Lipid Dynamics and Protein–Lipid Interactions in Integral Membrane Proteins: Insights from Solid-State NMR

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Integral membrane proteins are of great biomedical and biological interest but are challenging targets for structural biology. One of the challenges is the characterization of membrane protein interactions with a proper lipid bilayer that mimics their native environment. The membrane environment can directly affect the structure of integral membrane proteins and can also be crucial for optimal activity. Solid-state NMR is in a unique position to probe the protein–lipid interplay, by providing insight into both structure and dynamics of the protein as well as the lipids. Under native conditions, biological membranes are characterized by a dynamic and disordered 'liquid crystalline' structure. Solid-state NMR experiments are affected by dynamics, at times in negative ways, but the dynamics can also be leveraged to gain valuable information, including orientational constraints. This article discusses approaches to probe integral membrane protein interactions with lipids, and the role of lipid dynamics, through the use of static as well as magic-angle-spinning solid-state NMR.

Keywords: solid-state NMR, membrane proteins, molecular dynamics, protein structure, order parameters, relaxation, oriented samples, magic-angle-spinning NMR

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Introduction

The study of membranes has been one of the longest-running threads within biological solid-state nuclear magnetic resonance (SSNMR). It can reveal key characteristics of the lipids, while also providing site-specific information on peptides or proteins embedded in true lipid bilayers. Furthermore, one can characterize the interaction of proteins with the bilayer, in terms of topology, interactions with specific lipids, and the interplay between protein and lipid dynamics. These capabilities are of particular importance given that other structural and biophysical methods have difficulty in providing similar information.

Lipid Structure and Dynamics

Biomembranes in Context

Biological membranes delineate the borders of cells as well as intracellular compartments. The lipid composition of these membranes varies and even gets adjusted in response to external stimuli. The lipids that form the bulk of these membranes can spontaneously self-assemble into lipid bilayers, where hydrophobic acyl chains are sequestered away into a hydrophobic 'core' that is shielded from the solvent by an interface layer made up of the lipid headgroups.[1,2] Although phospholipids (featuring a phosphate-based headgroup) are commonly found in these membranes, there are important roles for other components, such as cholesterol (Figure 1a).

Membrane Fluidity and Disorder

A key feature of biological membranes is that normally a highly fluid 'liquid crystalline' state is maintained, reflected in the classic fluid mosaic model.[3] Within the core of the membrane, the acyl chains are extremely mobile and explore many different conformations. The lipids as a whole also undergo wobble motions. The ability of lipids to exchange between the two monolayers (flip flop) is limited, but they have a great ability to diffuse *within* the monolayers via fast Brownian motion within the plane of the bilayer. One consequence of all these dynamics is that there is an inherent distribution in the location (or depth) where particular lipid atoms are found. Figure 1b illustrates this inherent disorder for a liquid crystalline bilayer, based on a combination of experimental and computational studies.[1] Note that this disorder imposes an inherent limit on the precision with which protein–membrane interactions can be determined.

Membrane–Protein Interactions

There are large variations in the local composition of membrane components as a function of location within the cell (e.g., comparing the plasma membrane with the endoplasmic reticulum) or between cell types. In some cases, the presence or absence of specific lipids (e.g., phosphatidylinositols) is used to target proteins to specific membranes. More generally, composition differences cause variations in the structural and biophysical characteristics that may facilitate membrane protein trafficking and can induce changes in protein conformation and function.[4]

