Review

^{19}F NMR as a Powerful Tool for Revealing Disease- and Metabolism-Related Membrane Protein Dynamics

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Abstract: Membrane proteins are central mediators of cellular material transport, signal transduction, and intercellular communication. Their dysfunction is closely linked to a variety of major diseases. Although traditional structural biology techniques can achieve high-resolution global analysis of membrane proteins, the lack of understanding of their dynamic structural mechanisms severely hinders the in-depth comprehension of their physiological functions and the development of targeted therapeutic drugs. ^{19}F nuclear magnetic resonance (^{19}F NMR) spectroscopy, with its unique advantages including high sensitivity and minimal endogenous background interference, provides a powerful means of analyzing the conformational changes and interactions of membrane proteins. This review begins by comparing the main methods currently used in the study of membrane protein structure and function, clarifying the scope of application and the limitations of each technique. It then focuses on the unique advantages of ^{19}F NMR in characterizing the local structure of membrane proteins, analyzing their dynamics, and investigating protein–protein and protein–ligand interactions. Building on this, we summarize various ^{19}F labeling strategies and their applicable scenarios, and review cutting-edge applications of this technique in the study of disease- and metabolism-related membrane proteins, covering structural elucidation of key membrane proteins such as G protein-coupled receptors (GPCRs) and ion channels, as well as real-time monitoring of functionally related conformational changes. Finally, using proteins associated with sleep disorders, cancer, and metabolic diseases as examples, we review significant advances in the application of ^{19}F NMR in targeted drug screening and the development of allosteric modulators, highlighting the unique value of this technique in filling gaps in dynamic structural information, advancing the elucidation of metabolic disease mechanisms, and facilitating precision drug discovery.

Keywords: ^{19}F NMR; membrane proteins; site-specific conjugation; conformational dynamics; drug discovery; structural biology

1. Introduction

Biological membranes act as natural barriers to delineate functional compartments within and outside the cell, while membrane proteins effectively link different functional units together. It is estimated that around 20–30% of the genes in most genomes encode membrane proteins [1]. For instance, in the regulation of the immune system, membrane proteins participate in real–time regulation throughout T–cell activation, effector responses, and termination [2]. During the early phase of immune regulation, classical major histocompatibility complex (MHC) molecules on the cell surface process antigens and present the resulting peptides to T cells, thereby transmitting antigenic information [3]. During T–cell activation, the combined action of the T–cell receptor (TCR) and co–stimulatory receptors (such as CD28 and 4–1BB) promotes immune responses by regulating cell proliferation and the secretion of related immune factors in a timely manner [4]. In the later stages of the immune response, immune checkpoints such as programmed death protein–1 (PD–1) bind to their ligands to effectively inhibit T cell activation, thereby preventing excessive immune responses [5]. Furthermore, the transport functions of membrane proteins are essential for cellular metabolism. For example, the ATP–binding cassette (ABC) transporters facilitate the transmembrane transport of various substrates, ranging from ions to macromolecules, by binding to and hydrolyzing ATP, thereby maintaining cellular metabolic homeostasis [6].

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As key executors of cellular functions, membrane proteins are essential for maintaining cellular homeostasis. This also makes them highly attractive targets for therapeutic intervention. Current estimates indicate that membrane proteins constitute about 60% of all therapeutic targets, with nearly half of approved drugs acting on this protein class [7]. For example, the anti–OX40 antibody rocatinlimab effectively alleviates atopic dermatitis (AD) symptoms with a favorable safety profile [8]. In cancer treatment, immune checkpoint inhibitors target immune checkpoints such as CTLA–4 [9] and PD–1 [10], preventing T cells from receiving "shutdown" signals and thereby prolonging T cell activation to kill cancer cells. Furthermore, in the field of metabolic disease treatment, drugs targeting the Glucagon–like peptide–1 receptor (GLP–1R), such as GLP–1 receptor agonists (GLP–1 RAs), have been widely used to treat type 2 diabetes and obesity. They can promote insulin secretion in a timely manner while maintaining cardiovascular health [11,12]. Consequently, an in–depth analysis of the structural characteristics, functional mechanisms, and conformational dynamics of metabolism– and disease–related membrane proteins is of great scientific significance and holds considerable practical potential for elucidating the pathogenesis of diseases, identifying novel drug targets and developing precision treatment strategies.

However, due to their unique physicochemical properties and relatively low expression levels, the structural and functional analysis of membrane proteins faces distinct challenges compared to soluble proteins. First, their high hydrophobicity and transmembrane domains necessitate a native–like lipid bilayer environment to maintain proper folding, stability, and biological activity [13,14]. Thus, their physiological conformations are readily impacted by environmental factors, such as low temperature or high salinity. Second, the biological function of membrane proteins relies on rapid, reversible exchange among multiple functional states (e.g., active/inactive receptor states, open/closed ion channel states) across timescales ranging from microseconds to milliseconds. Most of these states are low–abundance, transient intermediates that are difficult to capture and characterize [15]. Furthermore, many membrane proteins function as large multimeric assemblies or form complexes with downstream signaling partners, further increasing the complexity of their structural and dynamic analysis [16]. These intrinsic features render membrane proteins among the most challenging targets in modern structural biology.

Membrane proteins play an irreplaceable role in maintaining homeostasis and in the treatment of related diseases. However, their unfavorable physicochemical properties and highly dynamic conformational changes significantly increase the difficulty of research. Although techniques such as X–ray crystallography and cryo–electron microscopy have made important contributions to the structural analysis of membrane proteins, challenges remain in capturing dynamic functions and in visualizing the screening of receptor–targeted drugs. ^{19}F NMR overcomes these limitations. Its advantages—no background interference, high sensitivity, and the capacity to quantitatively track protein–protein interactions—allow dynamic molecular analysis without disrupting protein function. In this review, we compare conventional techniques with ^{19}F NMR, highlighting the advantages of ^{19}F NMR for membrane protein analysis. We summarize the versatility of current ^{19}F labeling strategies and tags for tackling membrane protein challenges, and review both the achievements and current limitations of ^{19}F NMR in this field.

2. Advantages and Characteristics of ^{19}F NMR in Membrane Protein Research

The past decades have seen substantial progress in the characterization of membrane protein structural biology, largely driven by X–ray crystallography, cryogenic electron microscopy (cryo–EM), and nuclear magnetic resonance (NMR) spectroscopy. Nevertheless, each of these approaches suffers from specific limitations—precisely the gaps that ^{19}F NMR spectroscopy is well equipped to fill (Figure 1).

