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Current Applications of Bicelles in NMR Studies of Membrane-Associated Amphiphiles and Proteins[†,‡]

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ABSTRACT: This review covers current trends in studies of membrane amphiphiles and membrane proteins using both fast tumbling bicelles and magnetically aligned bicelle media for both solution state and solid state NMR. The fast tumbling bicelles provide a versatile biologically mimetic membrane model, which in many cases is preferable to micelles, both because of the range of lipids and amphiphiles that may be combined and because radius of curvature effects and strain effects common with micelles may be avoided. Drug and small molecule binding and partitioning studies should benefit from their application in fast tumbling bicelles, tailored to mimic specific membranes. A wide range of topology and immersion depth studies have been shown to be effective in fast tumbling bicelles, while residual dipolar couplings add another dimension to structure refinement possibilities, particularly for situations in which the peptide is uniformly labeled with ^{15}N and ^{13}C. Solid state NMR studies of polytopic transmembrane proteins demonstrate that it is possible to express, purify, and reconstitute membrane proteins, ranging in size from single transmembrane domains to seven-transmembrane GPCRs, into bicelles. The line widths and quality of the resulting ^{15}NH dipole−^{15}N chemical shift spectra demonstrate that there are no insurmountable obstacles to the study of large membrane proteins in magnetically aligned media.

Bicelles represent a class of membrane model systems whose properties may be optimized to suit a wide variety of spectroscopic techniques in the study of lipid bilayers, membrane-associated amphiphiles, and membrane proteins. For example, studies of membrane peptides by solution NMR benefit from bicelles in the sense that the lipid bilayer component better mimics a biological membrane than that of the detergent micelle, while the bicelle aggregate sizes are sufficiently small to give rise to well-resolved lines. Bicelles have proven to be equally useful in solid state NMR applications, where the system is tailored to align in the magnetic field. In such studies, orientational information about the membrane protein (or lipid amphiphile) is obtained from the anisotropic component of the chemical shift or dipolar interactions arising from ^{13}C, ^{31}P, ^{15}N, ^{2}H, and ^{19}F nuclear spin probes. High-quality spectra are often observed due in part to the highly liquid crystalline environment of the bicelle lipids and the relatively high rotational mobility of the amphiphiles and proteins within a well-aligned medium. Finally, a number of MAS[1] or off-magic angle sample spinning experiments have been perfected in which both anisotropic and isotropic information is obtained. Some of these experiments have been extended to the study of purely soluble proteins, where the bicelle in combination with sample spinning is simply used to scale the degree of anisotropic

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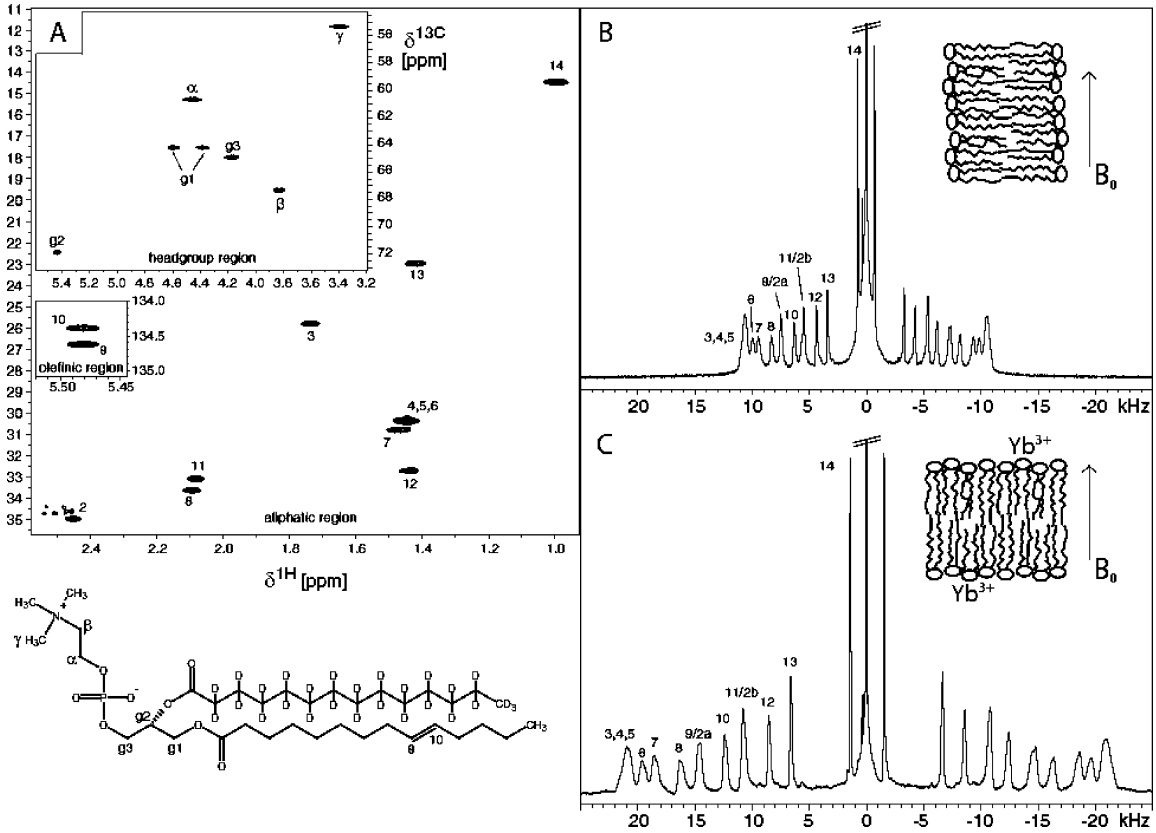