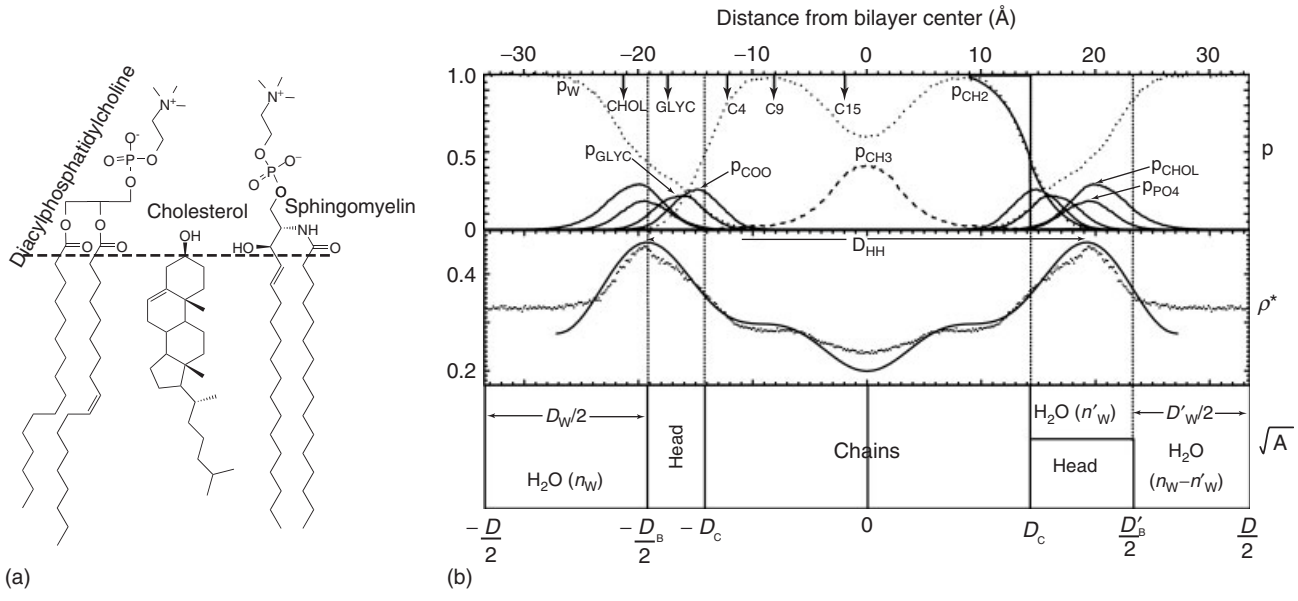

Figure 1. Lipid bilayer structure and composition. (a) Selected membrane components showing the range in chemical structures, resulting in differences in flexibility, preferred curvature, and phase behavior. (b) Structure of a liquid crystalline DPPC (dipalmitoylphosphatidylcholine) bilayer, showing the broad distribution of each individual atomic site, reflecting extensive dynamics within the membrane.[1] (Reprinted from Ref. 1, Copyright 2000, with permission from Elsevier.)

Membrane Studies by SSNMR

Membrane SSNMR Sample Conditions

SSNMR is generally compatible with a wide array of membrane-mimetic sample conditions. Nonetheless, the specific type of SSNMR experiment that one employs may limit the accessible lipid composition, and a careful choice of lipid composition and reconstitution methods is crucial (see also Membrane Proteins and Aligned Membrane Proteins: Structural Studies).[5] Another step in the SSNMR sample preparation usually involves the centrifugal separation of membranes from excess solvent, in order to maximize the amount of sample in the sample tube and thus maximize the signal. This can be facilitated by centrifugal devices that pellet membranes directly into the sample holder.[6] For optimal biological relevance, one would want to ensure that the lipid bilayers are in the liquid crystalline state and 'fully hydrated', which typically requires at least 40 wt% hydration. Most lipids or lipid mixtures undergo a transition into a nonnative, rigid 'gel' state upon cooling below the melting temperature, t_m (see also Lipid Polymorphism). As schematically shown in Figure 2a, this much more ordered state not only has significantly reduced mobility but also features a concomitant increase in membrane thickness. Different biophysical and structural characteristics of dehydrated, frozen, or gel-state membranes may change the conformation of embedded proteins and compromise the desired biological relevance.

In its most simple form, SSNMR measurements are performed on a (hydrated) membrane pellet, stationary in the magnetic field, to yield *static* SSNMR data that reflect the anisotropic NMR signals from all molecules (lipids + proteins) in the vesicles, modulated by any motional averaging that is present. Although such spectra reflect a superposition of multiple broad lines, one can often extract useful structural and motional data (Figures 2 and 3).

One approach to avoid much of the line broadening is to impose *alignment*, such that only a single orientation is observed. This can be accomplished through deposition of membranes on very thin slides, which can lead to alignment of the bilayers (and any protein embedded within). Alternatively, certain lipid mixtures can be made to form bicelles that undergo alignment within a strong magnetic field, thus achieving a similar effect. SSNMR on the aligned membranes then allows for the direct measurement of orientational constraints relative to the aligned lipid bilayer (discussed in Aligned Membrane Proteins: Structural Studies; see also Figure 4b).

Finally, reconstituted membrane samples can yield narrow lines without alignment when submitted to MAS NMR (Figure 4e). Here, the MAS removes the line broadening to yield peaks at the isotropic chemical shift. Although this most commonly avoids the need for preparation of aligned membranes, there have been some demonstrations of MAS on oriented membrane samples.