X–ray crystallography is the gold–standard technique for determining atomic–resolution structures of membrane proteins, provided that high–quality diffraction crystals can be obtained. For instance, in the structural study of channelrhodopsin (ChR), an X–ray crystal structure with a resolution of 2.3 Å was obtained, which not only revealed its fundamental architecture but also laid the foundation for subsequent functional investigations [17]. However, the success of this technique depends critically on the ability to obtain crystals capable of high–resolution diffraction, and most membrane proteins are difficult to crystallize to a high standard due to their hydrophobicity and conformational flexibility [18,19]. Furthermore, crystallization typically requires a considerable amount of time, during which membrane proteins may fail to maintain their native conformation. Additionally, the requirement for large sample quantities (milligrams) precludes the structural analysis of certain membrane proteins using this method. The advent of electron cryo–microscopy (cryo–EM) has greatly advanced the structural analysis of membrane proteins. Cryo–EM circumvents the need for protein crystallization by suspending the sample in vitrified ice through plunge freezing [20]. This approach enables the collection of large numbers of particle images, routinely achieving structural resolutions in the range of 4 Å [21]. While cryo–EM has advanced membrane protein structure determination, proteins below 100 kDa remain challenging for this technique, particularly within membrane protein systems [22]. Furthermore, while cryo-EM predominantly

captures static snapshots, conformational heterogeneity can be resolved through computational classification, yet it still cannot directly monitor dynamic activity in real time. This, to some extent, constrains our understanding of the dynamic functions of receptors [23]. Overall, X–ray crystallography and cryo–EM are both primary methods for obtaining structural information about proteins. Although cryo–EM avoids the complex crystallization experiments required in X–ray crystallography, its molecular weight limitations mean that it is not widely applicable to the study of low–molecular–weight proteins. More importantly, both methods face technical barriers when it comes to obtaining information on protein structural dynamics.

Conventional nuclear magnetic resonance (NMR) techniques offer certain advantages in protein structure determination, particularly for low–molecular–weight proteins [24]. Furthermore, solution NMR facilitates the maintenance of the receptor's physiological conformation. As a powerful biophysical tool, it can determine protein details at atomic resolution and obtain the three–dimensional structures of membrane proteins. Compared to X–ray crystallography and cryo–EM, it enables the analysis of protein kinetic characteristics. However, in conventional solution NMR, even with the widespread use of heteronuclear isotope labeling and multidimensional experiments, proton–detected spectra still suffer from severe spectral crowding for large macromolecular systems (such as membrane protein–detergent complexes), where overlapping resonances substantially complicate data interpretation [25]. Although isotope labeling (^{13}C, ^{15}N) and two–dimensional spectral analysis can eliminate most interfering signals, they may reduce protein expression, leading to difficulties in sample acquisition [26]. Furthermore, in vitro studies of membrane proteins often require the use of detergent systems or nanodiscs to solubilize and stabilize the protein. While these membrane–mimetic environments aim to preserve a near-native conformation, they do not necessarily fully recapitulate the receptor's physiological state. Under these conditions, the molecular weight of the system can reach hundreds of kDa [27], far exceeding the detection limit of conventional NMR and leading to rapid signal decay and line broadening [28]. Methods such as transverse relaxation–optimized spectroscopy (TROSY), uniform isotope labeling, and nanodisc–optimized structures are currently used for NMR analysis of high–molecular–weight proteins [29–31]; however, these methods still face a number of challenges in practical application. Firstly, the prolonged incubation required for efficient deuteration may interfere with the expression efficiency of membrane proteins, and may even affect their native folding state and functional activity [32]. Secondly, standard TROSY experiments are primarily based on $^{1}H–^{15}N$ HSQC measurements of amide protons. Because these experiments detect amide N–H correlations, they do not directly report on carbon-bound protons, including those in aliphatic side chains, methyl groups, or many aromatic rings. Consequently, structural and dynamic information about these non-amide proton sites is difficult to obtain directly, leaving gaps in our understanding of side-chain conformational dynamics and their contribution to the overall conformational dynamics of proteins [33]. Consequently, these limitations continue to restrict, to a certain extent, the applicability of traditional NMR techniques in membrane protein systems with complex dynamic behavior.

Compared with all the detection methods mentioned in this review, site–specific labeling coupled with fluorine–19 nuclear magnetic resonance (^{19}F NMR) enables the dynamic capture of receptor function under physiological conditions. Fluorine has been demonstrated to be an extremely useful element for studying biological systems. Fluorine atoms are almost absent in biological tissues [34], which effectively eliminates the severe background interference issues encountered in conventional NMR conditions. Although ^{19}F NMR cannot provide complete protein structures, its high chemical shift sensitivity captures local receptor conformational changes with precision, from subtle rearrangements to microsecond–to–millisecond dynamics. This allows for the characterization of minute structural differences during the dynamic changes of different receptors and can be used to probe various interactions, including ligand–receptor, receptor–membrane, and drug–receptor interactions [35]. This effectively fills the gap left by other techniques that cannot dynamically study receptor function. Furthermore, by correlating changes in peak patterns with time, it can provide precise kinetic information regarding different interactions [36]. Additionally, compared to conventional NMR techniques, ^{19}F NMR does not require elucidation of the entire structure. It accommodates the introduction of nanodiscs, thereby yielding high–quality ^{19}F NMR spectra [37]. It should be acknowledged that the introduction of a ^{19}F label can potentially perturb the native protein conformation. Consequently, rigorous control experiments are typically required to validate the labeling effects, which inevitably adds to the experimental burden.

In addition to the methods discussed above, techniques such as electron paramagnetic resonance (EPR), single–molecule FRET (smFRET), and molecular dynamics (MD) simulations also play significant roles in elucidating the structure and functional dynamics of proteins. However, the development and application of technologies are always mutually reinforcing. In practical research, one should carefully select the most appropriate techniques based on the specific research objectives and the characteristics of the protein itself. If the goal is to obtain a complete high–resolution three–dimensional structure, then based on the molecular weight, stability, and crystallization properties of the protein, a reasonable choice can be made among X–ray, Cryo–EM,

and conventional NMR techniques. In contrast, ^{19}F NMR has certain limitations in obtaining the overall folding information of proteins and cannot independently provide a complete global three–dimensional structural model. However, its unique chemical advantages make it valuable in local functional research. Specifically, ^{19}F NMR is particularly suitable for monitoring changes in the microenvironment of the catalytic centers, conformational transitions induced by ligands, and protein–protein or protein–ligand interactions, and can accurately capture these dynamic events with high temporal resolution. Therefore, ^{19}F NMR can be regarded as a powerful complement to traditional structural analysis techniques, and is particularly suitable for studying dynamic proteins, membrane proteins, or functional conformational changes in systems that are difficult to crystallize.