FIGURE 1: NMR spectra obtained in fast tumbling bicelles (A), aligned bicelles (B), and lanthanide-doped bilayers (C), from the identical lipid, MLMPC-d_{27} (i.e., *sn*-2-perdeutero-1-myristelaidoyl-2-myristoyl-*sn*-glycero-3-phosphocholine, shown in the figure), at 45 °C in each case. The spectrum in panel A represents a $^{13}C-^{1}H$ HSQC spectrum of the proton-coupled portion of the lipid in a $q = 0.8$ MLMPC-d_{27}/DHPC-d_{22} bicelle in 25% (w/v) PO_4^- buffer (pH 7), obtained with 256 t_1 increments, four scans per increment, and a spectral width of 12 000 Hz in the indirect dimension. The olefinic region was actually sampled in a separate HSQC experiment, using 24 increments, 44 scans, and a 400 Hz spectral width in the ^{13}C dimension. Panels B and C represent the respective ^{2}H NMR quadrupolar echo spectra from the (perdeuterated) *sn*-2 chain of the lipid in the $q = 3.0$ MLMPC-d_{27}/DHPC bicelles and $q = 3.2$ MLMPC-d_{27}/DHPC Yb^{3+}-doped bilayers, with a Yb^{3+}:MLMPC ratio of 1:100. Both ^{2}H NMR spectra were obtained using an echo refocusing time of 60 μs and either 256 scans (B) or 512 scans (C) and were processed with an exponential window function corresponding to 60 Hz line broadening. All spectra were obtained on a Varian Inova 600 MHz instrument, using a standard HNC probe (A) or a dual-channel HX probe (B and C).

information (*1*). A number of excellent reviews on the subject of bicelles with particular emphasis on concepts of alignment, reconstitution of membrane proteins, phase behavior, and general applications to spectroscopy, membrane protein crystallography, and separation exist (*2*−*7*). This review will focus on developments in isotropically and magnetically aligned bicelles, particularly with regard to NMR studies of membrane-associated amphiphiles and membrane proteins.

Phospholipids typically assemble into bilayers and adopt multilamellar structures in solution. The resulting aggregates are far too large for solution state NMR studies of membrane-associated proteins, although fast MAS has been shown to provide reasonable resolution when the peptide under study undergoes fast axially symmetric rotational diffusion in the bilayer (*8*−*11*). In contrast, bicelle aggregate sizes may be tailored for solution NMR or solid state NMR. Bicelles consist of one or more long chain phospholipids which self-assemble into a bilayer, in combination with an amphiphile such as CHAPSO or a short chain lipid such as DHPC. The amphiphile serves to coat the hydrophobic interface of the bilayer, creating a disk-shaped domain appropriate in size and mobility to be suitable for solution NMR when the ratio of long chain phospholipid to amphiphile (q) is between 0.10 and 1.0. A $^{1}H-^{13}C$ HSQC spectrum of an unsaturated lipid (MLMPC-d_{27}) in combination with chain perdeuterated DHPC, in a so-called isotropic or fast tumbling bicelle ($q = 0.8$), is shown in Figure 1A. The spectrum provides a spectroscopic snapshot of the lipid bilayer under solution NMR conditions. Note that nearly every carbon nucleus in the lipid can be detected at 600 MHz, allowing the possibility of monitoring chemical shift perturbations in studying the effects of membrane additives.

[1] Abbreviations: 2D, two-dimensional; CD, circular dichroism; CHAPSO, 3-(cholamidopropyl)dimethylammonio-2-hydroxyl-1-propanesulfonate; Δ^8-THC, (−)-Δ8-tetrahydrocannabinol; Me-Δ^8-THC, (−)-*O*-methyl-Δ8-tetrahydrocannabinol; DHPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; DIOMPC, 1,2-di-*O*-tetradecyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPE−DTPA, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine−diethylenetriaminepentaacetate; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; DMPS,1,2-dimyristoyl-*sn*-glycero-3-(phospho-L-serine); DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPM, 1,2-dioleoyl-*sn*-glycero-3-phosphomethanol; DQF-COSY, double-quantum-filtered correlation spectroscopy; GPCR, G protein-coupled receptor; MAS, magic angle sample spinning; MLEV, Malcolm Levitt's composite pulse decoupling; MLMPC-d_{27}, *sn*-2-perdeutero-1-myristelaidoyl-2-myristoyl-*sn*-glycero-3-phosphocholine; NOESY, nuclear Overhauser effect spectroscopy; DEPT, distortionless enhancement by polarization transfer; INEPT, insensitive nuclei enhanced by polarization transfer; HSQC, heteronuclear single-quantum correlation; HMBC, heteronuclear multiple-bond correlation; PISA, polarity index slant angle; TOCSY, total correlated spectroscopy; VASS, variable-angle sample spinning.

