the protein's transmembrane helix, providing atomic-level experimental evidence for the ligand-binding mechanism of multidrug transporters [51]. Despite its advantages, the liposome system has several technical limitations that restrict its broader application in NMR studies. The relatively large size of liposomes results in slow vesicle tumbling rates, rendering them incompatible with the fast molecular tumbling required for high-resolution solution-state NMR experiments. Furthermore, the preparation of liposome samples is labor-intensive and experimental conditions are difficult to optimize. The high fluidity of phospholipid molecules and the intrinsic heterogeneity of liposome size and curvature further complicate the interpretation of lipid-protein interactions [39].

Different membrane-mimetic systems have their own merits and drawbacks. Micelles are easy to handle but poor in native membrane simulation; bicelles are suitable for ssNMR but limited in loading capacity; nanodiscs provide excellent biomimetic environments yet require complicated preparation; SMALPs eliminate the need for detergents yet suffer from limited stability. Therefore, selecting the optimal membrane-mimetic system according to research objectives, protein properties and technical requirements constitutes the core direction for the methodological development of solution-state NMR in membrane protein research.

3.2. Solution NMR Techniques

In addition to providing resonance assignments and dynamics information, solution NMR can obtain atomic-level structural restraints through a variety of experimental approaches, thereby supporting high-resolution structure determination and interaction studies. Among these, chemical shift perturbation (CSP) offers a rapid means to map binding sites and quantify affinities, making it a primary tool for studying protein-ligand interactions. Meanwhile, Nuclear Overhauser Effect (NOE), Paramagnetic Relaxation Enhancement (PRE), and Residual Dipolar Coupling (RDC) provide complementary spatial restraints—covering short-range, long-range, and orientational constraints, respectively—that collectively constitute the core parameter set for membrane protein structure determination in solution

3.2.1. CSP

Chemical shift perturbation (CSP) is a core method for studying protein-ligand and protein-protein interactions in solution [52]. When a ligand or binding partner binds to the protein, the protein conformation and dynamic properties change, which causes changes in NMR signal frequency and relaxation behavior of the affected nuclei, ultimately manifested as shifts of specific spectral peaks [53]. CSP technology uses ^{15}N-labeled proteins and gradually titrates unlabeled ligands, collecting two-dimensional HSQC spectra at different ligand concentrations to track peak shifts [54]. With the aid of high-field NMR and cryogenic probes, a complete titration experiment can typically be completed within a single day. When combined with TROSY and fully deuterated proteins, this method can be extended to protein systems with molecular weights of several hundred kDa. [52,55].

By analysing the magnitude of spectral peak shifts, residues affected by binding can be identified, providing clues for potential binding interfaces, while fitting titration curves yields an apparent dissociation constant (Kd)

under appropriate experimental conditions. Thus, CSP is one of the few techniques that can simultaneously determine both binding sites and affinity [56]. This technology has now been extended to the study of protein interactions within living cells. However, it should be noted that signals in living cells are easily masked by interactions with other macromolecules, and the interpretation of chemical shift changes depends on the exchange regime between free and bound states [53].

In the study of the SARS-CoV-2 spike protein, researchers identified the binding sites of heparin and fondaparinux on the receptor-binding domain (RBD) through CSP and determined their affinities. They also confirmed that lifitegrast, a drug used to treat dry eye syndrome, binds to the highly conserved linoleic acid-binding pocket on the RBD [57]. Another study clarified, through CSP experiments, that the ABO(H) blood group antigen does not bind to the RBD, which provides a key basis for research into viral infection mechanisms [58].

3.2.2. Distance Restraints from NOE, PRE, and RDC

NOE, PRE, and RDC are key techniques for obtaining structural restraints between ligands and membrane proteins and for constructing binding modes. Among them, NOE and PRE provide distance information, while RDC provides orientational constraints that report on the angular relationships of internuclear vectors. They are essential for fragment-based drug design (FBDD) and are often used in combination, significantly enhancing the accuracy and reliability of membrane protein structure determination.

NOE provides fundamental distance restraints for membrane protein structure determination, directly reporting on spatial proximity between atoms and facilitating resonance assignment and structure calculation [37]. For instance, the high-resolution structure of pSRII, the first seven-transmembrane protein solved by solution-state NMR, was determined using long-range methyl NOEs combined with hydrogen bond restraints [59]. RDC provides unique orientation constraints, reflecting the overall angular relationship of internuclear vectors, which can effectively correct the structural deviation caused by insufficient local NOE constraints [14].

When the structural constraints provided by NOE and RDC are insufficient for the precise determination of protein structures, PRE serves as a valuable supplement, providing crucial long-range spatial information. By introducing paramagnetic spin labels at specific protein sites and taking advantage of the enhanced effect of unpaired electrons on the relaxation of surrounding nuclei, PRE can capture spatial proximity data up to 35 Å, making it particularly suitable for probing flexible regions within membrane proteins and low-abundance conformations [14]. In membrane protein-protein interactions, structural determination of proteorhodopsin has greatly improved the orientation accuracy of transmembrane helices via the combined use of PRE, NOE and RDC [60].