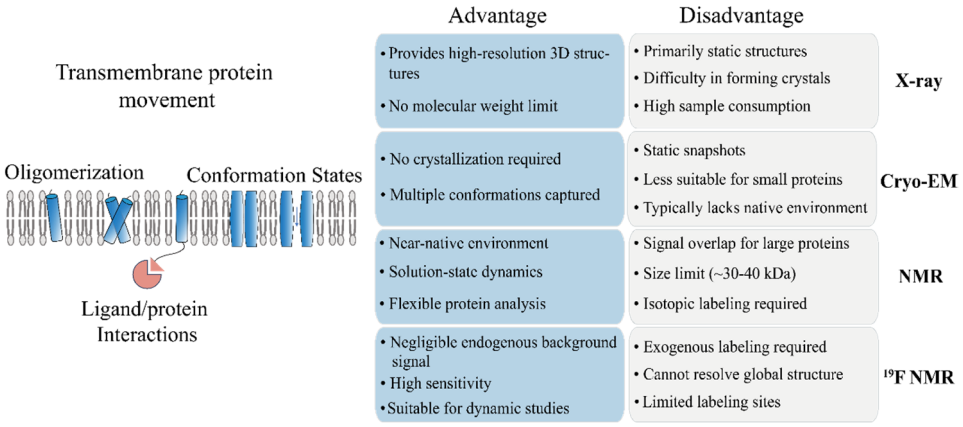


Figure 1. Comparative analysis of protein structure determination methods.

3. Functional Monitoring and Labeling Strategies of ^{19}F Tags

3.1. Fluorine Labeling Strategies

^{19}F is not naturally present in biological systems. Therefore, to study the dynamic changes in membrane proteins using ^{19}F NMR, the introduction of a ^{19}F NMR probe into the target protein is an essential prerequisite. To date, three well–established approaches have been developed to achieve ^{19}F labeling of membrane proteins, as detailed below:

(1) Post–translational Modification of Proteins

The core principle of this category of methods is to first express and purify wild–type recombinant proteins or recombinant proteins bearing specific reactive sites, followed by the covalent conjugation of fluorine–containing tags to the side chains of specific amino acid residues on the target protein via chemical or enzymatic reactions (Figure 2A). This represents a core strategy for achieving site–specific fluorine labeling of proteins, among which cysteine–targeted labeling is the most well–established and widely used technique [35]. The high nucleophilicity and unique reactivity of the cysteine thiol group enable specific conjugation of fluorinated tags to the protein surface via mild reactions, such as disulfide bond exchange and nucleophilic substitution. For example, in studies of the adenosine A_{2A} receptor ($A_{2A}R$) complex, the ^{19}F label 2–bromo–N–(4–(trifluoromethyl)phenyl) acetamide (BTFMA) was covalently attached to the thiol group of a cysteine residue via nucleophilic substitution, enabling the identification of the protein's active, intermediate, and inactive states [38]. This method typically uses site–directed mutagenesis to introduce labeling cysteines. However, reduced expression and structural perturbation caused by mutations limit its applicability for some proteins. To address this issue, Frère et al. proposed a method for single–point labeling of target proteins under non–denaturing conditions, utilizing a highly specific nucleophilic substitution reaction between a halogenated acetamide and the thiol group of a cysteine residue. The labeling efficiency of this method is significantly higher than that of conventional labels; more importantly, it does not require the introduction of any cysteine mutations into the target protein [39]. In the work of Sušac et al., an in–membrane chemical modification (IMCM) method was also proposed, which chemically modifies natural amino acids to introduce specific sites with minimal mutagenesis [40]. This method has been applied to GPCR–G protein systems to explore protein conformational changes under different membrane conditions [41]. In addition, the amino groups of lysine (Lys) residues can be labeled via Mannich–type reactions [42]. It has been reported that a labeling efficiency of up to 100% can be achieved under mild conditions at pH 7.2 and ambient temperature [43].

Overall, this method features mild reaction conditions, high site specificity, and excellent labeling efficiency. It offers flexible tag selection, which can be tailored to match experimental requirements for sensitivity and line

width, and single–site labeling can eliminate spectral peak overlap. However, the experimental results depend largely on the choice of tagging sites, which must be rationally selected based on the protein's three–dimensional structure.

(2) ^{19}F Labeling During Protein Expression

The key feature of this class of methods is the direct incorporation of fluorinated noncanonical amino acids into the nascent polypeptide chain via genetic and metabolic engineering approaches during heterologous expression of the target protein, representing a mainstream strategy for fluorine labeling of membrane proteins. Conventional methods use amino acid–deficient *E. coli* strains (e.g., BL21(DE3) and C43(DE3) derivatives) [44] or inhibitors like glyphosate to block the relevant biosynthetic pathways [45]. By supplementing fluorinated noncanonical amino acids (ncAAs) to replace the corresponding natural amino acids, the bacterial endogenous translation system is utilized to achieve fluorine labeling of the target protein (Figure 2B). Among fluorinated amino acid analogues, aromatic fluorinated amino acids are currently the most widely used, including fluorophenylalanine (e.g., 4–F–Phe [46]) and fluorotryptophan (e.g., 5–F–Trp [47], 6–F–Trp [48]). To date, these tags have been employed to study protein folding–associated conformational changes. For example, in studies of protein aggregation and misfolding, the 3–F–Phe label in the β–state SHaPrP allows measurement of enthalpy, entropy, and heat capacity differences among monomeric, octameric, and prefibrillar aggregate states. Concurrently, the exchange kinetics between different states provide equilibrium parameters, offering deeper insight into PrP misfolding at early stages [49].

It is noteworthy that fluorine labeling of solvent–exposed aliphatic amino acids can counteract chemical shift anisotropy (CSA) and enhance resonance signal mobility, thereby conferring advantages in NMR spectroscopy and resulting in narrowed line widths [50]. This method features a straightforward operational workflow, low cost of commercially available reagents, and high labeling homogeneity. Furthermore, due to the extremely low natural abundance of tryptophan in proteins, fluorine labeling of a single–copy tryptophan residue can directly achieve single–site detection, thus avoiding spectral peak overlap. However, this method has notable limitations: it requires screening of compatible auxotrophic strains, which is time– and labor–intensive during the early development stage, and high concentrations of fluorinated amino acids exert a growth inhibitory effect on the bacterial host.