Table 1: Bicelle Formulations for Magnetic Alignment

composition	% amphiphile; temperature (°C)	comments
3:1 DMPC/CHAPSO	30; ~40	may denature some proteins (*104*); similar formulations with CHAPSO and other detergents (*3*)
2.7−3.2:1 DMPC/DHPC	20−40; 35−40	most commonly employed (*64*); deuterated versions available
2.7−3.2 (DMPC + cholesterol or DMPA)/DHPC	20−25; 35−40	useful eukaryotic membrane mimics (*70*)
3.1−3.5 (DMPC + DMPS)/DHPC	25; 35−42	charged lipids stabilize bicelles (*105*, *106*); useful mimic of eukaryotic membranes
3.1−3.5 (DMPC + DMPG)/DHPC	25; 35−42	charged lipids stabilize bicelles (*105*, *106*); useful mimics of prokaryotic membranes
2.7−3.5 (DMPC + DMPE)/DHPC	20; 36−39	10% DMPE, DMPS, or DMPG can be added (*107*), giving a useful mimic of myelin and erythrocytes
3.3 (DMPC + cardiolipin)/DHPC	15; ~40	mitochondrial membrane mimic (*108*)
1.65−9.0 DIOMPC/DIOHPC	2−30; 27−50	100 mM KCl, also stable at low pH (*109*)
3.5 (DMPC/DHPC) + PEG	25−40; ~40	1% pegylated lipid-stabilized bicelles and improved alignment (*110*)
3.2−8.0 DMPC/DHPC + ~100:1 DMPC/Yb^{3+}	20−40; 35−90	a phospholipid chelate may also be substituted to complex the Yb^{3+} ions (*24*); avoids cylindrical distributions and associated inhomogeneous line broadening expected for immobile proteins (*111*); potential for low-angle diffraction and methods benefiting from uniaxially aligned transition dipoles
0.1−1.0 DMPC/DHPC	10−25; 5−45	standard formulation for solution NMR studies; most of the above bicelle formulations can be tailored to give good ^{1}H NMR resolution (*28*)
0.5 DMPC/CHAPSO	10; 37	standard formulation for solution NMR (*112*)

In solid state NMR experiments which measure orientational properties (*6*, *12*, *13*), phospholipid dispersions may be mechanically teased into a uniaxially aligned state via annealing on thin glass plates (*14*, *15*). The advent of bicelles has made the preparation of aligned membrane samples (and their resident proteins) routine. As suggested by Figure 1B, bicelles for which the long chain to short chain phospholipid molar ratio is between 2.8 and 6.5 will spontaneously align in a magnetic field such that the bilayer plane is parallel to the magnetic field. The short chain amphiphile (usually DHPC) plays a slightly different role in such aligned aggregates; in most cases, DHPC-rich torroidal defects are believed to be scattered throughout an extended bilayer resembling a perforated lamellar morphology (*16*, *17*). The DHPC-rich defects lend sufficient mobility to the overall aggregate that spontaneous alignment can occur. A great deal of work has been done in an effort to understand the morphology and phase behavior of bicelles over a wide range of temperatures and compositions (*18*−*21*), while a range of lipid mixtures are available so that the bilayer component of the bicelles mimics particular types of cell membranes, as summarized in Table 1. The ^{2}H NMR spectrum in Figure 1B of a magnetically aligned $q = 3.2$ lipid bilayer arises from the *sn*-2 chain of the identical lipid that was used to prepare fast tumbling bicelles for Figure 1A. The quadrupolar splittings reflect the order parameter gradient along the lipid chain, and such spectra have been used to study the influence of membrane penetrants or additives on lipid bilayer structure and dynamics.

A third variation on the bicelle theme involves the use of certain lanthanide additives, which complex with the phospholipid headgroups, conferring a positive magnetic susceptibility anisotropy to the lipid bilayers and causing them to align with the bilayer symmetry axis parallel to the field (*7*, *22*). A ^{2}H NMR spectrum of the lanthanide-doped or flipped bicelles is shown in Figure 1C, wherein a small amount of Yb^{3+} (i.e., MLMPC-d_{27}:Yb^{3+} ratio of 100) has been added to a bicelle. The system can be further optimized by introducing phospholipid chelates, which avoid nonspecific interactions between the lanthanide and the lipids or peptide additives (*23*, *24*). The lanthanide-doped bilayers were originally heralded as an ideal model membrane system for the study of orientational properties of membrane proteins (*25*) because the largest frequency dispersion is expected at this orientation. Moreover, cylindrical powder patterns and the corresponding line broadening which is expected for immobile membrane proteins in regular aligned bicelles should not occur in the parallel orientation. This will be discussed later in the context of the PISEMA experiments. One possible as yet untapped advantage of the parallel aligned bilayers would be in the study of orientational properties of peptides or membrane additives by techniques such as FTIR, fluorescence anisotropy, or linear dichroism, all of which benefit from uniaxially aligned membranes (and correspondingly aligned transition dipole moments). Since fields as low as 0.5 T can be used to maintain bilayer alignment (*26*), small rare-earth magnets could be used in conjunction with current optical spectrometers to measure orientational properties in uniaxially aligned lipid bilayers in a bulk hydrated phase. In the sections that follow, this article will highlight spectroscopic studies of membranes, small membrane additives, and membrane proteins, which take advantage of the three classes of bicelles described above. We will begin with an overview of solution state NMR studies, where fast tumbling bicelles provide a biologically mimetic environment for structural and topological studies of membrane additives and membrane peptides. In solid state NMR applications requiring aligned samples, orientational studies with both conventional bicelles and parallel aligned bicelles will be discussed.