Lipid Dynamics Studied by SSNMR

SSNMR provides many tools to probe lipid dynamics (see Bilayer Membranes: Deuterium and Carbon-13 NMR, Lipid Polymorphism, and Structural and Dynamics Studies of Lipids by Solid-State NMR). The *in-plane lipid diffusion* rate can be measured by pulsed field gradient NMR.[7] The diffusion is typically not rapid enough for molecules to circumnavigate liposomes within the NMR timescale, leading to static SSNMR spectra with broad lines. The observed width and shape reflect the CSA and other anisotropic interactions, and

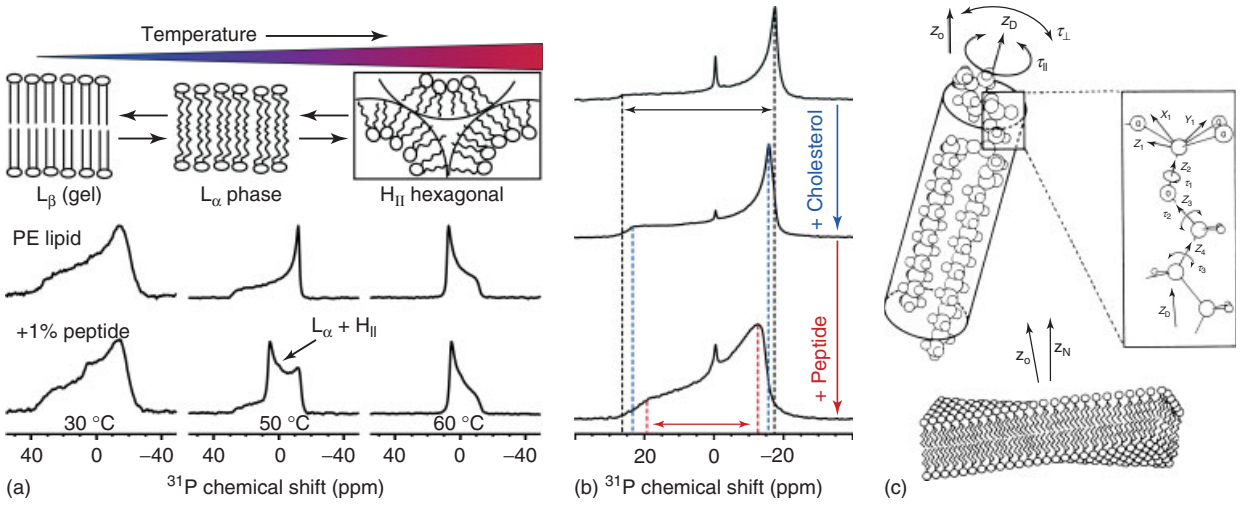

Figure 2. ^{31}P NMR and phospholipid dynamics. (a) Lipid phase behavior can be monitored by static ^{31}P NMR, permitting detection of, e.g., curvature modulation by proteins.[32] (Adapted with permission from Ref. 32. Copyright 2000 American Chemical Society.) (b) ^{31}P NMR lineshapes of fluid bilayers are sensitive to changes in lipid dynamics induced by cholesterol (middle) and bound peptides (bottom).[33] (Adapted with permission from Ref. 33. Copyright 2012 American Chemical Society.) (c) The exact lineshape of the ^{31}P spectra depends on a combination of lipid motions that separately affect the apparent CSA and the underlying linewidth (T_2 relaxation).[8] (Reprinted from Ref. 8, Copyright 1992, with permission from Elsevier.)