3.3. Solid-State NMR: Magic-Angle Spinning (MAS) and Oriented Samples

3.3.1. MAS NMR

Magic Angle Spinning (MAS) solid-state NMR (ssNMR) is a key method for resolving atomic-level information of membrane proteins. Its core principle is to spin the sample at a high speed at the 54.7° "magic angle", averaging out anisotropic nuclear spin interactions over a rotor period and yielding high-resolution spectra; meanwhile, a variety of supporting radiofrequency pulse sequences such as PDSD, TEDOR, and REDOR can respectively introduce homonuclear and heteronuclear interactions in a targeted manner, supporting homonuclear/heteronuclear correlation analysis and accurate distance measurement, thereby capturing key structural constraints such as distances and torsion angles [26,61].

To obtain high-resolution NMR structural information, efficient isotopic or spin labeling of membrane proteins is essential. Common labeling strategies include uniform labeling [31], selective labeling [62], site-directed spin labeling (SDSL) [32], and methyl labeling [63], among others (Figure 2).

Leveraging MAS NMR technology, researchers have accomplished large-scale resonance assignment of both backbone and sidechain resonances for the Voltage-Dependent Anion Channel (VDAC) reconstituted in DMPC lipid bilayers [64]. This achievement not only addresses the long-standing technical challenge of detecting loop-region residues in detergent-solubilized systems but also represents the first comprehensive acquisition of chemical shift data for the N-terminal 25 residues—critical for VDAC function. To overcome the dual limitations of insufficient resolution and sensitivity, MAS NMR technology has undergone continuous optimization. NMR signal-to-noise ratio (SNR) is directly related to the gyromagnetic ratio; ^{1}H has a much higher gyromagnetic ratio than ^{13}C and ^{15}N, providing an inherent sensitivity advantage [65]. Compared with traditional direct ^{13}C detection, indirect proton detection can enhance signal intensity by 3–5 fold under optimized conditions (e.g., high MAS frequencies and suitable deuteration schemes) [66]. Furthermore, strategies such as isotope dilution (including full or partial proton back-exchange) at moderate spinning speeds of 20–50 kHz [66], and increases in magnetic field strength up to 1.2 GHz [67] have further expanded the technical scope of application. For example, Weingarth et al. [68] studied the fully

protonated, membrane-embedded KcsA channel at 60 kHz MAS. They not only obtained a narrow ^{1}HN linewidth of 0.3–0.5 ppm and completed the first site-specific ^{1}H analysis of this protein family, but also captured conformational changes of the GYG motif, thereby offering a direct approach for investigating the link between buried water and protein function.