Solution State NMR Studies of Membrane Peptides in Fast Tumbling Bicelles

In micelles, detergents are known to be in fast exchange with the bulk solvent which can make interpretation of NOEs between peptides and the micelle and, in general, any topology experiments somewhat ambiguous. Furthermore, curvature effects are occasionally observed with small

peptides (*27*), and the absence of specific phospholipids or mixtures of phospholipids may cause small peptides to adopt entirely nonbiologically mimetic conformations. For this reason, a large number of solution NMR structural studies have been conducted on small membrane peptides using fast tumbling bicelles rather than detergent micelles (*28*). For example, a recent CD study of the 53-amino acid presequence of ATP synthase revealed a higher helical content in acidic phospholipid bicelles. However, in this case, NMR-based structure studies were subsequently performed in SDS micelles, where resolution was sufficient to make assignments and measure secondary shifts and NOESY-based distance constraints (*29*). In situations where the peptide under study is not uniformly labeled with ^{13}C and ^{15}N, complete assignments are usually possible for polypeptides consisting of ≤30 residues, by the traditional series of two-dimensional (2D) ^{1}H NMR homonuclear TOCSY (*30*), NOESY (*31*), and DQF-COSY (*32*) experiments. In one such study of a cell-penetrating peptide, transportan, incorporated into a $q = 0.33$ DMPC/DHPC bicelle, complete assignments were achieved for all backbone residues and 90% of the side chain resonances (*33*). Two hundred twenty distance constraints were obtained for this 27-residue peptide, with a GWTLNSAGYLLGKINLKALAALAKKIL-amide sequence, making it possible to propose a structure consisting of an amphipathic helix with well-defined structure at the C-terminal end and a slightly less well-defined helix toward the N-terminus. Another example of a cell-penetrating peptide is the 16-residue peptide Antp (*43*−*58*), which possesses a basic−aromatic motif, and in particular two key tryptophan residues allowing it to insert into the hydrophobic core of the membrane, as determined by lipid−peptide NOEs in $q = 1$ isotropic bicelles (*34*). A similar study was conducted with a small membrane peptide termed human motilin, with a FVPIFTYGELQRMQEKERNKGQ sequence (*35*). Comparisons of NMR structure and dynamics studies in SDS micelles, neutral $q = 0.5$ bicelles, and acidic $q = 0.5$ bicelles revealed that motilin consists of an ordered α-helical conformation between residues 9 and 20, while the N-terminus adopts two well-defined turns. This structure was found to be significantly different from that observed in SDS, while a model-free analysis of relaxation rates from a ^{13}C-enriched Leu10 probe indicated that the peptide is considerably less ordered in $q = 0.25$ acidic bicelles than in SDS. Mobility (or lack thereof) certainly determines the quality of the line widths in solution NMR spectra. For example, a recent two-dimensional ^{1}H NMR study of a 30-residue homologue of an encoded prion protein in $q = 0.25$ DMPC/DHPC bicelles only showed signals from the less structured and dynamic termini, while the structured hydrophobic region believed to lie in the lipid bilayer interior remained obscured (*36*). A complete structure based on 263 NOE distance constraints was possible in DHPC micelles, while $^{2}H_2O$ exchange experiments suggested the peptide adopts a transmembrane conformation.