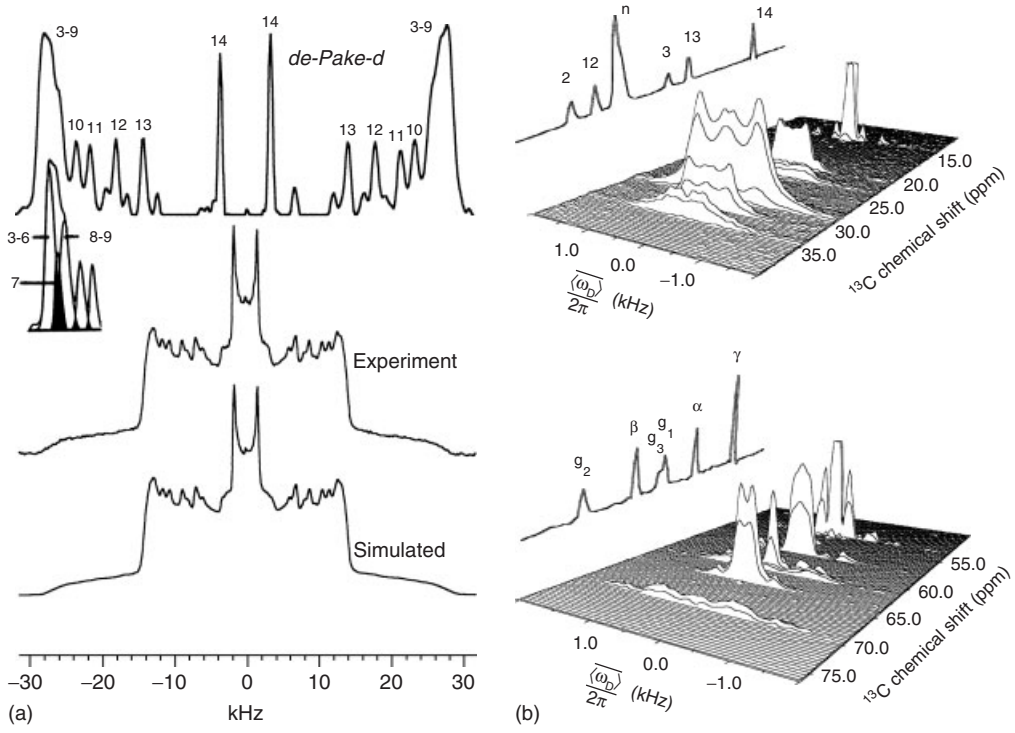

Figure 3. Lipid acyl chain dynamics. (a) 1-D ^{2}H NMR on aligned or nonaligned lipids featuring perdeuterated acyl chains allows the determination of C–D order parameters that reflect the local (dis)order.[34] One can infer changes in bilayer thickness from such data, for instance, upon incorporation of a transmembrane α-helix (shown here). Experimental spectra (middle) can be simulated (bottom) to obtain site-specific order parameters, sometimes facilitated by de-Pake-ing (top) to show enhanced resolution of an oriented spectrum. (Reprinted with permission from Ref. 34. Copyright 1998 American Chemical Society.) (b) MAS SSNMR enables the site-specific measurement of C–H dipolar couplings, to yield analogous order parameters, without the need to incorporate deuterated lipids.[11] (Reprinted with permission from Ref. 11. Copyright 1997 American Chemical Society.)

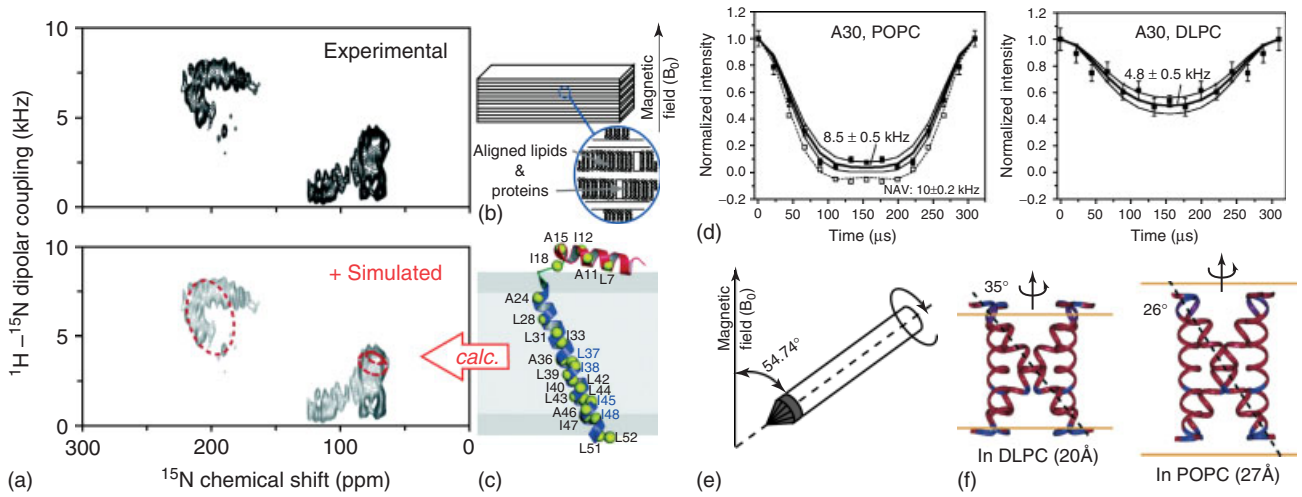

Figure 4. Orientational constraints in lipid bilayers. (a) Static SSNMR on macroscopically oriented samples (here on glass slides; (Reprinted with permission from Ref. 35. Copyright 2006 American Chemical Society.) see (b)) permits the extraction of orientational constraints relative to the aligned membrane. (Reprinted with permission from Ref. 35. Copyright 2006 American Chemical Society.) Here, PISEMA was used to study the orientation of two helical segments of phospholamban (c).[35] (Reprinted with permission from Ref. 35. Copyright 2006 American Chemical Society.) (d) DIPSHIFT experiments measuring local one-bond ^{1}H–^{15}N dipolar couplings in the M2 transmembrane peptide in an unoriented sample under MAS (e), for two lipids with different membrane thicknesses.[24] (Adapted with permission from Ref. 24. Copyright 2007 American Chemical Society.) Changes in the N–H coupling reflect different orientations of the dipolar vectors relative to the axis of averaging (the membrane normal), and thus imply different conformations in each lipid (f).[24] (Adapted with permission from Ref. 24. Copyright 2007 American Chemical Society.)