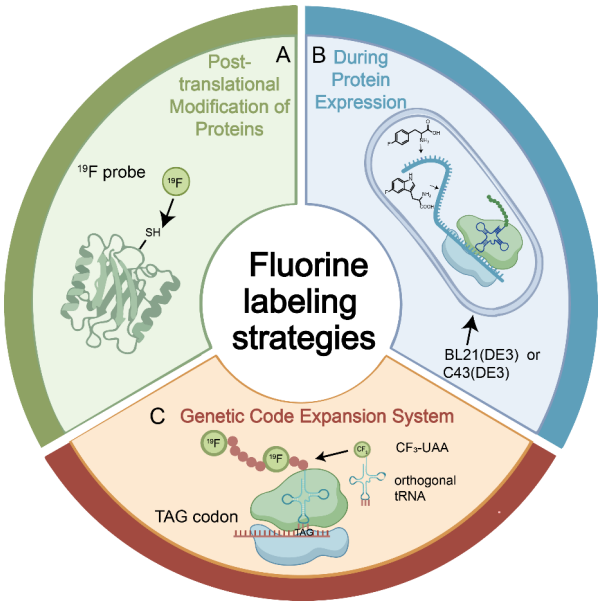


Figure 2. Three different fluorine labeling methods. (**A**) Post-translational modification of proteins: after the protein has been expressed and purified, the fluorine-containing probe is chemically conjugated to residues such as the cysteine thiol group (–SH). (**B**) ^{19}F labeling during protein expression: during protein expression, fluorinated noncanonical amino acids (such as trifluoromethylphenylalanine) are recognized by aminoacyl–tRNA synthetases and incorporated into the polypeptide chain. (**C**) ^{19}F labeling by genetic code expansion system: By utilizing orthogonal aminoacyl–tRNA synthetases and tRNA pairs that recognize the amber stop codon (TAG), fluorinated noncanonical amino acids, such as trifluoromethyl-containing noncanonical amino acids (CF_3–UAAs), are specifically introduced at predetermined positions within the target protein.

(3) ^{19}F Labeling by Genetic Code Expansion System

This method can also be used for site–specific ^{19}F labeling during protein translation. Non–canonical amino acids (ncAAs) are introduced using an orthogonal tRNA/aminoacyl–tRNA synthetase pair. This approach employs

amber stop codon (TAG) suppression to precisely and site–specifically incorporate fluorinated noncanonical amino acids into any predefined position of the target protein, without interfering with the translation of native amino acids [51,52] (Figure 2C). This method predominantly uses trifluoromethyl (CF_3)–containing noncanonical amino acids, such as tfm–Phe and tfm–Tyr. Wang et al. genetically incorporated the noncanonical amino acid 3′–trifluoromethyl–phenylalanine (mtfF) into cannabinoid receptor 1 (CB_1) expressed in the baculovirus expression system [53]. Using ^{19}F NMR spectroscopy and X–ray crystallography, they discovered that the allosteric modulator Org27569 and agonists synergistically stabilize a previously unrecognized pre–active state, and proposed an allosteric modulation model to explain the unique behavior of Org27569. Meanwhile, the new noncanonical amino acid para–pentafluorosulfanyl phenylalanine (SF_5Phe) can also be site–specifically incorporated into proteins with high yield. This tag can enable the measurement of ^{19}F–^{19}F NOEs in a protein, supporting further exploration of protein–protein and protein–membrane interactions [54]. Overall, this method possesses absolute site specificity, allowing the selection of labeling sites on demand and fundamentally eliminating spectral peak overlap. The rapid internal rotation of the CF_3 group can counteract line broadening caused by chemical shift anisotropy (CSA), making it perfectly compatible with high–molecular–weight proteins, membrane proteins, and in–cell NMR systems. It also supports a wide range of fluorinated noncanonical amino acids, providing extremely high flexibility in tag selection. However, in practical applications, this method requires the construction of a two–plasmid expression system, which involves high operational complexity, and the expression level of the target protein is often reduced when multi–site insertion is performed.

This section concludes the main labeling strategies used in ^{19}F NMR studies. Post–translational chemical modification offers simple procedures, flexible tag selection, and mild reaction conditions; however, it provides a limited observational perspective and requires careful selection of suitable labeling sites. ^{19}F labeling during protein expression is straightforward, cost–effective, and provides high labeling uniformity; nevertheless, it lacks site specificity, which may lead to multi–site labeling and peak overlap, and high concentrations of fluorinated amino acids are toxic to host cells. Genetic code expansion systems (e.g., amber codon suppression) enable site–specific labeling and precise control of probe positioning. However, this approach requires the construction of orthogonal tRNA/synthetase pairs, involves complex procedures, and typically results in lower protein expression yields when multi–site labeling is employed. These three strategies are complementary; in practice, the appropriate labeling method should be selected according to specific research requirements.

3.2. Common Types of ^{19}F Probes

The ^{19}F nucleus exists in 100% natural abundance [55,56], and its near–absence from biological tissues provides an effective zero–background environment for NMR detection. Monitoring subtle protein conformational changes typically requires introducing an exogenous ^{19}F tag at a defined site to create a sensitive NMR observable window. Currently, many commercially available fluorinated tags serve as powerful NMR probes and are widely applied to study protein conformational dynamics, protein–membrane interactions, and quantitative metabolic analysis. According to the labeling method, ^{19}F probes can be classified into two categories: chemically modified tags and biosynthetic tags. Each type of tag has its own advantages in chemical shift response and applicable research scenarios. In this section, we list different tags and discuss their advantages and application limitations.