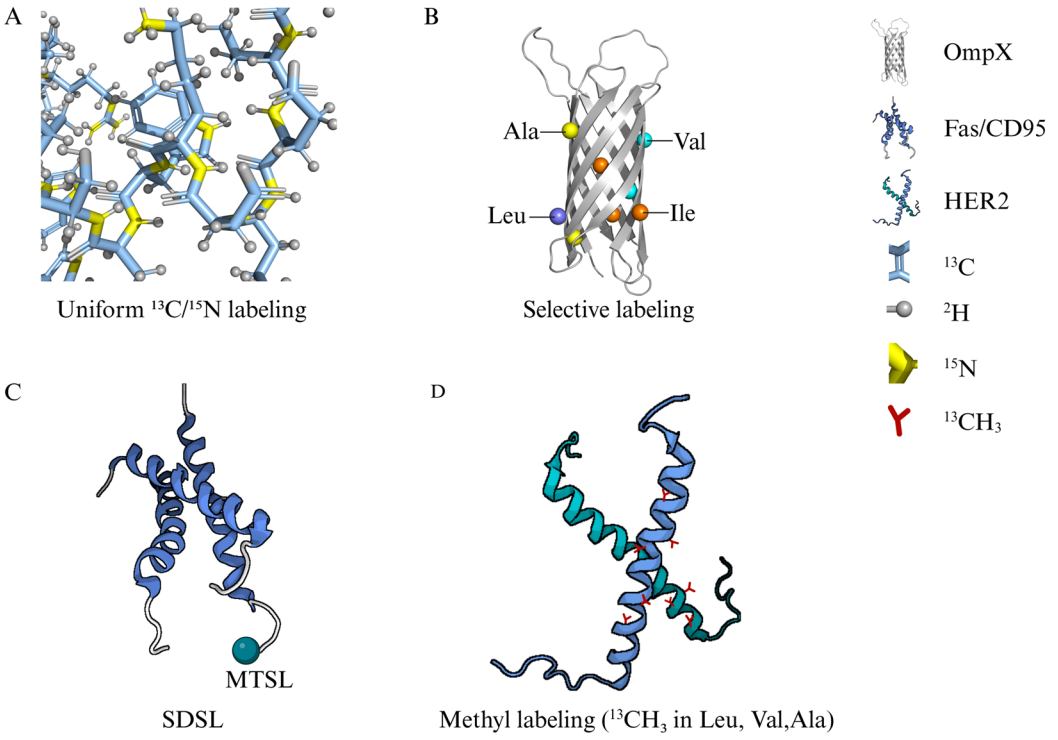


Figure 2. Representative labeling strategies for membrane protein NMR. (**A**) Uniform ^{13}C/^{15}N labeling on a part of the MPER-TMD of HIV-1 gp41 (PDB: 6V4T) (gray: deuterated ^{2}H background; yellow: ^{15}N; blue: ^{13}C). (**B**) Selective labeling on OmpX in nanodiscs (PDB: 2M06) (yellow: Ala; cyan: Val; purple: Leu; orange: Ile). (**C**) Site-directed spin labeling (SDSL) on the Fas/CD95 TMD trimer (PDB: 2NA7) (dark green: nitroxide, e.g., MTSL; other spin labels include Gd^{3+}, trityl, and ncAA) (**D**) Methyl labeling (^{13}CH$_3$ in Leu, Val, Ala) on the HER2 TMD dimer (PDB: 5OB4) (red: ^{13}CH$_3$ groups).

With the continuous increase in MAS frequency, the resolution problem of proton detection has been gradually solved. Lalli et al. employed 100 kHz ultrafast MAS combined with a 23.5 T high magnetic field, which allowed them to acquire well-resolved ^{1}H signals of the transmembrane domain and detect long-range ^{1}H-^{1}H contacts using less than 0.5 mg of fully protonated rhodopsin [69]. Spinning speeds of MAS exceeding 160 kHz have recently matured and become technically feasible for membrane protein research, as exemplified by aquaporin Z (AqpZ), with which a proton linewidth below 70 Hz can be achieved in native-like lipid bilayer systems, enabling nearly complete observation of protein backbone signals using only submilligram amounts of sample [70]. Using proton-detected MAS NMR, Forster et al. [71] solved the β-barrel structure of Opa60 in lipid bilayers. This work not only laid the foundation for functional studies of Opa60 in gonorrhea infection but also represented the latest advance in proton-detected MAS NMR for atomic-resolution structure determination of membrane proteins.

In the future, with the continuous advancement of higher-field magnets, ultra-fast MAS probes, and novel pulse sequences, MAS NMR is expected to achieve breakthrough progress in frontier areas such as high-complexity structural analysis of multi-spanning membrane proteins and in-situ dynamic studies of membrane proteins under physiological conditions, thereby providing more powerful technical support for the in-depth elucidation of membrane protein functional mechanisms.

3.3.2. OS-ssNMR

Oriented sample solid-state NMR (OS-ssNMR) is one of the core technologies for the study of membrane protein structural biology. Its core principle is to achieve macroscopically uniform orientation of membrane proteins within the lipid bilayer by using mechanical fixation (e.g., fixing the sample on glass slides [72]) and

other self-orienting membrane-mimetic systems such as aligned bicelles [73]. Structural constraints are then obtained by utilizing anisotropic nuclear spin interactions (e.g., ^{15}N chemical shift anisotropy and ^{15}N-^{1}H dipolar coupling) [26,61]. OS-ssNMR does not require membrane protein crystallization or removal from the native lipid environment, and can retain protein functional integrity under physiologically relevant conditions. It has been successfully applied to various membrane proteins, including bacteriorhodopsin [74], the HIV-1 Vpu transmembrane domain [75], and the multidrug resistance transporter EmrE [76], effectively revealing the *in situ* orientation and conformational dynamics of proteins within their native membranes.

MAS NMR and OS-ssNMR offer highly complementary approaches for membrane protein structural studies. MAS NMR uses magic-angle spinning to remove anisotropic broadening, enhancing spectral resolution, and employs radio-frequency (RF) pulses to capture spin interactions, yielding distance and torsion angle constraints that enable secondary to quaternary structure determination. Conversely, OS-ssNMR exploits anisotropic parameters to directly determine the spatial orientation of membrane protein domains within the lipid bilayer, including tilt and rotation relative to the membrane normal [61]. The structure of CrgA was determined by combining both techniques. OS-ssNMR provides the tilt and rotational orientation information of transmembrane helices, while MAS NMR supplements interhelical distance constraints. The combination of the two methods, together with dynamic simulation, ultimately elucidated the three-dimensional packing pattern of its transmembrane domain [77]. By integrating both data types with dynamic modeling, the strategy yields high-resolution structures while preserving *in situ* conformations in a near-native lipid environment, offering a powerful route to tackle large membrane proteins.