their averaging by the various modes of motion that the lipid experiences. Figure 2 shows *^{31}P NMR* 1-D spectra of naturally abundant ^{31}P in the phospholipid headgroups, acquired under ^{1}H decoupling, with a lineshape dominated by the ^{31}P CSA, modulated by dynamical averaging. As the static ^{31}P CSA of phosphate groups is known, we can use the partially averaged lineshape to extract information on the orientation of motional averaging processes. In its most basic form, this can be used to establish *lipid phase behavior* because different phases have different rates and axes of motional averaging (Figure 2a; see Lipid Polymorphism). It can also be used to disentangle the complex motions in the fluid lipid bilayer (illustrated in Figure 2c).[8]

To probe the lipids' acyl chains, one can use static *^{2}H NMR* on liposomes containing lipids with deuterated acyl chains (see Bilayer Membranes: Deuterium and Carbon-13 NMR). Here, the widths of the spectral components reflect the averaged quadrupolar coupling constants (QCCs), yielding site-specific *order parameters* of the various CD_3 and CD_2 groups along the acyl chain (Figure 3a). These indicate local disorder and thus the membrane's (*hydrophobic*) *thickness*, which is a biophysical parameter that can affect the folding of membrane proteins (Figure 4). Analogous order parameters can be extracted from MAS NMR spectra in which one measures site-specific, one-bond ^{1}H–^{13}C dipolar couplings (Figure 3b). Reduction of measured values relative to static values once again yields the local order parameter. Note that these experiments do not require lipid deuteration, which is (i) more expensive and (ii) easily available only for select lipids. *Relaxation measurements* can also provide important insight into site-specific motion of the lipids and their acyl chains and are, for instance, used for validation of molecular dynamics (MD) models of lipid bilayers.[9]

Consequences of Lipid Dynamics for SSNMR

The dynamic nature of lipid membranes has significant consequences for studies by SSNMR, in terms of sensitivity, recoupling or excitation methods, relaxation behavior, as well as (^{1}H) decoupling efficiency. Traditionally, SSNMR is associated with the use of *dipolar recoupling*, for instance, by use of CP for heteronuclear polarization transfer and signal enhancement. Indeed, CP-based experiments have found extensive usage for studying lipid bilayers, e.g., for heteronuclear transfer in 2-D ^{1}H/^{13}C spectra.[10] However, the lipid dynamics in the liquid crystalline state can reduce the effective dipolar couplings, reducing the efficiency of CP for the most mobile sites. At the same time, this reduction in the dipolar coupling strength also reduces the need for high-power *^{1}H decoupling* and may result in T_2 relaxation times that are much longer than typical for rigid solids. In fact, in liquid crystalline bilayers, ^{1}H/^{13}C 2-D spectra can often be obtained without ^{1}H–^{1}H homonuclear decoupling.[10–12] The mobility-enabled long T_2 values also pave the way for an effective application of *scalar-coupling*-based experiments that are normally of more limited use for SSNMR, such as (refocused) INEPT (insensitive nuclei enhanced by polarization transfer) spectroscopy.[12] They also enable direct ^{1}H detection, for instance, in 'high-resolution' magic-angle spinning (HR MAS) NMR (see High-resolution MAS for Liquids and Semisolids).

Membrane Proteins by SSNMR

SSNMR methods for studying membrane proteins have been discussed in several recent reviews[13–15] (see also Aligned Membrane Proteins: Structural Studies and Membrane Proteins).

SSNMR on Aligned Membrane Proteins

Static SSNMR on oriented bilayers (see preceding sections) can provide direct insight into the orientation (tilt) of protein secondary structure elements. From measuring ^{2}H QCC, ^{15}N chemical shifts, ^{15}N–^{1}H dipolar couplings, or combinations thereof,[16–18] one can obtain residue-specific orientational restraints, which can be combined with known secondary structures to constrain the structure of proteins within the membrane (see Aligned Membrane Proteins: Structural Studies).

