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Nanodiscs and solution nuclear magnetic resonance

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Although membrane proteins constitute a significant portion of the genomes of all species and represent well-validated targets for numerous therapeutic interventions, high-resolution structural knowledge of this class of proteins still falls behind that of their soluble counterparts. Despite serious technological developments in the methods presently available for structural characterizations, as well as decades spent on such investigations, membrane proteins remain notoriously difficult to study. This is particularly true for environments which mimic native membranes well enough to maintain their proper functional states. This mini review covers the most recent advances in the structural and dynamic characterization of membrane proteins through the utilization of solution nuclear magnetic resonance methods applied to lipid nanodiscs.

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Introduction

Membrane proteins (MPs) are essential components of cell and organelle membranes, responsible for a myriad of different functions. These include intercellular signaling and intracellular signal transduction, ion and metabolite transport, cell structural integrity maintenance, and the support of enzymatic reactions and energy production. Although comprising only about 30% of the human genome, MPs serve as targets for about 70% of the currently available drugs [1]. Within these, G protein-coupled receptors (GPCRs) represent the most sought-after subclass of proteins in drug development [2].

Historically, two methods have been utilized for high-resolution structure determination of biomacromolecules (including MPs), with most structures being obtained by X-ray crystallography and the remainder by solution nuclear magnetic resonance (NMR). This distribution highlights the molecular size limitations of the latter technique. In the recent decades, however, significant technological developments have allowed for the extension of the available toolkits for high-resolution structural studies. This has been achieved through the inclusion of cryo-EM and solid-state NMR techniques, and by pushing the size limits of solution NMR. This review will focus on solution NMR methodology as this technique allows the investigation of not only structural but also dynamic features of the target, with the goal of better understanding its function within a cell.

Several different types of membrane mimetics have been introduced over the years to support structural studies of MPs, each with specific pros and cons. However, no media provides a silver bullet for all cases. Other sources provide a thorough description of the approaches available for MP extraction and solubilization, the process necessary for the application of solution NMR methods [3]. Hence, the extensive classification of these membrane-mimicking environments is not a major focus of this review. This review covers a single class of membrane mimetics—lipid containing nanodiscs—which are further separated into subclasses as discussed below.

Lipid nanodiscs

i) Membrane scaffold protein-based

Lipid nanodiscs were originally envisioned by Sligar and et al. [4–6] through the manipulation of a high-density apolipoprotein ApoA1 derivative presently known as membrane scaffold protein (MSP), to form a belt surrounding hydrophobic edges of the lipid bilayer. This belt comprises two antiparallel multicomponent amphipathic helixes, with the hydrophobic faces of the helixes interacting with acyl lipid tails while the hydrophilic portions are exposed to aqueous solution. The diameter of the formed discs is determined by the length of the MSP variant, stretching from ∼7 to ∼13 nm depending on the number of helical components within a single MSP construct [3,7]. Smaller discs, with diameters less than 7 nm, are unstable and tend to shift to lager complexes or aggregates [8,9]. Larger discs, with diameters of ∼17 nm, are designed for the incorporation of larger MPs, and have been developed through the linear combination of two copies of MSP proteins [10]. However,

these constructs are not optimal for solution NMR applications due to the intrinsic size limitations of the method. Incorporated MPs replace the lipid molecules within the bilayer, thus the optimization of the ratios among MP, MSP, and lipid are essential for effective nanodisc formation. The overall molecular weight (MW) of the complex reflects the MW of the incorporated MP, two molecules of MSP, and the remaining lipids. The hydrocarbon thickness of the discs depends on the lipids used to form a bilayer. For phosphatidylcholines (PCs), for example, it varies from ∼2.5 nm in dimyristoyl-PC (DMPC) to ∼3.2 nm in distearoyl-PC (DSPC) discs [3]. The physicochemical and biological properties of the discs also depend strongly on the lipid composition. Further, nanodisc stability and homogeneity are improved through circularization, by covalently linking the N- and C-termini of MSP construct. For example, Sortase A-mediated MSP ligation produced a better NMR spectral quality in two tested cases, namely VDAC-1, a β-barrel membrane protein, and NTR1, a GPCR [11]. Another approach to connect the N- and C- termini of MSP constructs is *in vivo* split intein splicing [12]. The properties of several circularized nanodiscs were further analyzed by a collection of biophysical techniques, with the spectroscopy proven possible at elevated temperatures beneficial for high-resolution NMR studies, and a confirmed improved quality of solution NMR spectra of VDAC-1, in comparison with linear nanodiscs [13].