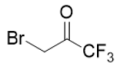
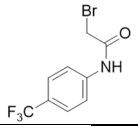
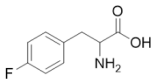
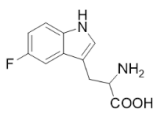
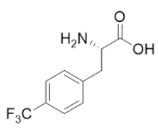
Most chemically synthesized tags are attached to target proteins via site–specific conjugation, which facilitates the capture of different protein conformations and enables dynamic analysis of various interactions. For example, 3–bromo–1,1,1–trifluoroacetone (BTFA) [56–62], 2–bromo–N–(4–(trifluoromethyl)phenyl)acetamide (BTFMA) [57,63], and 2,2,2–trifluoroethanethiol (TET) [57,64,65] are currently the most widely used probes for various membrane proteins. These tags all contain a trifluoromethyl (CF_3) group. The rapid internal rotation of the CF_3 group effectively averages chemical shift anisotropy (CSA), significantly reducing line broadening—a common issue in large proteins. This property makes these tags particularly suitable for ^{19}F NMR detection of membrane proteins and high–molecular–weight proteins. One study systematically compared the chemical shift sensitivity of six CF_3–containing NMR probes. The results showed that the chemical shift dispersion of BTFMA was significantly superior to that of traditional labels such as BTFA and TET, providing theoretical support for the screening and optimization of ^{19}F NMR probes for membrane proteins and other large biomolecules [63]. Next–generation probes are also under development. Frère et al. designed pyridinone–based CF_3 probes that exploit intramolecular conformational effects to amplify chemical shift responses to microenvironmental changes, improving conformational sensitivity. Nevertheless, these probes still have limited solvent–polarity discrimination capability [39]. Huang et al. developed a highly sensitive monofluoroethyl (mFE) probe that covalently labels cysteine sites and precisely distinguishes multi–conformational protein aggregates [66].

Most biosynthetic tags share structural homology with their natural amino acid counterparts, allowing them to be introduced co–translationally during protein synthesis. These tags are mainly composed of fluorinated phenylalanine (Phe) and fluorinated tryptophan (Trp), featuring a wide range of chemical shift distributions and high sensitivity to local electrostatic and hydrogen bond environments, making them suitable for capturing subtle conformational changes of proteins [50]. Commonly used monofluorine labels currently include 4–fluorophenylalanine (4–F–Phe) [67–69], 5–fluorotryptophan (5–F–Trp) [70–75] and 4–trifluoromethyl–L–phenylalanine (tfmF) [76–80]. These labels have numerous applications in areas such as protein conformational changes, protein–membrane interactions, and protein folding. Among these, 5–F–Trp exhibits the most pronounced environmental sensitivity, particularly in response to changes in the internal hydrogen–bond network of proteins [80]. Several trifluorinated labels are also widely used. Researchers have utilized AlphaFold and molecular dynamics simulations to predict the optimal labeling site of the tfmF [70]. Wang et al. used mtfF (3′–trifluoromethyl–phenylalanine) and other trifluoromethyl–containing noncanonical amino acids as probes, combined with genetic code expansion technology, to achieve site–specific labeling of GPCR membrane proteins at defined positions. However, this system is complex to construct and often leads to reduced expression levels of the target protein [53]. The tag structure and its application are shown in Table 1.

In addition to the above two types of core probes, there are also multi–amino acid–reactive small molecule ^{19}F probes such as lysine and glutamine, which can overcome the dependence on cysteine and achieve simultaneous labeling of multiple residues, but they are prone to peak overlap and low site recognition [42]. Chai et al. [81] developed a thiol–specific ^{19}F probe with strong specificity, which can simultaneously detect various small molecule thiols, but it is only applicable to small molecule systems and cannot be directly applied to complex protein conformation studies. Chen et al. [82] constructed a biorthogonal metabolic ^{19}F probe based on the SPAAC click chemical strategy, which can achieve targeted labeling of live tumor cells and ^{19}F MRI imaging of deep tissues, but it can only act at the cellular whole level and cannot precisely label single membrane proteins or soluble proteins at specific sites.

In summary, both chemically synthesized and biosynthetic ^{19}F tags offer distinct advantages for membrane protein studies. Chemically synthesized CF_3–containing tags, such as BTFMA, benefit from rapid internal rotation that averages chemical shift anisotropy and reduces line broadening, making them particularly suitable for large proteins and membrane systems. Biosynthetic tags, including fluorinated phenylalanine and tryptophan derivatives, resemble natural amino acids and can be co–translationally incorporated, minimizing structural perturbation. However, genetic code expansion often increases system complexity and reduces expression yields. Overall, the complementary properties of these two tag families provide flexible options for probing membrane protein dynamics, and future probe optimization guided by computational predictions will further expand the capabilities of ^{19}F NMR in structural biology.

Table 1. Chemical structures and applications of commonly used ^{19}F NMR probes.

^{19}F Tag	Structure	Labeling Target	Features and Applications	Reference
BTFA		Cys	Protein conformational changes Protein folding Protein–ligand binding	[56–62]
BTFMA		Cys	Protein conformational changes Reported to provide greater chemical-shift dispersion	[57,63]
TET		Cys	Protein conformational changes Protein structure determination	[57,64,65]
4–F–Phe		Phe	Protein conformational changes Protein–membrane interaction Protein folding	[67–69]
5–F–Trp		Trp	Protein conformational changes Protein–membrane interaction Protein folding Most significant response to the changes in the hydrogen bond network within the protein	[70–75]
tfmF		Phe	Protein conformational changes Protein–membrane interaction Protein–protein interactions within living cells	[76–80]

4. ^{19}F NMR Applications in Disease- and Metabolism-Related Membrane Protein Dynamics

^{19}F NMR enables real–time monitoring of conformational dynamics in membrane proteins under near–physiological conditions, which is particularly crucial for understanding metabolic regulation and disease mechanisms. This section summarizes key advances in the use of this technique to study the structures and activation mechanisms of GPCRs, ion channels, and metabolic transporters, with a focus on how ^{19}F NMR can be used to capture conformational dynamics induced by ligand binding and allosteric regulation, thereby revealing the molecular logic underlying protein function regulation. The section also highlights its applications in the development of drugs targeting membrane proteins (e.g., for diabetes and breast cancer), covering the entire process from lead compound screening to pharmacological measures, and demonstrating its unique value in accelerating drug discovery.

4.1. Applications of ^{19}F NMR in Elucidating Structures and Activation Mechanisms of Disease-Associated Membrane Proteins

Membrane proteins play a significant role in regulating cellular viability, and their function depends on various factors, including conformational changes, membrane environment effects, and assembly of functional complexes. In this section, we discuss in detail the application of ^{19}F NMR to various membrane proteins and introduce the exploration of in situ ^{19}F NMR for the characterization of membrane proteins (Table 2).