Structural Measurements by MAS SSNMR

In MAS SSNMR, one typically prepares and pellets nonoriented liposomes or vesicles, which can be of almost arbitrary lipid composition. Structural studies of membrane proteins by MAS SSNMR usually employ very similar methods to those applied to other biological solids. Multidimensional heteronuclear assignment experiments follow a by now well-established process, which where needed can be supplemented with judicious isotopic labeling strategies. Obtained chemical shifts provide insight into the localization of secondary structure motifs and estimates of backbone torsion angles. Many structural constraints are accessible via MAS SSNMR[19] (see Dipolar Recoupling: Heteronuclear, Dipolar Recoupling: Homonuclear Experiments, Dipolar-Based Torsion Angle Measurements for Protein Structure Determination, and Structural Studies of Protein Fibrils and Misfolded Proteins by Solid-State NMR). The response of membrane protein structures (as reflected in, e.g., chemical shifts) to changes in the membrane environment may report on the surrounding membrane (Figure 5).

Membrane-induced Protein Dynamics

The dynamics of proteins (or peptides) bound to fluid membranes are sometimes dictated in part by the membrane environment.[20] This is particularly true for smaller peptides or proteins that lack a very robust self-contained structure. The principles discussed in context of lipid dynamics apply similarly here. Intermediate timescale dynamics in membrane-bound polypeptides interferes with the efficiency of ^{1}H decoupling, leading to broadened lines.[20] This *line broadening* in membrane-bound proteins or peptides can present a major challenge.

Another aspect is that one often needs to account for these (anisotropic) dynamics in the *interpretation* of SSNMR experiments. This is manifest in helix tilt measurements, for instance. The observed NMR parameters are time-averaged values, and a detailed analysis may require a structural and dynamical model of some complexity. The precise effect of the anisotropic motion depends on the measured NMR parameters (e.g., ^{2}H QCC vs ^{15}N–^{1}H dipolar coupling), not only in terms of the timescale but also given the different orientations of the applicable tensors relative to the axes of motion. This then requires the measurement of complementary constraints, and increasingly detailed motional models that may be informed by MD simulations.[18,21] Similarly, dynamics can modulate dipolar couplings (possibly in an anisotropic fashion!) and thus impact the acquisition and analysis of structural dipolar-coupling-based measurements by MAS SSNMR.

Given the abovementioned negative effects of sample motion, one may want to *modulate* or eliminate these dynamics, e.g., by adjustment of lipid compositions and temperature.[22] One may need to check for changes in protein structure (and function) under nonnative conditions, for instance, upon transitioning into the gel phase.

Characterizing Protein Dynamics by SSNMR

Various approaches exist to qualitatively or quantitatively probe local dynamics in proteins.[14,23] Some of these experiments are similar to methods used for the characterization of lipid dynamics (explained in preceding sections). Combinations of *scalar and dipolar* recoupling experiments can be used to probe mobility of membrane-bound proteins, revealing secondary structure and topology.[23] Quantitative measurements of *relaxation* rates and various *order parameters* can provide more detailed insights, for instance, by extracting anisotropic dynamical information. Figure 4d shows one example of using the classic DIPSHIFT (dipolar chemical shift correlation) experiment for measuring dipolar couplings to *determine helix tilt* by MAS SSNMR.[24] Fluid membranes cause a rapid uniaxial reorientation of membrane-embedded proteins about the membrane normal. The orientation of the NMR tensor (e.g., ^{2}H QCC, ^{1}H–^{15}N dipolar coupling) relative to the averaging axis (the membrane normal) can be determined by comparing the residual anisotropy to the corresponding parameter in absence of motion. This then yields orientational constraints relative to the lipid bilayer without the need for preparing aligned membranes, both in static SSNMR and under MAS.[24–26]

Protein–Lipid Interactions by SSNMR

Effects on Lipid Behavior

The association, topology and localization of membrane-bound proteins can be probed by detecting *changes induced in the lipids*. Even without labeling the polypeptide, one can detect induced changes in bilayer thickness, dynamics of acyl chains and headgroups, and phase behavior. These parameters inform on the induced curvature of the membrane and disruption of the bilayer structure (both of biological interest). The changed lipid dynamics often affect the acyl chains and headgroups in different ways, thus indicating the site of interaction. For example, a surface-bound amphipathic helix may insert between the headgroups, reducing the available space for the headgroups and increasing the spacing of the acyl chains. This may be detected as decreased motion of the phosphate group (^{31}P NMR), along with increased motion of the acyl chains (order parameters from ^{2}H QCC or ^{1}H–^{13}C dipolar couplings). In some studies, small but detectable changes in the (^{1}H) chemical shifts of the lipids can be seen and attributed to proximity of, e.g., aromatic side chains.