ii) Saposin A

In the search for smaller lipid-incorporated discs, another protein has shown promising properties. Two molecules of saposin A (SapA), a lysosomal lipid transfer protein, joined together around lipids forming a nanodisc [14]. Original studies with lauryl-dimethyl-amine-oxide detergent have shown that SapA discs, also known as Salipro nanoparticle, are ∼3.2 nm in diameter with MW weight of ∼43 kDa, which is smaller than that of the smallest nanodisc (∼62 kDa for D7 or ∼70 kDa for ΔH4-H5 constructs). For SapA-DMPC discs, MW is found to be similar (37 kDa) [15]. However, the dynamic nature of SapA allows for the accommodation of MPs of varying size through the utilization of multiple SapA molecules. The additional advantage of using SapA for discs formation is its applicability within a wider range of pH values [15]. The disadvantages, however, include an increased risk of reconstituted MPs spontaneously oligomerizing into non-native complexes, as well as the potential for a forged interaction between the MP and SapA due to the limited lipid content surrounding the reconstituted MP [16].

iii) Copolymer discs

SMALPs are discoidal lipid—polymer aggregates, where a belt surrounding hydrophobic edges of the lipid bilayer is formed by styrene—maleic acid (SMA) composed of styrene and maleic acid in ratios of 2:1 or 3:1 [17]. SMALPs assemble spontaneously in aqueous solutions and can directly solubilize MPs, with the endogenous lipids surrounding it from the native membrane. However, the narrow pH range at which experiments should be performed (about 6.8—7.5) is disadvantageous for solution NMR applications. This limitation is due to SMA pKa (6.5), and to overcome this, the polymer must be modified. Indeed, maleimide quaternary ammonium (SMA-QA)-derivatized copolymer discs show an improved stability at low pH and, in addition, better size control [18]. Introduction of an SMA derivative with a negatively charged taurine moiety, SMA-tau, also demonstrated that this polymer can form nanodiscs with a patch of lipid bilayer [19].

Styrene-free alternatives have been sought as a cheaper alternative to SMA. As the result, an aliphatic copolymer with alternating diisobutylene/maleic acid, DIBMA, appears to form slightly larger heterogeneous nanoscale discs [20]. As alternative approach, a library of amphiphilic poly[styrene-*co*-(sodium 4-styrene sulfonate)] copolymers (termed SSS) was synthesized. Out of it, two copolymer compositions (SSS-L30 and SSS-L36) were shown to solubilize membranes to an extent similar to SMA; however, in contrast to SMA, both remained soluble at low pH and in the presence of Mg^{2+} ions [21]. The polymer-based macro-nanodiscs, composed, for example, of negatively charged SMA-EA polymers and DMPC lipids [22], with diameters in an excess of 20 nm, generate sufficient magnetic susceptibility anisotropy to allow alignment in an external magnetic field. These are useful as an alignment medium to measure residual dipolar couplings (RDCs) of soluble biomolecules. The charge of the belt-forming polymer, however, instils a major limitation for functional reconstitution of MPs possessing an opposite net charge to that of the polymer. To abate this limitation for functional reconstitution of a membrane-bound redox complex constituting a cationic cytochrome P450 (CYP450) and an anionic cytochrome P450 reductase, Ramamoorthy lab has developed nonionic inulin-based polymer discs [23]. One of the most recent additions to the copolymer discs toolkit comes in the form of SMA block copolymers with acrylic acid and methacrylic acid used to widen the operational pH range of SMA in regard to maintaining its water solubility at low pH and stabilizing its MP assemblies in solution [24].

iv) Peptides-based

An 18-residue amphipathic peptide, derived from the ApoA1 single helical component, has been shown to form discoidal lipid particles similar to MSP nanodiscs [25]. Interestingly, these peptides have the ability to solubilize the membrane; therefore, MP extraction can

be achieved in the absence of detergents [26]. However, since these peptides do not form a continuous belt around the rim of lipid bilayers, the nanodiscs formed are endogenously heterogenous, dynamic, and relatively unstable, looking more like classical lipid bilayers (with rims formed by detergent)—bicelles [27]. Larger discs, called macrodiscs, with the diameter within a 30 nm range, have been developed by combining multiple copies of another 14-residue amphipathic peptide and are used as an alignment media for RDC measurements [28].

v) DNA scaffolds

An interesting approach, namely formation of DNA-encircled lipid bilayers (DEBs), requires DNA modification through selective alkylation. In this case, an amphipathic rim surrounding hydrophobic edges of the lipid bilayer is formed by alkylated DNA, with the diameter of the discs being a direct reflection of DNA length: for example, a 147-nucleotide-long DNA circle results in nanodiscs of ∼14.7 nm in diameter [29]. Since DNA strand length can be modulated at will, nanodiscs assembly of various sizes, including small, beneficial for solution NMR, becomes possible.