3.3.3. DNP NMR

Although ssNMR exhibits unique advantages in membrane protein research, its application is still constrained by inherently low sensitivity. The weak thermal equilibrium polarization of nuclear spins forces conventional experiments to rely on milligram quantities of sample and days to weeks of signal averaging, making it challenging to study low-abundance membrane proteins, transient interactions, or complex cellular environments [26,78]. The development of dynamic nuclear polarization (DNP) provides a critical solution to this limitation. Free electrons possess a much higher gyromagnetic ratio than atomic nuclei, allowing them to achieve greater thermal equilibrium polarization in a magnetic field. DNP boosts NMR signals by transferring high electron polarization to nuclei via microwave irradiation. Under ideal conditions, proton signals can be amplified by up to 658 times; in actual biological samples, signal gains of 10-100-fold can be achieved. This significantly reduces experimental time (from several weeks to a few hours) and lowers the sample requirement to the sub-milligram level [78]. Researchers used DNP to investigate the outer membrane protein Ail of *Yersinia pestis*, achieving not only high-resolution NMR spectra in the native bacterial outer membrane environment but also elusive interactions between this protein and the surrounding lipopolysaccharide layer [79].

DNP-MAS ssNMR has become a leading technique for membrane protein structure determination, combining complementary methods that synergistically boost sensitivity and resolution. In studies of the *E. coli* multidrug transporter EmrE, this combined technique achieved a 19-fold signal enhancement, confirming for the first time the direct interaction between the substrate and the key residue E14 in a liposomal environment [51]. In studies of the ABC transporter MsbA, this integrated method accurately captured the protein's conformational changes under diverse functional states and revealed the dynamic response mechanism of drug transport [80]. These advances enable ssNMR to probe more complicated, physiologically compatible biosystems and offer a robust technical foundation for structural biology.

4. Applications of NMR in Membrane Protein Structure Determination

4.1. Small Membrane Peptides and Single-Transmembrane Proteins

Single-spanning transmembrane peptides and small membrane proteins have long served as model systems in membrane protein NMR. In 2005, Jaroniec et al. used solution NMR to solve the structure of the HIV-1 gp41 N-terminal fusion domain in micelles, revealing its α-helical secondary structure and membrane-mimetic positioning [81]. Subsequent studies further determined the atomic structures of the complete transmembrane domain of gp41 and the membrane-proximal external region (MPER) in bilayer micelles, which more closely resemble physiological conditions, elucidating the assembly mechanism of the transmembrane trimer and its key role in viral membrane fusion and immunogen design [31].

OS-ssNMR and MAS NMR enable the determination of key structural parameters of transmembrane helices, including tilt angles relative to the bilayer normal, membrane immersion depths, and molecular assembly characteristics. Additionally, these techniques facilitate the analysis of how specific lipid components modulate

transmembrane structures—for instance, by regulating helix tilt angles and oligomerization efficiency [75,77]. For example, studies on the transmembrane domain of HIV-1 Vpu have clearly delineated its in-situ orientation within native lipid membranes using OS-ssNMR [75]. Furthermore, NMR-based structural characterization has been successfully applied to the transmembrane domains of other single-transmembrane proteins, such as the Fas/CD95 death receptor and DR5. These studies revealed that these proteins achieve intra-membrane oligomerization through proline motif-induced helix kinking or specific inter-helical interfaces, thereby regulating downstream signal transduction cascades [32,82]. Collectively, these investigations not only elucidate the structure-function relationships of single-transmembrane proteins in membrane environments but also lay a crucial methodological foundation for the structural determination of larger membrane proteins in subsequent research.

4.2. Multitransmembrane Proteins and Complexes

Building on the methodological foundation laid by single-transmembrane protein research, with the development of high-field magnets, advanced isotope labeling strategies (such as full-protein ^{2}H, ^{13}C, ^{15}N labeling, site-specific amino acid isotope labeling, and deuteration) and core NMR technologies including TROSY and NOESY, NMR has been successfully applied to the structural and conformational studies of multitransmembrane proteins and membrane protein complexes.

β-barrel multitransmembrane proteins: The three-dimensional structures and dynamic characteristics of the outer membrane proteins OmpA and OmpX from Gram-negative bacteria have been resolved by solution NMR in micelle systems, both of which were studied using full-protein ^{2}H, ^{13}C, ^{15}N labeling combined with TROSY-based experiments. The transmembrane domain of OmpA is an 8-stranded β-barrel structure with ion channel function; NMR relaxation analysis confirmed that its barrel core is highly rigid, while significant nanosecond-scale dynamic changes exist in the extracellular loops and C-terminus [83]. OmpX was reconstituted in DHPC micelles, and its solution structure was consistent with its crystal structure, confirming the 8-strand antiparallel β-barrel fold [84].

Ion Channels and Transporters: Using solution NMR combined with whole-protein labeling and TROSY techniques, the structure of VDAC-1 was determined in LDAO micelles, confirming that its transmembrane domain adopts a unique 19-strand β-barrel topology. NMR chemical shift mapping identified its binding sites for β-NADH, cholesterol, and Bcl-xL, providing a structural basis for the regulatory mechanism of this ion channel [85].