Topology. In micelles, membrane protein topology and immersion depth have been approached by a variety of techniques. For example, chemical shift deviations from random coil values for the Hα, $^{13}C\alpha$, and HN nuclei provide direct information about peptide secondary structure and the hydrophobicity of the local environment (*33*, *37*). Chemical shift deviations are also prominent for ^{19}F nuclei upon binding or interacting with membranes. This was shown to be the case in a ^{19}F NMR study of binding of a 20 kDa actinoporin to $q = 0.25$ bicelles (*38*). Biosynthetic incorporation of 5-fluorotryptophan enabled the monitoring of regions of the protein believed to interact with the membrane which consisted of either DMPC or DMPC and sphingomyelin. Detergent−protein intermolecular NOEs also help to define the transmembrane regions of the protein, in addition to regions of the protein in contact with the headgroup or hydrophobic interior (*39*−*41*). Line broadening from paramagnetic additives such as Mn^{2+} or chelated Gd^{3+} defines regions of the protein in contact with the solvent or detergent headgroup, thus complementing NOEs to the micellar interior (*41*, *42*). Similarly, detergents or lipids possessing doxyl spin-labels at specific sites on the chain give rise to localized paramagnetic line broadening of protein ^{1}H resonances and thus provide information about immersion depth (*40*−*42*). In addition, water amide exchange measurements are extremely useful in corroborating membrane protein topology since the detergent hydrophobic interior offers a gradient of protection effects which are strongest in the micelle interior, while steric effects are also reflected in such experiments (*43*, *44*). More recently, paramagnetic effects from oxygen in detergent micelles and bicelles have been used to obtain information about steric effects and immersion depth of probe nuclei in amphiphiles, peptides, and membrane proteins (*45*−*48*). Oxygen exhibits a pronounced concentration gradient in both micelles and lipid bilayers, which translates into a significant paramagnetic gradient in terms of spin−lattice relaxation rates for ^{1}H and ^{19}F nuclei and chemical shift perturbations for ^{19}F nuclei. Often, these paramagnetic gradients provide Angstrom resolution in immersion depth, although such experiments require equilibration of samples at oxygen partial pressures between 20 and 30 bar, using specialized NMR tubes (*45*). The arsenal of methods described above is useful in the study of topology and immersion depth of membrane peptides in fast tumbling bicelles. For example, upon determination of the solution NMR study of transportan in bicelles, amide proton secondary shifts and line broadening effects from doxyl-labeled phospholipids helped to delineate a distinct region of the amphipathic helix that was in contact with the hydrophobic domain of the bilayer. Similar paramagnetic effects from both the 5- and 12-doxyl spin-labels were attributed to the fact that lipids possess a high degree of surface mobility. Note that a distinct periodicity of peak amplitude was evident from this analysis, consistent with the notion of an amphipathic helix, while the strongest effect appeared at Asn15.

Binding. Topology and immersion depth studies of membrane-associated peptides must be considered carefully in light of the possibility that the peptide may be weakly bound to the membrane and in fast exchange with the solvent. Accurate measurements of binding constants are possible via monitoring of chemical shifts (*49*), average diffusion rates (*50*), or ^{15}N relaxation rates as a function of peptide:lipid ratios, which was done recently with the cell-penetrating peptide penetratin, with a RQIKIWFQNRRMKWKK sequence (*51*). In this study, 95% of the peptides were found to be fully bound to the bilayer in a 90:10 DMPC/DMPG mixture. The situation in which a peptide or amphiphile is in fast exchange between a lipid bilayer and bulk solvent not only is fortuitous from a spectroscopic point of view,

since the resolution resulting from increased mobility is often dramatically improved, but also may be biologically relevant. In one study of a class of glycosylated β-endorphin analogue peptides designed to have blood−brain barrier association and penetration properties, amide proton secondary shifts and NOEs helped to define tertiary structure and average membrane interactions (*52*). NMR and other evidence pointed toward a model in which the peptides converted from a nascent helical structure in solution to an amphipathic helical conformation in the presence of zwitterionic and acidic lipids. Amphipathic binding of the glycopeptides to the membrane was further suggested to correlate with blood−brain barrier transport properties via an endocytotic mechanism. In this model, the authors postulated that the glycoside moiety on the peptide served to establish an appropriately weak membrane binding constant so that the blood−brain barrier penetrating peptide was thought to undergo transitions from helix to random coil as it exchanged between the membrane and bulk solvent, and in so doing could "hop" between membranes.

Small Molecules. A number of studies involving interactions of small molecules with bicelles have been performed. In many cases, the appropriate biologically mimetic membrane is the key to understanding the conformation, orientation, positioning, and dynamics of the amphiphile, all of which factor into its mode of action. Though isotropic bicelles are usually prepared so that q ranges between 0.10 and 1.0, a recent ^{1}H NMR solution study of two lipophilic cannabinoids, Δ^8-THC and its *O*-methyl ether analogue, Me-Δ^8-THC, was performed in a $q = 2.0$ DMPC/DHPC bicelle system (*53*). Using standard two-dimensional homonuclear measurements, the conformations of both cannabinoids were found to differ within the membrane mimetic media due to the more amphipathic-like properties imparted by the phenolic hydroxy group of Δ^8-THC compared to those of Me-Δ^8-THC. Such differences in conformation could not be detected in detergent micelles. Similar homonuclear ^{1}H NMR studies were performed on the endogenous opioid, methionine-enkephalin (Menk) in $q = 0.5$ DMPC/DHPC and DMPC/DMPG/DHPC fast tumbling bicelles (*50*). Pulsed-field gradient diffusion measurements revealed that approximately 60% of the peptides were bound to the membrane, while $^3J_{HN-H\alpha}$ scalar couplings and NOEs provided torsion angle and distance constraints. These constraints suggested that the dominant conformations of the neuropeptides made it possible to bind both μ- and δ-opiate receptors. Topology measurements, as discussed above, may also be combined with standard homonuclear ^{1}H NMR experiments to determine average positioning of small molecules in the bicelle. For example, positioning of small amphiphiles such as caffeine, nicotine, and imipramine in lipid bilayers may be assessed by water NOEs, oxygen-induced relaxation rate enhancements, or chemical shift perturbations upon transfer from water to a bicelle (*48*). Wang et al. examined binding of adamantine to a number of fast tumbling bicelle formulations. Pulsed field gradient diffusion measurements provided an estimate of the average diffusion rate of adamantine and its partition coefficient into the bilayer, which ranged between 27.6 in DMPC and 37.8 in POPC bilayers (*54*). Though intermolecular NOESY and ROESY measurements revealed very weak contact between the lipid and drug, saturation transfer difference measurements, in which resolved lipid resonances were selectively saturated for a range of mixing times, helped to position the drug at the membrane−water interface.