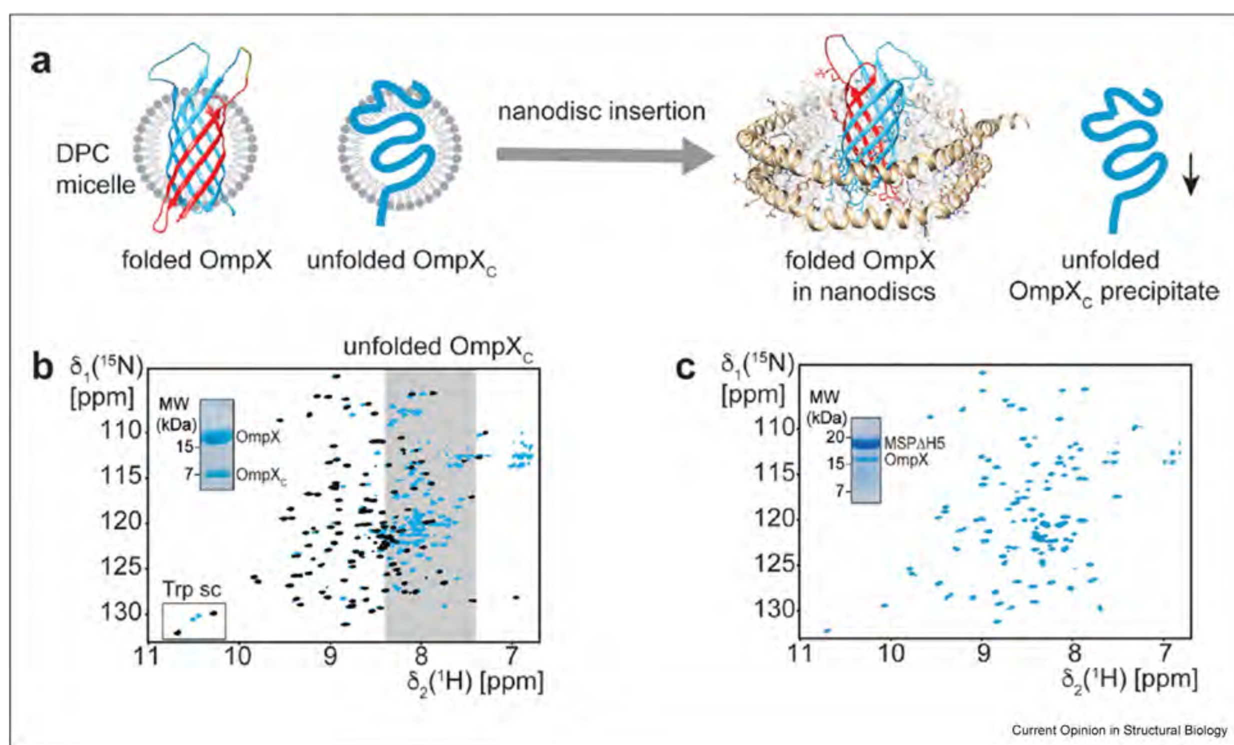
Recent solution NMR studies of membrane proteins in lipid nanodiscs

i) Segmental labeling

Segmental isotope labeling is often a suitable strategy to overcome broad resonance and peak overlap, enabling high-resolution structure determination of large targets by solution NMR. However, protein ligation within integral membrane proteins is complicated by the insolubility of the hydrophobic protein fragments and problems with the removal of ligation side-products. A stabilized split intein system has been shown to resolve these issues. Consequently, high-quality NMR spectra of OmpX (Figure 1) and MPV17, with a β-barrel and α-helical topology, respectively, were collected in lipid nanodiscs with markedly reduced complexity [30].

ii) Transverse relaxation optimized spectroscopy-based structural investigations

Figure 1



(a) After intein splicing and refolding, properly folded full-length and a misfolded $OmpX_C$ fragment are copurifying in DPC detergent micelles, giving rise to a TROSY spectrum containing signals of both species (blue spectrum in panel **(b)**. The black spectrum in **(b)** is a reference with folded labeled OmpX in DPC micelles. Insertion into lipid nanodiscs selects for the properly folded species and efficiently removes the $OmpX_C$ fragment, indicated by TROSY spectrum lacking signals in the unfolded region **(c)**. Reproduced from [ref 30]—Melina Daniilidis, Laura E. Sperl, Benedikt S. Müller, Antonia Babl, and Franz Hagn, "Efficient Segmental Isotope Labeling of Integral Membrane Proteins for High-Resolution NMR Studies", J. Am. Chem. Soc. 2024, 146, Figure 3, page 15,406. Available under a CC-BY 4.0 license. Copyright 2024 the Authors. DPC, dodecylphosphocholine; TROSY, transverse relaxation optimized spectroscopy