G Protein-Coupled Receptors (GPCRs) and Their Signaling Complexes: Using ssNMR, researchers determined the three-dimensional structure of the chemokine receptor CXCR1 in a phospholipid bilayer, revealing its seven-transmembrane α-helical fold [86]. In addition, solution NMR found that different functional states of rhodopsin bind to distinct elements of arrestin-1, inducing unique conformational changes and regulating signal transduction [87].

Other α-Helical Multitransmembrane Integral Membrane Proteins: Using solution NMR, researchers solved the three-dimensional structure of sensory rhodopsin II (pSRII) from a *haloalkaliphilic archaeon* in DHPC micelles. This was the first NMR structure of a detergent-solubilized seven-transmembrane protein, demonstrating the feasibility of this approach for studying similar membrane proteins [59]. Concurrently, by combining residual dipolar coupling and paramagnetic relaxation enhancement techniques, researchers determined the structure of mitochondrial uncoupling protein 2 (UCP2) in the GDP-bound state, revealing its unique channel conformation [88].

5. NMR Studies on Membrane Protein-Lipid/Protein/Ligand Interactions

5.1. NMR Characterization of Membrane Protein-Lipid Interactions

Membrane protein-lipid interactions are crucial for the structural stability and functional regulation of biological membranes. NMR affords *in situ* resolution of their dynamic features under physiological conditions, overcoming constraints of crystallographic and alternative structural methodologies. A variety of characterisation strategies have been developed for NMR across different systems.

MAS ssNMR is the core tool for studying interactions in lipid bilayers [89]. It can detect intermolecular contacts from Ångström to nanometer scales over a wide temperature range and under different membrane composition conditions [90,91]. For instance, through ^{13}C-^{13}C cross-peak analysis, the binding interface between the influenza virus M2 protein and cholesterol was successfully located, revealing that cholesterol binding induces membrane curvature and mediates M2 clustering to the neck of the budding virus for membrane scission [92]. Solution NMR uses CSP to screen for binding sites and determine dissociation constants [52]. For example, the study of C99 used this method to identify its cholesterol-binding site and determine its binding affinity [93]. DNP enhances NMR signals by 10-100-fold, and when combined with ^{13}C-labeled cholesterol, it substantially boosts the sensitivity for detecting weak interactions [92]. Moreover, ^{2}H NMR affords precise characterization of lipid dynamics and phase transitions. In VDAC1 two-dimensional crystals, variations in lipid order parameters identified coexisting annular

and bulk lipid fractions and protein-governed modulation of lipid phase transitions [94]. The combined use of these technologies enables deeper insight into how key lipids regulate membrane protein function, offering critical support for uncovering the biological significance of membrane protein-lipid interactions.

5.2. NMR Analysis of Membrane Protein Complex Assembly and Oligomerization

Membrane proteins often function as complexes or oligomers, with their assembly and oligomerization state directly modulating activity. Owing to its high sensitivity, NMR resolves assembly pathways and oligomeric properties under physiological conditions, tracing transient conformational changes and subtle intermolecular interactions [26].

Solution NMR spectroscopy enables analyses in conjunction with tailored membrane-mimetic systems. Unlabeled membrane proteins can be stably reconstituted in detergent micelles, lipid nanodiscs, or liposomes [38]. Notably, for liposome samples, CS and TCS experiments report on the soluble binding partners, which undergo fast tumbling in solution, circumventing the slow tumbling limitation of liposomes; when combined with perdeuterated soluble binding partners and complemented by selective labeling of membrane proteins, techniques such as cross-saturation (CS) and transferred cross-saturation (TCS) not only facilitate the mapping of specific interaction interfaces in membrane protein complexes [38,95] but also indirectly reveal the temporal binding sequence and binding affinity between proteins during oligomeric assembly. For instance, solution NMR has been applied to delineate the binding interface between the chemokine SDF-1α and the GPCR CXCR4 using the TCS approach. This study clarified the molecular binding mode wherein SDF-1α engages CXCR4 through multivalent interactions involving its N-terminal segment and extracellular loops, providing structural insights into GPCR-ligand recognition [96].

MAS ssNMR spectroscopy is not restricted by the tumbling rate or molecular weight of membrane protein complexes [53]. Integrating uniform $^{13}C/^{15}N$ labeling or site-specific isotope labeling with specialized pulse sequences—including proton-driven spin diffusion (PDSD) and dipolar-assisted rotational resonance (DARR)—at high MAS frequencies, it enables direct detection of interprotomer contacts within complexes, acquisition of inter-subunit distance restraints, and delineation of the structural basis for oligomeric assembly [26]. Combined solution and ssNMR has experimentally validated the native assembly of the BAM pentamer in outer membranes [97,98]. Moreover, TROSY coupled with protein deuteration supports high-resolution structural characterization of large-sized complexes [52,55]; nanodiscs preserve native conformational integrity [39], and combining these two tools is anticipated to capture transient conformations. ^{19}F NMR, which tracks conformational changes via fluorine-labeled probes, offers a new approach to studying interfacial interactions [18]. Although current research mainly focuses on interface and structural characterization, it has laid the foundation for studying the dynamic mechanisms of assembly and oligomerization.