GPCRs, the largest family of human membrane proteins, regulate nearly all life processes, including neurotransmitter transmission, hormone secretion, and cardiovascular homeostasis [83,84]. Currently, more than 30% of Food and Drug Administration (FDA)–approved drugs act on GPCRs [85]. Their signal transduction relies on sophisticated conformational dynamics. In recent years, with the widespread application of X-ray crystallography and cryo-EM, high-resolution structures of various GPCRs have been obtained in both inactive and active states. However, these techniques are predominantly limited to capturing static structural snapshots, and although conformational heterogeneity can be partially resolved through computational classification, they cannot directly monitor real-time dynamic processes, thus providing an incomplete picture of the conformational landscape and activation dynamics of GPCRs. ^{19}F NMR technology fills the gap by providing further insights into local conformational changes and kinetic information [57]. Furthermore, ^{19}F NMR technology enables quantification of transitions between different conformational states, providing clear insights into the four conformational states of the adenosine A_{2A} receptor ($A_{2A}R$) in the absence of ligands and under various ligand–binding conditions. This holds profound significance for understanding the function of G protein–coupled receptors in both health and disease [86]. Moreover, in interaction studies, ^{19}F NMR was used to map the conformational dynamics of the entire activation pathway of the $A_{2A}R$, revealing for the first time the key role of the Gβγ subunit in allosteric signal transduction [38].In research on metabolic receptors, ^{19}F NMR technology has successfully revealed the real–time dynamic processes of interaction between class B G protein–coupled receptors—the glucagon–like peptide–1 receptor (GLP–1R) and the glucagon receptor (GCGR)—and their respective ligands. These two receptor types are activated by glucagon–like peptide–1 (GLP–1) and glucagon (GCG), respectively, and play key but oppositely acting regulatory roles in the maintenance of blood glucose homeostasis. Using ^{19}F NMR techniques, the researchers successfully assigned the ^{19}F signals to the transmembrane domains of GLP–1R and GCGR and monitored in real time the conformational changes in these domains induced by ligand binding. This work overcomes the limitations of traditional static structural analysis and deepens our understanding of the molecular mechanisms of blood glucose regulation at the solution–dynamic level, and provides important structural–dynamic insights for the development of anti–diabetic drugs targeting Class B GPCRs [87].

In addition to the GPCR family, ^{19}F NMR technology is also widely applied to other receptor proteins involved in regulating metabolism and immunity. Transient receptor potential melastatin family member 8 (TRPM8) is an ion channel that controls the body's perception of cold temperatures and is believed to be associated with physiological processes such as pain and obesity [88]. Wade D. Van Horn and colleagues used ^{19}F NMR to investigate the functional dynamics of TRPM8, linking dynamic structural changes of the receptor to its activation levels and providing new strategies for the efficient development of ion channel modulators [89]. The ^{19}F NMR technique also enabled high–precision, real–time, in situ monitoring of the conformational dynamics of the neurotransmitter transporter homologue LeuT under the influence of ions and substrates [90]. The results revealed that Na^+ ions caused the extracellular vestibule to close, whereas K^+ ions shifted the conformational equilibrium toward an inward–facing intermediate state. These findings provide detailed mechanistic insights into ion–coupled regulation of receptor proteins. Furthermore, ^{19}F NMR represents a novel, structurally resolving analytical method for investigating the activation mechanisms of immune receptors. In studies of the co–stimulatory receptor OX40 involved in T–cell activation, ^{19}F NMR technology has revealed a "tug–of–war" regulatory mechanism. Visualization of changes in ^{19}F spectra clearly demonstrates the opposing forces between receptor oligomerization

and membrane–receptor interactions, providing new insights into the OX40 activation mechanism [91].In addition to investigating conformational changes induced by receptor–protein interactions, ^{19}F NMR can also be applied to quantitative studies of the function of metabolism–related transporters. Taking glucose transporter 1 (GLUT1) as an example, researchers used ^{19}F NMR to track the transmembrane exchange of fluorinated glucose molecules in real time. By analyzing the intensities of cross–peaks between intracellular and extracellular signals in the two–dimensional exchange spectrum (EXSY), they quantified the transporter's transport efficiency and the specificity constants for different fluorinated glucose substrates. This method does not rely on protein purification or reconstitution and allows for the direct acquisition of kinetic parameters in situ while maintaining the native structural and functional state of red blood cells. The established experimental framework and quantitative data provide critical support for the design and screening of specific inhibitors targeting GLUT1 [92].

Another important area of development for ^{19}F NMR is in situ studies of living cells. While traditional NMR techniques face significant background interference and insufficient sensitivity when applied inside cells, ^{19}F NMR—due to the near–absence of endogenous fluorine signals in living organisms—enables direct detection of target signals under near–zero background conditions [80]. This enables real–time tracking of protein conformational changes, ligand binding, and diffusion kinetics under physiologically relevant conditions [93]. As demonstrated with benchmark proteins (GB1 and ubiquitin), high–quality ^{19}F NMR signals can be rapidly and efficiently detected within living cells [94]. Furthermore, ^{19}F paramagnetic relaxation enhancement (PRE) measurements have been successfully implemented in mammalian cells, providing valuable distance constraints for structural characterization under physiological conditions [94,95]. For example, Kigawa and colleagues employed in–cell ^{19}F NMR spectroscopy to investigate the signal transduction mechanisms of Ras proteins directly in living cells [96]. By incorporating *p*–trifluoromethoxyphenylalanine (OCF_3Phe) into H–Ras, they characterized the conformational features of the protein within its native cellular environment. This study demonstrated for the first time that H–Ras exhibits conformational polymorphism on the plasma membrane in the native environment of living cells, providing a new approach to understanding the activation mechanism of Ras and its oncogenic transformation. Okamura et al. used real–time in–cell ^{19}F NMR spectroscopy to successfully observe and quantify the complete dynamic process by which the arginine–rich cell–penetrating peptide—octaarginine (R8)—penetrates the cell membrane of living cells and enters the cytoplasm, thereby providing a physicochemical basis for the spontaneous penetration of cell–penetrating peptides (CPPs) through the cell membrane [97].

Thanks to its unique conformational sensitivity, high sensitivity, and near–zero background interference, ^{19}F NMR has demonstrated exceptional value in elucidating mechanisms ranging from "static structures" to "dynamic mechanisms" across diverse membrane protein systems, including GPCRs, ion channels, transporters, and immune receptors. However, most current studies are still conducted using in vitro reconstituted systems such as liposome or detergent systems and have not yet fully achieved true physiological conditions (Table 2). Although recent studies have attempted to use ^{19}F NMR to investigate in situ conformational changes in membrane proteins, the application of intracellular ^{19}F NMR still faces multiple challenges. For example, probe delivery efficiency is low and intracellular distribution is uneven; target protein concentrations within cells are low; high viscosity leads to severe signal attenuation; and fluorinated probes and their delivery vehicles may exhibit cytotoxicity and interfere with normal physiological functions. These issues hinder, to some extent, the precise in situ analysis of membrane protein structure and function. Thus, continued innovation in novel labeling strategies, the development of low–perturbation, high–sensitivity probes, and the integration of complementary biophysical techniques will unlock new frontiers for ^{19}F NMR in elucidating membrane protein structural dynamics and functional regulation, particularly for in situ applications.