Structural and dynamic studies of human GPCRs in a lipid bilayer environment are of key importance for understanding structure–function relationships in continued drug development. Transverse relaxation optimized spectroscopy (TROSY)-based methods on partially (70%) deuterated samples may also provide sufficient spectral resolution. For example, it was confirmed that the complex of human A_{2A} adenosine receptor (A_{2A}AR) with an inverse agonist adopts its global fold in lipid nanodiscs in solution at physiological temperature (Figure 2). The lower-than-expected number of peaks can most probably be explained by a combination two reasons, a long overall correlation time and conformational exchange at intermediate timescale. Since the spectrum of A_{2A}AR looks sharper in nanodiscs, it seems that the receptor is better stabilized in lipid environment, reducing molten-globular type of motion. The NMR signal of five individually assigned tryptophan indoles, located in different regions of the receptor structure, further enabled a detailed assessment of the impact of nanodiscs on the local structure and dynamics of A_{2A}AR [31].

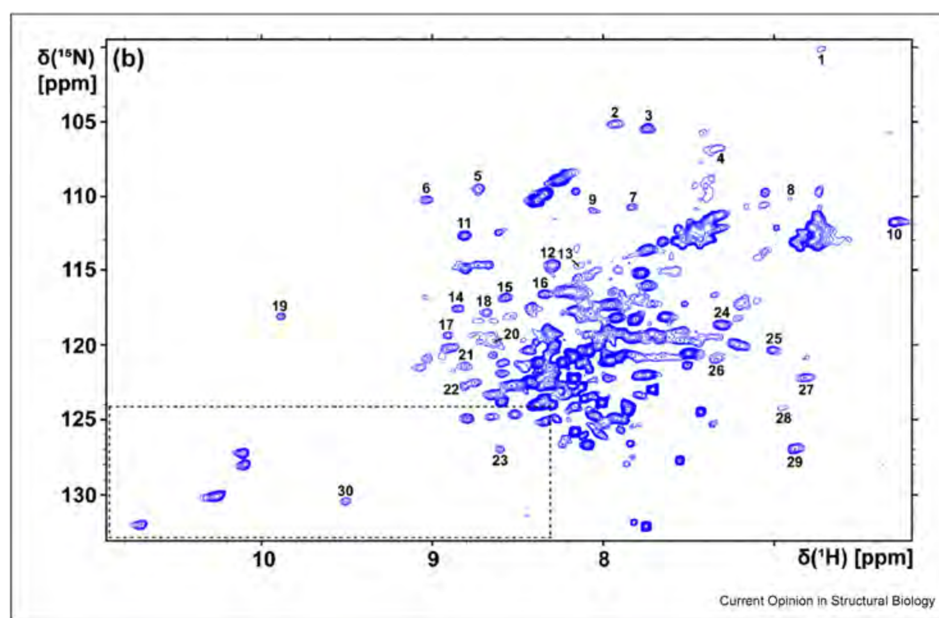
iii) Fluorine-19 nuclear magnetic resonance /dynamics

A full high-resolution structural analysis of a particularly large nanodisc-incorporated MP is not always possible, even with the help of TROSY-based experiments and smart isotope labeling schemes. This is often due to extreme broadness and/or complexity of the spectra in classical triple-resonance approach. In these cases, however, many specific questions can still be addressed, particularly by fluorine nuclear magnetic resonance spectroscopy.

As an excellent example, fluorine-19 nuclear magnetic resonance (^{19}F-NMR) allowed delineation of key functional states of the A_{2A}AR complexed with heterotrimeric G protein. An ensemble of inactive states, a G-protein-bound activation intermediate, and distinct nucleotide-free states associated with either partial or full agonist-driven activation have been illuminated [32]. Other group used ^{19}F-NMR spectra across a wide temperature range (Figure 3) to correlate functional data with ensemble population distributions, showing the best fit at physiological conditions: A_{2A}AR complexes with partial agonists and full agonists have shown large increases in the population of a fully active conformation with increasing temperature [33].