6. NMR Analysis of Membrane Protein Dynamics and Conformational Changes

Membrane protein function relies on conformational dynamics rather than a single static structure [99]. During the processes of receiving signals, binding ligands, and transporting substances, they undergo structural motions on different time scales. NMR enables atomic-level resolution of dynamic events spanning fast local fluctuations to slow functional transitions, serving as a core tool bridging membrane protein structure and function [38,99]. Based on the timescale of molecular motion, membrane protein dynamics are classified into three regimes: fast (ps-ns), intermediate (μs-ms), and slow (ms-s).

6.1. Fast Local Dynamics (ps-ns): Local Structural Flexibility and Conformational Entropy of Membrane Proteins

Picosecond-to-nanosecond fast motions arise mainly from side-chain rotations, loop fluctuations, and subtle backbone vibrations, underpinning membrane protein stability and function [100]. Conventional NMR relaxation experiments (R_1, R_2, heteronuclear NOE) are routinely used to characterize dynamics on this timescale and to assess regional rigidity and flexibility [38]. Model-free analysis distinguishes global molecular tumbling from internal local motions on the ps–ns timescale. For example, in the β-barrel membrane protein OmpA, the intensity of backbone motion gradually increases as residues move further away from the center of the membrane [83].

Studies have demonstrated that fast dynamics of membrane proteins are distinctive. A comparison of helical pSRII and barrel-shaped OmpW revealed their methyl side-chain motions markedly exceed those of soluble proteins, correlating with very high residual conformational entropy. In membrane environments devoid of robust hydrophobic interactions, such high entropy is essential to preserve the folded protein conformation [101].

Rapid motions directly influence function. For instance, the extracellular L1 loop of the bacterial outer membrane enzyme PagP is extremely flexible on the ps-ns timescale, providing the structural flexibility needed for substrate entry into the β-barrel core and supporting catalysis [102]. It is worth noting that while the choice of membrane mimetic can influence fast dynamics to some extent, rapid motions are often observed to be relatively unaffected by micellar or lipid bilayer environments and appear to be an intrinsic feature of membrane proteins in many cases [101]. For high-molecular-weight membrane proteins like hVDAC1, NMR has revealed that backbone rigidity is confined to the transmembrane regions, whereas flexibility resides in the loops [103].

6.2. Intermediate-Time-Scale Dynamics (μs-ms): Conformational Equilibrium of Channel Gating and Transporters

Microsecond-to-millisecond motions directly correlate with essential membrane protein functions, including ion channel gating, transporter conformational rearrangements, and receptor activation [38]. CPMG and $R_{1\rho}$ relaxation dispersion techniques sensitively detect conformational exchange on this timescale, allowing rates and energy barriers to be extrapolated from relaxation data [38,101]. The study of the ADP/ATP carrier shows that although the protein has a threefold symmetric structure, conformational exchange is concentrated in specific domains, and substrate binding regulates the conversion rate by altering the stability of the transition state without changing the conformational populations [38,104].

In ion channels, intermediate-timescale dynamics is the core of the gating mechanism. The activation and inactivation of KcsA potassium channels are mainly dominated by μs–ms conformational changes in the selectivity filter region, rather than the movement of the intracellular gate [68,100]. Experiments on the PRE of the OmpG pore protein indicate that its outer loops adopt three interconvertible conformations, corresponding to the open, closed and intermediate states, respectively [105]. NMR not only allows observation of this motion but can also be combined with structural calculations to improve the accuracy of three-dimensional models of membrane proteins, thereby providing a structural basis for elucidating their function.

6.3. Slow Conformational Exchange (ms-s): Functional State Transition and Physiological Regulation

Millisecond-to-second (ms-s) slow conformational exchange drives functional transitions in membrane proteins and strongly correlates with signaling, enzymatic catalysis, drug–target interactions and pathogenic mutations [38]. ZZ-exchange experiments serve as a reliable approach to quantify rates on this timescale, allowing direct measurement of interconversion rates between distinct conformations [38]. Studies on EmrE have revealed that the rate of conformational exchange in the unbound state is significantly higher than that in the bound state [106], and is unaffected by the size of the environment, suggesting that slow motions are an intrinsic property of transporters [38]. The slow dynamics of the KcsA potassium channel directly determine the inactivation process. The selectivity filter toggles between conductive and non-conductive conformations at a rate that closely matches the electrophysiological inactivation rate, and the lipid environment markedly shifts the conformational distribution [68,100]. Abnormal slow kinetics are also closely associated with disease. The Val-408-Ala mutation in the human Kv1.1 channel stabilizes the closed conformation, reducing K^+ permeability and causing paroxysmal ataxia. NMR is capable of quantifying the proportions of different functional conformations and their transition rates, revealing the pathogenic mechanism of the mutation at the atomic level [100,107].