Typical samples feature a substantial excess of lipids to protein, meaning that the disturbance of the thickness or chemical shifts of lipids near a protein may be hard to detect in a background of lipids that are distant from the protein. Fortunately, biophysical membrane characteristics can be quite sensitive to the presence of bound proteins, even for relatively low amounts of protein.

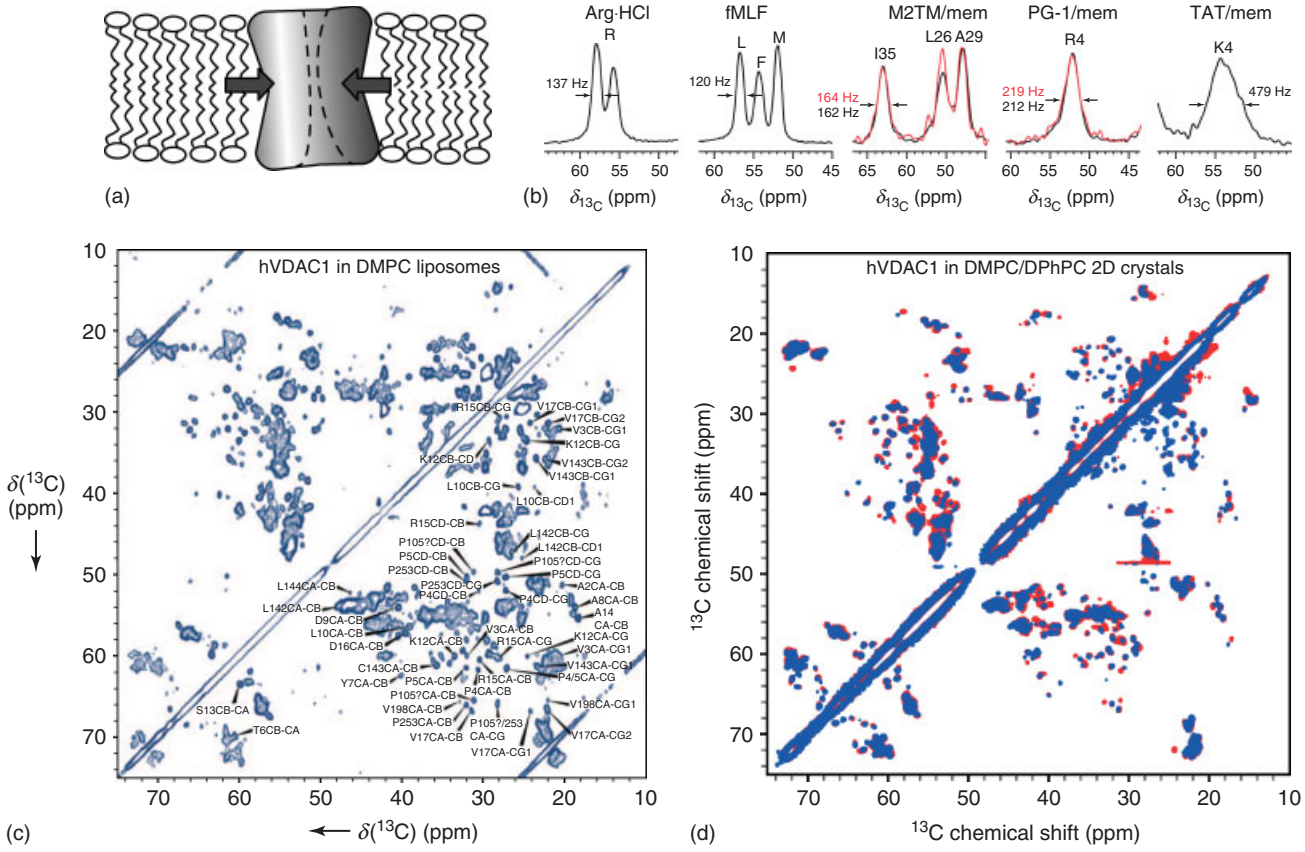

Figure 5. (a) The interaction with the membrane environment can have a notable effect on the structure and dynamics of embedded proteins. (b) This in turn can lead to line broadening: Cα peaks from ^{13}C-labeled residues in various peptides: crystalline Arg-HCl and fMLF-OH compared to membrane bound M2 peptide, protegrin-1, and HIV TAT peptides. In particular, small peptides can be highly sensitive to the fluidity and composition of the membrane.[22] (Reprinted with permission Ref. 22. Copyright 2011 American Chemical Society.) For larger proteins, such as the 32 kDa voltage-dependent anion channel, hVDAC1, the effects may be smaller, but still detectable by MAS SSNMR: (c) 2-D ^{13}C–^{13}C PDSD (proton-driven spin diffusion) spectrum in DMPC (dimyristoylphosphatidylcholine) liposomes; (Reproduced with permission from Ref. 36. © WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.) (d) ^{13}C–^{13}C RFDR spectra in DMPC (red) and DPhPC (blue) 2-D crystals.[37] Although many peaks are unchanged, and different polarization transfer schemes complicate direct comparison, residue-specific spectral changes can be observed in different membrane environments. (Adapted with permission from Ref. 37. Copyright 2012 American Chemical Society.)