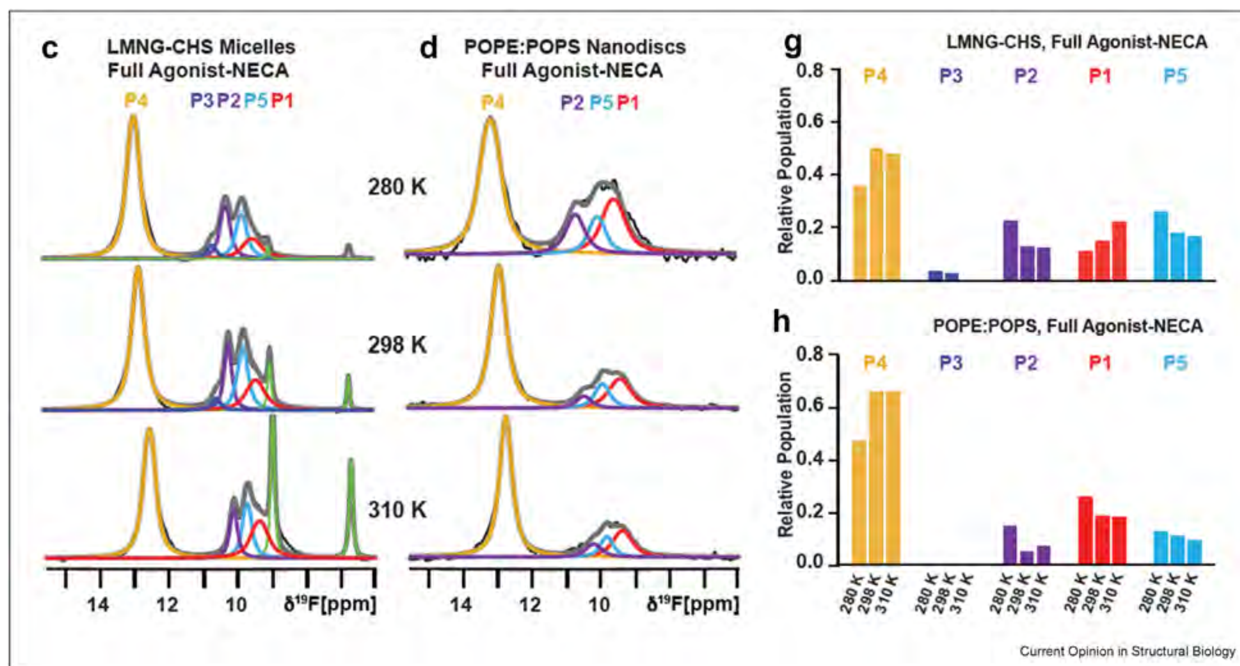
The importance of membrane lipid composition should not be underestimated. It has been shown that direct receptor–cholesterol interactions can drive activation of agonist-bound A_{2A}AR in membranes containing zwitterionic phospholipids. The presence of anionic lipids has been shown to attenuate the impact of cholesterol [34]. However, another study of fluorinated cholesterol analogs suggested transient interactions with A_{2A}AR, indicating a lack of high-affinity binding or direct allosteric modulation. The observed allosteric effects were proposed to be mostly indirect and originated from

Figure 2



TROSY spectrum of the complex of A_{2A}AR with the inverse agonist ZM241385 in MSP1D1ΔH5 (POPC/POPS 7:3) nanodiscs **(b)**. Reproduced from ref [31]—Canyong Guo, Lingyun Yang, Zhijun Liu, Dongsheng Liu and Kurt Wüthrich, "Two-Dimensional NMR Spectroscopy of the G Protein-Coupled Receptor A2AAR in Lipid Nanodiscs", Molecules 2023, 28, page 5419(5). Available under a CC-BY 4.0 license with authors' permission. Copyright 2023 the Authors. A_{2A}AR, A_{2A} adenosine receptor; TROSY, transverse relaxation optimized spectroscopy

Figure 3



Temperature-dependent variations in the conformational equilibria of $A_{2A}AR$: variable temperature ^{19}F NMR spectra of the complex with the agonist NECA, N-ethylcarboxamidoadenosine reconstituted in the LMNG-CHS micelles **(c)** and POPE:POPS nanodiscs **(c)**. Relative populations of each state over the range of studied temperatures, determined from the fitted data, are shown in a bar chart format (g and h for the micelles and nanodiscs, respectively). Reproduced from ref [33]—Naveen Thakur, Arka Prabha Ray, Beining Jin, Nessa Pesaran Afsharian, Edward Lyman, Zhan-Guo Gao, Kenneth A. Jacobson, and Matthew T. Eddy, "Membrane mimetic-dependence of GPCR energy landscapes", Structure 2024, Figure 4c,d,g and h, page 530. Available under CC-BY-ND 4.0 license with authors' permission. Copyright 2024 the Authors. $A_{2A}AR$, A_{2A} adenosine receptor; CHS, cholesteryl hemisuccinate; ^{19}F NMR, fluorine-19 nuclear magnetic resonance; LMNG, lauryl-maltose-neopentyl-glycol.