In summary, NMR systematically covers multi-timescale dynamics from picoseconds to seconds, comprehensively analyzing membrane proteins from local flexibility and functional motions to state transitions. It provides unique information for understanding the structure-dynamics-function relationship that is difficult to replace with other techniques. This capability renders NMR irreplaceable in deciphering the interplay between structure, dynamics, and function.

7. *In Situ* and Intracellular NMR: Toward Physiologically Relevant Environments

7.1. In Situ Solid-State NMR

Biomembranes are complex, dynamic and heterogeneous nanoscale interfaces [108]. Membrane mimics such as micelles, nanodiscs, and liposomes cannot replicate the complexity and asymmetry of natural membranes and may even destabilize proteins or mask key conformations [31,109].

In situ ssNMR enables direct observation of membrane protein structure and dynamics in native-like membrane patches and vesicles, avoiding detergents and artificial reconstitution while largely preserving native structure and function [109].

MAS technology alleviates spectral line broadening and underpins high-resolution spectral acquisition. By integrating proton detection, DNP and optimized isotope labeling, this methodology successfully overcomes the early-stage bottlenecks of low target-protein abundance and severe background interference, driving leapfrog progress of *in situ* NMR [109]. Figure 3 illustrates the principle of *in situ* DNP-enhanced ssNMR and its ability to boost signal sensitivity (typically 50–100 fold) for membrane proteins embedded in their native lipid environment. These advancements have collectively driven unprecedented progress in *in situ* NMR studies of membrane proteins.

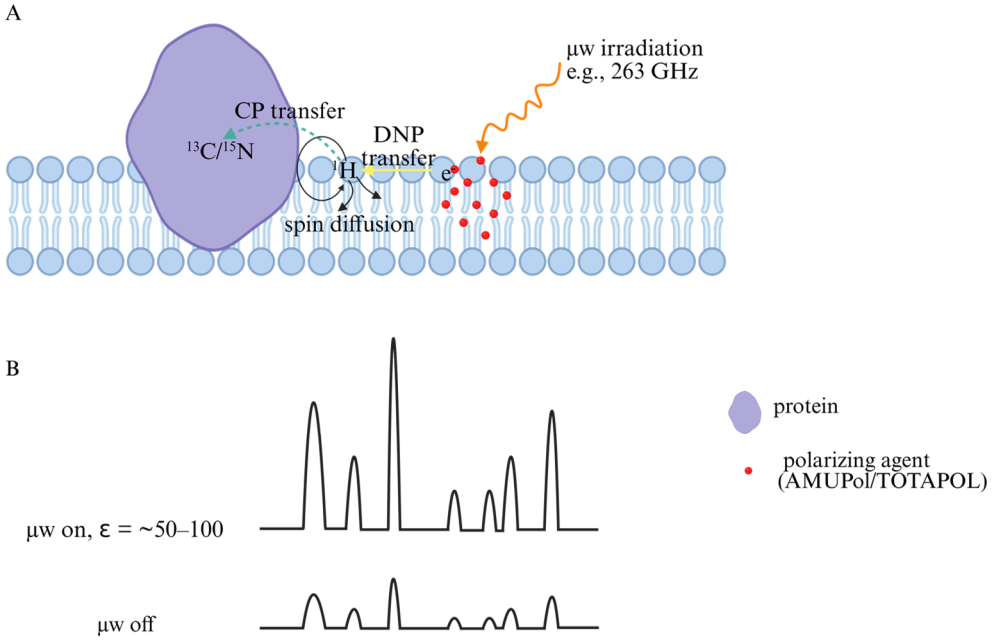


Figure 3. *In situ* DNP-enhanced ssNMR on membrane proteins in their native lipid environment. (**A**) Polarization transfer pathway: Microwave irradiation (μw, 263 GHz) saturates electron spins (e^-) of the polarizing agent (PA, red dots; e.g., AMUPol or TOTAPOL). DNP transfers polarization from e^- to nearby ^1H nuclei (yellow arrow). The hyperpolarization spreads through the ^1H network via spin diffusion (curved dashed arrow) and is then transferred to $^{13}C/^{15}N$ of the membrane protein via cross-polarization (CP, green arrow). (**B**) Schematic ssNMR spectra without (μw off, dashed line) and with (μw on, solid line) microwave irradiation. The DNP enhancement factor ε (typically 50–100) is indicated.

In the study of the seven-transmembrane photoreceptor ASR, MAS NMR directly resolved its structure in the inner membrane of *E. coli*, revealing that ASR formed a square lattice in native membranes, which differed significantly from the hexagonal packing observed in liposomes, thereby confirming the regulatory role of lipid composition in protein assembly [110]. In erythrocyte ghost membranes, static and 10 kHz MAS NMR revealed two distinct membrane perturbation mechanisms for antimicrobial peptides, providing direct evidence for assessing eukaryotic toxicity [111]. In studies of bacteriorhodopsin (bR), two-dimensional MAS NMR captured the conformational changes of Y185 during the photocycle, elucidating its mechanism as a “rotamer switch” regulating proton pump function [112]. These results make *in situ* NMR a key tool for studying membrane protein structure and dynamics under physiological conditions and have great potential in research on complex eukaryotic membrane proteins and complexes.