Table 2. Representative applications of ^{19}F NMR to membrane proteins.

Membrane Protein	Milieu	Applications	Reference
β_1AR	LMNG detergent	Characterization of receptor conformational states	[57]
A_{2A}R	Nanodiscs	Conformational change Receptor–protein interaction Lipid regulation of receptor function	[38,41,86]
NK1R	Nanodiscs	Conformation changes	[98]
TRPM8	LPPG detergent	Small–molecule ligand–receptor interaction	[89]
Na^+/Ca^{2+} exchanger proteins (NCXs)	Nanodiscs	Ion-dependent conformational changes Conformational rearrangement	[99]
LeuT	DDM detergent	Ion–dependent conformational changes	[90]
OX40	Nanodiscs	Receptor–signaling–partner interactions Lipid–receptor interaction	[91]
Microsomal cytochrome b5 (cytb5)	Nanodiscs	Conformational-state characterization	[100]
GltPh	DDM detergent	Conformational-state characterization	[64]
Ras	Native Cellular Environment	Structural feature characterization	[96]

In this table, LMNG is the abbreviation for Lauryl maltose neopentyl glycol, LPPG is the abbreviation for lyso–palmitoyl phosphatidylglycerol, DDM is the abbreviation for n–dodecyl–b–D–maltoside.

4.2. ^{19}F NMR in Drug Discovery

Receptor membrane proteins not only maintain body homeostasis by regulating various physiological functions but also serve as key therapeutic targets, as drugs exert their effects through recognition and interaction with these proteins. Drug discovery is a time–consuming and labor–intensive process. Accurate and high–throughput methods are crucial for facilitating drug discovery. Compared to other techniques, ^{19}F NMR demonstrates distinct advantages in drug screening, efficacy assessment, and mechanism of action studies. Depending on the site of ^{19}F labeling, screening can be conducted in two ways (Figure 3A). Statistical analysis indicates that many drugs, particularly small–molecule drugs, contain a fluorine atom [101]. Consequently, methods such as FAXS (fluorine chemical shift anisotropy and exchange for screening) or n–FABS (n–fluorine atoms for biochemical screening) enable the rapid and efficient identification of key drug candidates through spectral changes [102]. Membrane–bound fatty acid amide hydrolase (FAAH) is a class of enzymes that break down endogenous bioactive lipids and other substances, and is involved in regulating various physiological processes, such as pain and emotional states [103]. Drugs targeting this protein can be used to treat conditions such as pain and sleep disorders [104]. The work by Claudio Dalvit and colleagues utilized the n–FABS assay for fragment–based screening of membrane proteins. The n–FABS assay yielded a substantial hit rate of 16.5%. Nineteen previously unidentified small–molecule inhibitors of FAAH were discovered, exhibiting IC_{50} (half maximal inhibitory concentration) values ranging from the micromolar to millimolar range [105]. Furthermore, Chojiro Kojima's work has demonstrated that the use of ^{19}F NMR can effectively improve the accuracy of drug screening, enabling the screening of various targets, including protein kinases and membrane proteins [106].

Another approach to ^{19}F NMR screening involves placing the label on the receptor protein. For example, in studies of GPCR transmembrane receptors, when a ^{19}F label is attached to position 289 of the receptor, the addition of different agonists or antagonists allows clear observation of spectral changes and their intensities [107]. Overall, ^{19}F NMR spectroscopy records protein–drug interactions via one–dimensional (1D) spectra, and by revealing clear binding–induced changes, it offers greater convenience for drug screening.

In addition to screening, kinetic parameters such as binding affinity (K_d), efficacy, and drug residence time are also important factors to be considered during drug development [108]. Due to the high sensitivity of ^{19}F NMR detection and the marked chemical shift changes that occur before and after binding, this technique also plays a significant role in characterizing drug–target binding kinetics and conformational dynamics (Figure 3B) [91,109]. For example, in the work by Angela M. Gronenborn and colleagues, ^{19}F NMR was used to analyze the binding of cyclosporine A (CsA) to fluorinated cyclophilin A (CypA), and the lifetime of the CypA/CsA complex was measured directly in a cellular environment (k_{off} value of $5.4 \pm 0.9\ s^{-1}$), establishing a precedent for studying drug binding under native conditions [110]. Furthermore, in studies of the A_{2A} adenosine receptor, ^{19}F NMR was used to calculate the exchange rates between different activation states of the receptor, thereby enabling quantitative assessment of differences in drug activation potency and efficiency [107].

In addition to poor intrinsic activation efficacy, an unclear mechanism of action can compromise drug efficacy and may even lead to toxic side effects. Due to the flexibility of ^{19}F NMR labeling, which enables detection at different sites across the entire receptor, it is possible to observe changes in the intracellular structure of the receptor while the drug is bound (Figure 3C). Human epidermal growth factor receptor 2 (HER2) is overexpressed in more than 20% of breast cancers and in various other malignancies, making it a key target for cancer therapy [111]. By constructing the HER2 receptor structure in vitro and titrating a single–domain antibody (sdAb), researchers observed significant conformational changes before and after antibody binding, revealing the dynamic behavior of the tumor target HER2 in a near–physiological membrane environment and the antibody–induced conformational rearrangements [112]. Similarly, in studies of the β_2AR, comparing the ^{19}F spectra of the receptor in its resting state with those obtained after the addition of the inverse agonist carazolol, the partial agonist salmeterol, and the full agonist BI–167107 revealed distinct spectral changes for each ligand [113]. This enabled a detailed comparison of the effects of different drugs on receptor conformation and demonstrated the sensitivity of ^{19}F NMR technology for exploring the interaction mechanisms between therapeutically valuable drugs and their receptors.

^{19}F NMR technology demonstrates unique value throughout the entire drug discovery and development process: it enables high–throughput drug screening through ligand or receptor labeling; it allows quantitative assessment of pharmacological parameters such as affinity, potency, and complex lifetime via chemical shift changes; and it facilitates simultaneous monitoring of intracellular conformational changes in receptors through multi–site labeling, thereby providing deep insights into drug mechanisms of action. Collectively, these attributes

position ^{19}F NMR as a versatile platform that integrates drug screening, binding and functional characterization, and mechanistic interrogation, thereby providing a valuable toolkit for accelerating drug development, guiding rational drug design, and elucidating the functional mechanisms of membrane protein targets.