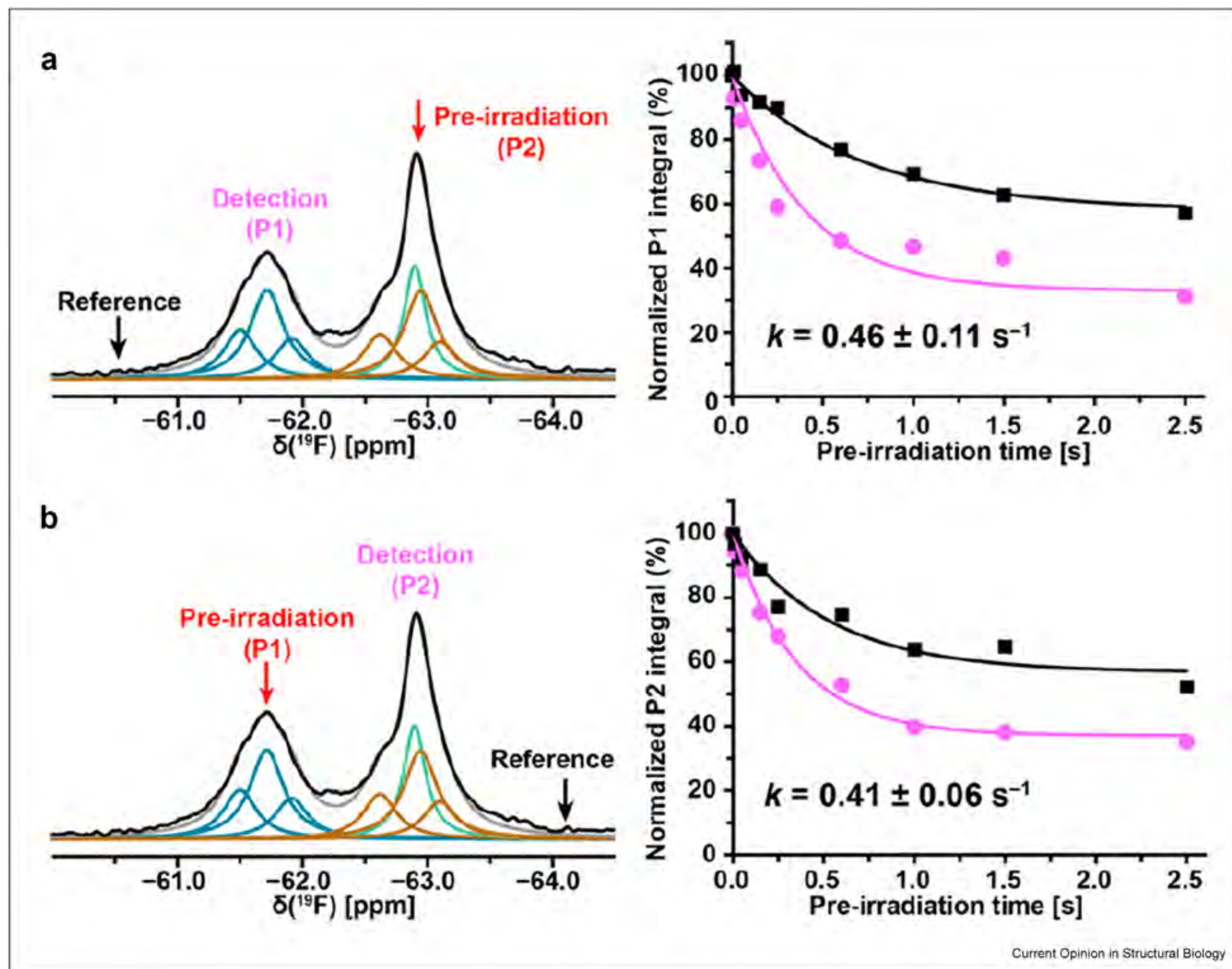
cholesterol-mediated changes in membrane properties as shown by membrane fluidity measurements and high-pressure NMR [35].