New Trends in Studies Involving Fast Tumbling Bicelles. Thus far, the majority of studies of membrane peptides in fast tumbling bicelles have been confined to homonuclear ^{1}H NMR experiments. Uniform ^{13}C and ^{15}N labeling should improve dispersion and thus possibilities for assignment of larger membrane peptides in bicelle media. Furthermore, $^{13}C\alpha$ and $^{13}C\beta$ chemical shifts help to define side chain torsion angles, while ^{13}C- and/or ^{15}N-edited experiments remove ambiguities in ^{1}H−^{1}H NOESY measurements. Nakamura et al. (*55*) recently revisited the positioning of the 14-residue amphiphilic peptide, mastoparan, in fast tumbling bicelles. The peptide uniformly labeled with ^{13}C and ^{15}N was successfully overexpressed in *Escherichia coli* by first creating a decahistidine-tagged ubiquitin−mastoparan fusion protein (*56*). The purified peptide was then incorporated into a $q = 0.5$ DMPC/DHPC bicelle medium. Sequential assignments could be easily made via HNCACB and CBCA(CO)NH experiments, and amide−water exchange rates gave an indication of the residues most buried in the bilayer and thus protected from water exchange. Using 95% deuterated buffer and a perdeuterated version of mastoparan, it was also possible to perform a cross saturation experiment to determine the extent of immersion of the backbone amide residues in the bilayer. Irradiation of the lipid methylenes for relatively long mixing times resulted in attenuation of the amide intensities in a ^{15}N−^{1}H HSQC spectrum, roughly in accordance with their contact to the bilayer. However, dynamics at the N-terminal end was found to play a major role in the extent of cross saturation, and thus, a quantitative determination of immersion depth was not possible. ^{13}C and ^{15}N labeling also facilitate detailed peptide dynamics studies. In particular, ^{15}N T_1, T_2, and ^{1}H−^{15}N heteronuclear NOEs closely mirror global and segmental motions over a broad range of time scales (*57*−*60*). Structure refinement may also be achieved in the form of residual dipolar couplings (RDCs) via bicelles. If the peptide−bicelle complex is introduced into an anisotropic medium such as a strained gel (*61*, *62*), the bicelle acquires collisionally induced order suitable for the measurement of RDCs (*27*). These orientational constraints were obtained through scalar couplings ($^1D_{HN}$, $^1D_{C\alpha C'}$, and $^1D_{C'N}$) in the strained gel medium. In addition, peptide−lipid NOEs and torsion angle measurements from $^{13}C\alpha$, $^{13}C\beta$, and $^{13}C'$ secondary shifts and three bond couplings, $^3J_{NC\gamma}$ and $^3J_{C'C\gamma}$, revealed that a peptide fragment from the HIV-1 envelope protein, gp41[282−304], exhibits a strained or bent conformation in micelles and a significant rigid helix conformation in $q > 0.25$ fast tumbling bicelles (*27*). Thus, the fast tumbling bicelles provide a versatile biologically mimetic membrane model, which in many cases is preferable to micelles, both because of the range of lipids and amphiphiles that may be combined and because radius of curvature effects and strain effects common with micelles may be avoided. Drug and small molecule binding studies stand to gain a great deal from fast tumbling bicelles. A wide range of topology and immersion depth studies have been shown to be effective in bicelles, while residual dipolar couplings add another dimension to structure refinement possibilities, particularly for situations in which the peptide is uniformly labeled with ^{15}N and ^{13}C.

Aligned Bicelles for Structure and Orientational Studies of Small Membrane Additives and Transmembrane Proteins by Solid State NMR