Intermolecular Recoupling and Polarization Diffusion

Site-specific interactions with the membrane can be determined via *intermolecular dipolar recoupling* experiments, e.g., ^{13}C–^{31}P REDOR involving a ^{13}C-labeled polypeptide and the phosphate group of phospholipids. Alternatively, one can use ^{19}F-labeled lipids for ^{19}F-based REDOR, which provides longer distances due to ^{19}F's higher gyromagnetic ratio. The analysis of such data can be complex due to proximity of multiple ^{31}P, inherent disorder in the membrane (Figure 1), and the effect of dynamics (in the case of a fluid, nonfrozen sample).

Constraints on the topology of membrane proteins and their interactions with surrounding lipids can be obtained by detecting *polarization transfer* between the lipids or solvents and the protein. Multidimensional correlation spectroscopy may allow polarization transfer to be detected between lipid and protein, e.g., via ^{1}H–^{1}H spin diffusion or ^{1}H–^{1}H RFDR (radiofrequency-driven recoupling) methods. Particularly, mobile protons in the solvent (or membrane hydrophobic core) can be selected via a T_2 filter.[27] These kinds of measurements can also serve to detect preferential proximity of different lipids, an experiment that may benefit from use of deuterated or otherwise isotopically labeled lipids.

Effects of Lipids on Proteins

As the mobility of surrounding lipids can modulate the dynamics of interacting proteins, one may be able to delineate such interactions based on a correspondence of lipid and protein relaxation parameters.[23,28] Furthermore, SSNMR spectra obtained in different membrane preparations (in terms of, e.g., lipid composition; Figure 5) can reveal changes in protein structure and dynamics. A systematic comparison to correlated effects on protein function may be used to elucidate the mechanism of interaction with the membrane and specific lipids within the bilayer.

Overview and Future Directions

As gradually more structural information on membrane proteins is obtained, by NMR and otherwise, it is clear that interactions with the lipid bilayer can be crucial for both structure and function.[4] SSNMR has been demonstrated in model systems and is increasingly applied to membrane-bound proteins of biological and biomedical interest. This review has examined many different SSNMR methods and studies, but there are many more methods that are available. Given this wealth of SSNMR methods, it is important to consider the respective strengths and weaknesses. For instance, orientational information can be obtained from aligned bicelles, slide alignment, or MAS NMR experiments. Sample alignment via magnetic alignment of bicelles can be less time-consuming than the painstaking use of slides. However, as of now, stable bicelles can only be made from a select subset of lipid compositions, while slide alignment accommodates a wider range of lipid compositions. Use of nonoriented samples and reliance on 'rotational alignment' allows for even more lipid compositions, but is strictly dependent on the fast uniaxial rotation that may not always be present (e.g., in cholesterol-rich membranes or in the case of protein oligomerization).

Currently, modern SSNMR offers a rich and expanding toolkit that facilitates unique insights into the molecular and dynamical processes that govern the function of integral membrane proteins. Going forward, aside from continued developments in SSNMR, there will be an important role for increased integration with complementary data, as already embodied in studies that integrate SSNMR with solution NMR data and MD simulations.[21,29–31]

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Related Articles

Bilayer Membranes: Deuterium and Carbon-13 NMR; Lipid Polymorphism; Membrane Proteins; Aligned Membrane Proteins: Structural Studies; Structural and Dynamics Studies of Lipids by Solid-State NMR

Biographical Sketch

Patrick C.A. van der Wel. b 1972. BSc, 1991, PhD, 2002, Fayetteville, AR. He started in solid-state NMR by its application to (bio)membranes at Utrecht University, studying lipid polymorphism and peptide–lipid interactions. He has worked on determination of protein structure and dynamics using ^{2}H-NMR on slide-aligned membranes during his PhD research in the laboratory of Roger Koeppe II and using magic-angle-spinning SSNMR as a postdoc in the group of Bob Griffin. Current research at the University of Pittsburgh focuses on amyloid structure and formation, as well as the conformation and dynamics of membrane-bound proteins.

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