7.2. In-Cell/On-Cell NMR

In-cell/on-cell NMR enables studies to be conducted within living cells or on cell surfaces, revealing receptor-ligand binding, conformational changes and protein-protein interactions at atomic resolution, thereby providing a powerful tool for drug discovery and signal transduction research [53,113].

In-cell NMR focuses on the interior of living cells and analyzes the structure, dynamics, and interactions of intracellular proteins [53]. It enables direct measurement of drug-target binding kinetics and affinity, facilitating early screening of compounds with superior membrane permeability [53]. The technique can map ligand binding

sites via CSP and, when combined with DNP-assisted ssNMR, study targets that are challenging to observe by solution NMR [114]. In-cell NMR with ^{19}F labeling has revealed conformational diversity of H-Ras on the plasma membrane [115]; DNP-enhanced ssNMR discovered the multi-target effect of antimicrobial peptide Mac1 on *E. coli* [116]; and intracellular ssNMR advances are further bolstering membrane protein structural analysis [117].

On-cell NMR interrogates cell-surface receptors within intact cells, employing exogenous ligands or antibodies to identify binding sites and measure binding affinities in the absence of membrane-protein purification. Solution NMR (STD, trNOE) infers binding indirectly from altered free-ligand signals, while high-resolution MAS ssNMR directly interrogates the conformation and dynamics of large membrane protein complexes. In the study of the GPCR GRP-R, on-cell STD NMR successfully resolved the binding epitope of GRP-R with bombesin on the surface of tumor cells overexpressing GRP-R, providing key structural information for anticancer drug design [118]. Both in-cell and on-cell NMR are advancing rapidly and are already used to study receptor-drug interactions, quantify binding affinities, screen compounds, and optimize antibodies, holding great promise for drug discovery and precision medicine.

8. Conclusions and Future Perspectives

NMR spectroscopy has become one of the core techniques for studying membrane protein structure and function. It enables atomic-resolution determination of three-dimensional structures, multi-timescale dynamics, and complex molecular interactions of membrane proteins in native-like and *in situ* membrane environments. The synergistic innovations in solution-state NMR, ssNMR (including MAS and oriented samples), and DNP-enhanced techniques have significantly overcome traditional sensitivity and resolution bottlenecks, making it possible to study multi-spanning membrane proteins, dynamic conformational transitions, and *in situ* cellular membrane environments.

Despite the substantial progress made in NMR technology, this field still faces key challenges. Membrane-mimetic systems cannot fully recapitulate the native membrane environment, and most currently available detergent-free systems possess inherent limitations [23]. NMR still faces technical bottlenecks in sensitivity, resolution and dynamic characterization, preventing efficient, *in situ* precise analysis of proteins in complex systems and native membrane environments [26]. Moreover, laborious sample preparation and low drug-screening throughput partially impede research expansion.

To address current bottlenecks, future membrane protein NMR research will focus on recapitulating physiological environments, developing technical innovations, and fostering interdisciplinary integration. Developing more stable and compatible peptide nanodiscs and novel SMA derivatives will further improve detergent-free membrane mimetics, allowing efficient extraction and stable encapsulation of native membrane proteins [119,120].

At the technical level, the combination of methods such as ultrafast magic-angle spinning, ^{19}F NMR, and rotational-echo double-resonance (REDOR), along with AI-assisted analysis and non-uniform sampling strategies, can effectively accelerate data acquisition and improve experimental throughput, while optimizing dynamic nuclear polarization conditions to achieve a balance between signal enhancement and dynamic characterization [18,121–123].

Building on this foundation, future work should promote the integration of NMR with techniques such as cryo-electron microscopy and single-molecule fluorescence, thereby facilitating *in situ* studies of native cell membranes and at the whole-cell level. NMR fragment screening platforms will accelerate the development of membrane protein-targeted drugs, particularly for metabolically relevant targets such as GPCRs [124], transporters [125], and channels [126] implicated in diabetes, obesity, and non-alcoholic fatty liver disease. Recent advances in solid-state NMR have enabled direct observation of drug binding at membrane-embedded targets [121], while ^{19}F NMR and fragment-based approaches have further expanded the toolkit for ligand discovery against membrane proteins [127]. The integration of NMR structural insights with drug discovery pipelines holds promise for accelerating the development of therapeutics targeting membrane proteins involved in metabolic disorders [26,119,127]. Driven by interdisciplinary convergence, NMR technology will continue to overcome current limitations, propelling membrane protein research toward higher resolution, greater physiological relevance, and enhanced translational potential.

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