Spectroscopic Studies of Lipids and Small Membrane Additives. Bicelles and lanthanide-doped bilayers, whose spectra are shown in panels B and C of Figure 1, respectively, provide a convenient means of studying small membrane additives and membrane proteins of virtually arbitrary size by solid state NMR. Such studies of bicellar lipids commonly utilize ^{2}H, ^{13}C, or ^{31}P NMR to probe either segmental orientational properties (*63*, *64*), whole body orientational and dynamic properties (*65*−*68*), or the phase behavior of the system. Deuterium NMR studies are advantageous since aligned spectra of perdeuterated lipids generally give complete resolution of all deuterated sites, while a single anisotropic or orientation-dependent nuclear quadrupolar interaction dominates. The principal axis of the interaction tensor typically lies along the CD bond, which facilitates interpretation of the resultant quadrupolar splittings in terms of orientational order parameters. Similarly, lipid headgroup and acyl chain order parameters may be assessed from the measurement of ^{13}C NMR and ^{31}P NMR chemical shift anisotropies (CSAs) and dipolar couplings. The CSA may be determined for a given resonance with the knowledge of the chemical shift under isotropic conditions and in the magnetically aligned state. Since aligned bicelles may be effectively converted to an isotropic state by addition of short chain lipids, the chemical shift resonances in the aligned state may be traced to their isotropic values and thus assigned by traditional means (*69*, *70*). Such scaling procedures also allow determination of the sign of the dipole coupling, thereby removing ambiguities in the determination of possible conformations in studies of aligned systems. Alternatively, off magic angle spinning experiments may be used to scale the overall orientational order in the bicelle toward the isotropic limit for assignment and determination of dipole couplings (*71*−*75*). When the bicelle is spun at kilohertz spinning rates about an axis that makes an angle ($0° < \Omega < 54.7°$) with respect to the magnetic field, the director is distributed in a plane perpendicular to the rotation axis, and the resulting anisotropic interactions are scaled by a predictable factor (*71*). A second means of scaling anisotropic interactions involves very slow sample spinning (i.e., 0.5−2 Hz) about an axis orthogonal to the field, using lanthanide-doped flipped bicelles. In this case, a stepping motor may be used to rotate a cylindrical sample tube, housed in a horizontal solenoidal coil to obtain a spectrum of any sample orientation. These experiments are most easily performed by rotating the sample by 180° on the time scale of 0.5−2 s and subsequently acquiring the NMR spectrum at a designated time after the initiation of rotation. At such slow spinning rates, there is no evidence of disordering over a period of 24 h, after repeated rotation cycles with standard repetition times. Spectra of uniaxially aligned membranes as a function of sample orientation are in principle useful for determining the sign of dipole couplings or performing assignments without the complexities of spinning sidebands or centrifugation artifacts. To demonstrate this principle, Figure 2 shows a series of experimental ^{2}H NMR spectra of DMPC-d_{54} from a $q = 6$ Yb^{3+}-doped parallel aligned bilayer system, which have been acquired by rotating the sample by 180° in 1 s and sampling 124 discrete orientations. The rotation rate is such that the sample orientation changes by less than a degree during each of the 124 distinct acquisition times. In a given rotation, key orientations (sample times) may be chosen to prevent saturation of the signal.

Amphiphiles uniformly enriched with ^{13}C may also be assigned in the aligned states using INADEQUATE sequences designed to connect adjacent ^{13}C nuclei, coupled via scalar and dipolar interactions (*76*, *77*). Much of the earlier work in peptide− or protein−lipid interactions from the perspective of solid state NMR studies of the lipid may be found in excellent reviews (*3*−*5*, *16*). A good deal of current research on lipid−protein interactions using aligned bicelles involves the study of cell-translocating and antibacterial peptides. Sizun et al. (*78*) combined ^{2}H NMR spectra and relaxation studies to analyze the effect of addition of a 35-residue transmembrane domain of a receptor tyrosine kinase, NeuTM35, in aligned bicelles. Using a completely perdeuterated lipid probe, it was possible to study changes in the bilayer orientational order and dynamics at a peptide:lipid ratio of only 1:1800. The transmembrane peptide was observed to induce ordering of the lipids, particularly in the so-called plateau region with the concomitant onset of slow-frequency motions. A similar study of lipid−peptide interactions was conducted on the 16-residue peptide Antp (*43*−*58*), whose positioning in the membrane was studied by solution NMR using lipid−peptide NOESYs, as discussed in the previous section (*34*). Substitution of two key tryptophan residues with phenylalanines resulted in much weaker membrane penetration. In aligned $q = 4$ DMPC/DMPG/DHPC bicelles, ^{31}P static and MAS NMR spectra revealed a change in the DMPG headgroup orientation with the addition of peptide, while ^{2}H NMR spectra revealed a slight increase in acyl chain ordering at a lipid:peptide ratio of 100:2. A mechanism of interaction of the peptide with the membrane is proposed. The ^{13}C and ^{31}P NMR chemical shift signature in combination with orientational features or order parameters from the measurement of $^{13}C-H$ or $^{13}C-^{31}P$ dipole couplings of the resident lipids represent a powerful tool for the study of interactions between membrane-associated amphiphiles or peptides and lipids. Recently, a heteronuclear dipolar solid state NMR experiment has been elegantly demonstrated in $q = 3.5$ DMPC/DHPC bicelles, where $^{13}C-^{1}H$ dipole couplings are correlated with ^{13}C CSAs for resolvable ^{13}C resonances of DMPC (*79*). Figure 3A features the two-dimensional spectrum of the magnetically aligned lipid bilayer at 37 °C, obtained using the SAMMY pulse sequence (*80*). Figure 3B shows local changes in the ^{13}C CSA and $^{1}H-^{13}C$ dipole couplings of DMPC upon introduction of very small amounts of a surface-associated antimicrobial peptide $(KIGAKI)_3$ and an integral membrane peptide, containing the transmembrane segment of phospholambin. Similar experiments have recently been performed using a novel pulse sequence scheme which has less stringent needs for homonuclear decoupling (*113*).