When micelles are utilized to study GPCRs, detergent screening to choose the best possible option is recommended. As an example, it has been shown that the populations of locally different substates are markedly different in dodecyl-D-maltoside/cholesteryl hemisuccinate (CHS) and lauryl-maltose-neopentyl-glycol (LMNG)/CHS micelles, whereas the $A_{2A}AR$ conformational manifold in LMNG/CHS micelles is closely similar to that of the lipid bilayer nanodiscs [36]. Considering that nanodiscs represent a better mimetic of the natural lipid bilayer, LMNG/CHS micelles might be a good choice for reconstitution trials of class A GPCRs.

The ^{19}F-NMR also allows observing dynamic conformational intermediaries that remain elusive from rigid snapshots obtained by predominant methods such as X-ray crystallography or cryo-EM. The study of aprepitant, an approved drug that targets the neurokinin 1 receptor for the treatment of chemotherapy-induced nausea and vomiting, revealed 180° flipping motions of the bis-trifluoromethyl-phenyl ring about a single bond that attaches it to the core of the molecule (Figure 4). This reflects transient structure fluctuations with amplitudes up to at least 6 Å, indicating that the GPCR transmembrane helices undergo large-scale floating motions in the lipid bilayer [37].

Conformational changes in nanodisc-incorporated Na^{+}/Ca^{2+} exchanger (NCX) were investigated by Khiem et al. [38]. Consecutive interactions of $3Na^{+}$ or $1Ca^{2+}$ in these transporters are known results in an alternative exposure (access) of the cytosolic and extracellular vestibules to opposite sides of the membrane, where ion-induced transitions between the outward-facing (OF) and inward-facing (IF) conformational states drive a transport cycle. Multiple substates of apo and ion-bound species were observed in nanodisc-reconstituted (but not in detergent-solubilized) NCX, meaning that the lipid−membrane environment preconditions multiple substate populations toward the OF/IF swapping. Importantly, ion-induced substate redistributions occurred within each major state, where substate interconversions may precondition the OF/IF swapping. In contrast with large changes in population redistributions, the sum of substate populations within

Figure 4



Large-amplitude structure fluctuations of NK1R in nanodiscs detected by ^{19}F NMR observation of the bound drug aprepitant (Lorentzian deconvolution of its NMR spectra revealing the following substates: P1a, P1b, and P1c (blue) represent one of the two $-CF_3$ groups; M (green) represents micelle-associated "free" aprepitant; P2a, P2b, and P2c (brown) represent the second $-CF_3$ group; the sum of P1, M, and P2 is shown in gray). ^{19}F saturation transfer between the two trifluoromethyl groups was investigated. **(a and b)** The red arrow indicates the carrier position for the pre-irradiation, and the black arrow the position of the reference irradiation; the detection position is indicated with purple lettering. Lorentzian deconvolution of the 1D ^{19}F-NMR spectra is used to identify a minimal number of overlapping signals that provide a quantitative fit of the experimental data. The plots on the right show the normalized integrals of the observed peak at different pre-irradiation times on resonance and at the reference position. The Bloch−McConnell equation was used to fit the experimental data and derive the exchange rates. Reproduced from ref [37]—Benxun Pan, Dongsheng Liu, Lingyun Yang, and Kurt Wuthrich, "GPCR large-amplitude dynamics by 19F NMR of aprepitant bound to the neurokinin 1 receptor", PNAS 2022, 119, Figure 3, page 5. Available under a CC-BY-NC-ND 4.0 license. Copyright 2022 the Authors. ^{19}F NMR, fluorine-19 nuclear magnetic resonance; NK1R, neurokinin 1 receptor.

each inherent state (OF or IF) remained nearly unchanged upon ion addition.

iv) Binding/mechanistic studies

Investigation of MPs related to disease states in lipid nanodiscs provides a more reliable connection between structure and function. In the case of Alzheimer's disease, the fragmentation of the membrane-bound amyloid precursor protein (APP) results in the production of amyloid-β peptides, the starting point for amyloid toxicity. The 99-residue C-terminal domain of APP was successfully reconstituted into polymer nanodiscs and characterized using size-exclusion chromatography, mass spectrometry, solution NMR, and magic-angle spinning solid-state NMR. The feasibility of using lipid-solubilizing polymers for isolating and characterizing APP in the native *E. coli* membrane environment was demonstrated as well [39]. Misfolding of α-synuclein, a key marker in Parkinson's disease, depends upon lipid environment. The effects of zwitterionic vs anionic lipids and cholesterol content on α-synuclein binding to lipid bilayer, as well as its oligomerization and

fibrillation, have been investigated in SMA nanodiscs [40]. Solution NMR in this study allowed observing residue-specific effects.