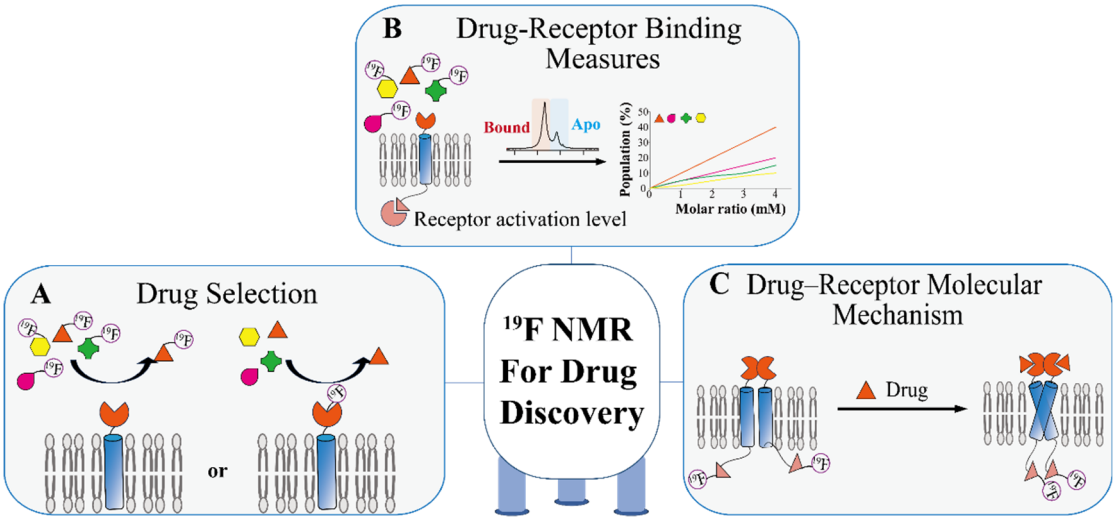


Figure 3. Schematic diagram of the ^{19}F NMR drug discovery and pharmacological evaluation platform (**A**) Drug selection using ^{19}F NMR. Two strategies for high–throughput drug screening using ^{19}F NMR technology. For fluorinated drugs, changes in the spectra of the free and bound forms can be directly compared to identify binding events (**left**); for non–fluorinated drugs, a ^{19}F label can be specifically introduced into an appropriate site on the target protein to indirectly monitor signal changes caused by drug binding (**right**). (**B**) Quantitative analysis of receptor activation levels and drug-receptor binding parameters. By analyzing NMR spectral changes upon drug–receptor binding, the concentration–dependent binding and conformational response can be quantitatively characterized. (**C**) Research on drug–receptor molecular mechanisms. By introducing ^{19}F labels into key functional domains of receptors, real–time monitoring of local conformational rearrangements induced by drug binding is achieved. Analyzing chemical shift perturbations and changes in relaxation behavior enables elucidation of the molecular logic linking drug binding to functional regulation, thereby providing mechanistic guidance for structure–based drug design and the development of allosteric modulators.

5. Summary

As central mediators of cellular signal transduction and material transport, membrane proteins exhibit intrinsic conformational dynamics that fundamentally challenge conventional structural biology approaches. This review identifies ^{19}F NMR as a powerful tool for elucidating the molecular mechanisms of membrane proteins. It highlights the unique physicochemical advantages of ^{19}F, including 100% natural abundance and near–zero endogenous fluorine background, and elaborates on the selection, labeling, and practical applications of ^{19}F probes across various membrane protein systems.

Overall, the application of ^{19}F NMR has revealed functional conformational intermediates hidden behind static high–resolution structures, promoting the transition of structural biology from “snapshot” observation to “movie”–like dynamic analysis. Whether studying GPCR biased signaling or ion channel gating kinetics, dynamic detection and analysis bring us closer to physiological reality. Currently, ^{19}F NMR technology is still evolving. Beyond the content discussed in this review, existing studies have achieved kinetic quantification via one–dimensional ^{19}F lineshape analysis, providing an efficient and convenient alternative for high–throughput drug screening [36]. ^{19}F–^{19}F TOCSY experiments have clearly detected couplings beyond the van der Waals radius [114]. Furthermore, dynamic nuclear polarization (DNP)–enhanced magic–angle spinning solid–state NMR has enabled atomic–resolution structural characterization of specific proteins in mammalian cells [115]. The integration of artificial intelligence (AI) is another trend in the development of ^{19}F NMR. Research has already led to the development of a method called LEFshift, based on the random forest machine learning algorithm, which can efficiently predict the ^{19}F chemical shifts of molecules in a compound library without the need for experimental measurements. Combined with the ^{19}Focused screening strategy, this method enables direct deconvolution of mixed NMR signals, thereby accelerating the drug discovery process for disease targets [116]. At a higher level of precision, some studies have employed large–scale QM/MM calculations and successfully reproduced the changes in ^{19}F chemical shifts upon binding of covalent inhibitors to target proteins, fully demonstrating the utility

of high–precision calculations in elucidating binding modes of drugs [117]. Collectively, these methodological innovations have propelled ^{19}F NMR beyond its traditional role as a supplementary probe, establishing it as a versatile quantitative platform that simultaneously delivers structural restraints, dynamic parameters, and interaction information.

Looking ahead, with the development of more sensitive fluorinated probes and continued advancements in multi–modal ^{19}F NMR technology, the scope of application for this technique is expected to expand from in vitro reconstitution systems to in situ dynamic observations at the level of living cells and even tissues, playing an increasingly important role in the study of metabolic– and disease–related membrane proteins. In the field of metabolic regulation, ^{19}F NMR holds promise for real–time tracking of substrate transport processes in nutrient transporters, as well as the allosteric conformational dynamics of metabolically active GPCRs and ion channels, thereby deepening our understanding of the molecular mechanisms underlying metabolic diseases such as diabetes, obesity, and cardiovascular disease. In drug discovery, this technology may contribute to the development of allosteric modulators and covalent inhibitors targeting membrane proteins, providing critical dynamic structural information for precision drug discovery. Furthermore, the deep integration of ^{19}F NMR with machine learning will advance the precise prediction of chemical shifts and the automated interpretation of spectra, significantly enhancing its applicability and throughput in complex biological systems. These advancements will collectively position ^{19}F NMR as a powerful analytical tool linking the dynamic conformational changes of membrane proteins to the mechanisms underlying metabolic diseases.

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