In coagulation cascade, activated factor VIII (FVIIIa) binds to activated platelet membranes, forming the intrinsic tenase complex with activated factor IX. Avery et al. proposed a membrane-binding model for the FVIIIa C1 and C2 domains and used NMR to identify residues that interact with soluble phosphoserine and lipid nanodiscs. In addition, increasing phosphatidylethanolamine and decreasing phosphatidyl-serine content has been shown to decrease overall FVIII affinity for membrane surfaces [41].

Bak is a pore-forming Bcl2 protein that induces apoptosis at the outer mitochondrial membrane via oligomerization. This process, however, can be inhibited by anti-apoptotic Bcl2 proteins, such as BclxL. BclxL is very efficient in inhibiting Bak pore formation, but the mechanistic basis of this preferred interaction has remained enigmatic. Leitl et al. utilized NMR to identify Bak-α1 as a second binding site for nanodiscs-associated BclxL and showed that it specifically interacts with the Bcl2-homology 3 binding groove of BclxL, contributing to a better mechanistic understanding of the fine-tuned interactions between different players of the Bcl2 protein family [42].

The KRAS gene plays a pivotal role in numerous cancers by encoding a GTPase that, upon association with the plasma membrane, activates the MAPK pathway, promoting cellular proliferation; it is mutated in a quarter of human cancers. Cornilescu et al. [43], using nanodiscs as a membrane mimetic, demonstrated through NMR and fluorescence resonance energy transfer studies the ability of the small molecule graveoline to perturb KRAS–membrane interaction. Lee *and* Lee [44] utilized size-tunable nanodisc platforms and paramagnetic relaxation enhancement (PRE) analyses to reveal the structural basis of the cooperative assembly of fully processed KRAS. The cooperativity was shown to be modulated by the mutation and nucleotide states of KRAS and the lipid composition of the membrane. Real-time NMR demonstrated that higher-order oligomers retain higher numbers of active GTP-bound protomers in KRAS GTPase cycling, providing a deeper understanding of the nanocluster-enhanced signaling in response to the environment. Gu et al. [45], through the quantitative interpretation of PRE data arising from membrane mimetics with spin-labeled lipids, mapped binding orientation of KRas4B and revealed four distinct orientational states that are close but not identical to reported previously.

Ghanam et al. employed NMR and biophysical methods to characterize Tat_{86}, a human immunodeficiency virus type1 transactivator of transcription, interactions with phospho-inositol diphosphate ($PI_{4,5}P_2$) and lipid nanodiscs [46]. Using NMR relaxation techniques, Lu et al. found that the ligand binding affects not only conformational exchanges between major and minor states but also the affinity of human intestinal fatty acid binding protein to nanodiscs [47]. Sugiki et al. used solution NMR and Isothermal Titration Calorimetry (ITC) to clearly detect a specific interaction between the pleckstrin homology domain of ceramide transport protein and phospho-inositol monophosphate (PI_{4P}) embedded in the lipid nanodisc, and to distinguish the specific binding from nonspecific interactions with the bulk surface of the lipid nanodisc; therefore, providing detailed characterization of the protein–lipid membrane interface [48].

Smoothened (SMO) protein is a member of the GPCR family that is involved in the Hedgehog signaling pathway. Zhu et al. used SMA copolymer to directly extract SMO transmembrane domain and surrounding lipids. The obtained SMA nanodiscs showed high homogeneity and maintained the physiological activity of SMO protein, enabling the measurement of the dissociation constant for SMO ligands using ligand-based solution NMR spectroscopy [49]. Wang et al. introduced a combinational approach integrating NMR and homogenized membrane nanodiscs preparation to characterize the ligand–GPCR interactions. This approach has potential for drug screening as it benefits from minimal receptor preparation with reduced nonspecific binding. Maintaining receptor structural heterogeneity is essential for functional diversity, which improves feasibility for a more reliable probing of ligand–GPCR interaction necessary for faithful ligand discovery [50].

Concluding remarks

A deeper understanding of structural and dynamic properties of MPs still constitutes a major challenge. However, technological developments and scientific advances over the last couple of decades have enabled the visualization of several important members of this diverse collection of proteins. In these efforts, solution NMR applications have been particularly valuable in addressing MP dynamics and conformational diversity. While lipid nanodiscs have superior properties as compared with other membrane mimetics, they are yet to become a true first choice for such studies.

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Credit author statement

Olga Vinogradova contributed to conceptualization, writing–original draft preparation, reviewing and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sbi.2025.103067.

Data availability

No data was used for the research described in the article.

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** of outstanding interest

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