Spectroscopic Studies of Large Membrane Proteins in Magnetically Aligned Bicelles. The study of larger integral membrane proteins by solid state NMR brings new challenges in expression and reconstitution of appropriately labeled proteins in membranes, preparation of aligned samples, and execution of the appropriate one-, two-, or three-dimensional NMR experiment which gives separation

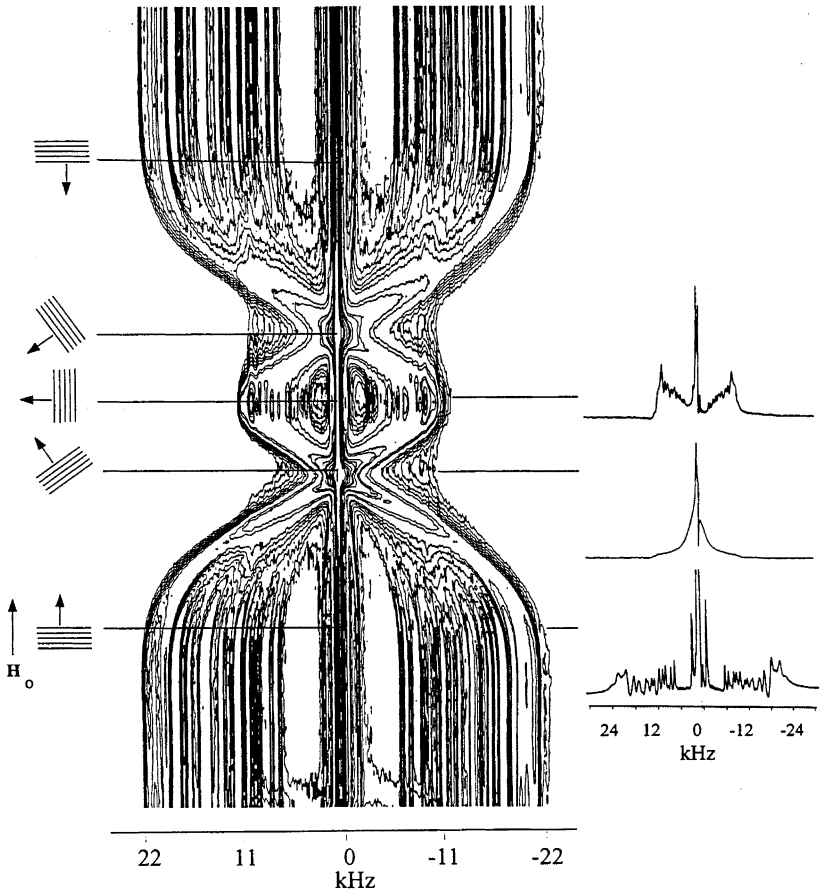

FIGURE 2: Contour plot of deuterium NMR spectra vs time (vertical axis) as a 40% (w/v) $q = 6.0$, DMPC/Yb = 90 aligned bilayer rotated from the equilibrium orientation (i.e., the bilayer normal, $\hat{n}$, parallel to the field) to an angle of 180°, as shown by the contours at the top of the figure. The entire contour involves a total of 124 unique slices in the vertical (time or, effectively, orientation) dimension and 1000 scans. A 3 s equilibration time was inserted between rotations. Experiments were performed using a home-built double-resonance probe, consisting of a 5.5 mm seven-turn solenoid. A programmable stepping motor was interfaced with the probe and the Bruker DMX 400 console (V. B. Volkov and R. S. Prosser, unpublished results).

and assignment of resonances associated with distinct residues. This section will focus on the PISEMA (polarization inversion spin exchange at the magic angle) experiment since the application of PISEMA to membrane proteins in bicelles has been met with the greatest success in studies of oriented samples (*81*−*85*).

The successful expression and purification of integral membrane proteins invariably involve the use of a fusion protein, while inclusion bodies seem to be the preferred target for overexpression of the protein (*6*). Purification and reconstitution typically involve affinity chromatography in the presence of lipids, and it has been shown that arbitrarily large integral membrane proteins such as the polytopic α-helical membrane proteins can be refolded and ultimately transferred to a bicelle medium (*6*). The homotrimer of the 121-residue polytopic α-helical membrane protein, DAGK, has been shown to regain activity upon reconstitution of DAGK into multilamellar POPC vesicles after the detergent had been dialyzed out of mixed micellar mixtures (*86*), and such methods can be applied to the preparation of bicelles for the purposes of magnetic alignment. Recently, a 350-residue G protein-coupled receptor, termed CXCR1, was heterologously expressed with high yields and purified and reconstituted into DMPC vesicles, whereupon appropriate amounts of DHPC were added to produce a bicelle medium suitable for solid state NMR studies (*87*). Thus, the procedures for reconstituting membrane proteins into magnetically aligned bicelles involve optimization steps which need to be determined for each system; invariably, these procedures are less challenging than the task of crystallizing a membrane protein.

^{15}N-labeled membrane proteins in oriented media provide two spectroscopic features which are useful for defining the orientational properties of the protein: the ^{15}N CSA and the ^{15}N−^{1}H dipole coupling. Both features may be simultaneously revealed for an arbitrary number of residues in the two-dimensional separated local field experiment (*88*), wherein ^{1}H−^{15}N dipolar evolution occurs under ^{1}H−^{1}H homonuclear decoupling, while the ^{15}N resonances can be uniquely observed under ^{1}H decoupling. In studies of magnetically aligned membrane proteins, significant improvements on this theme have been introduced by using either polarization inversion spin exchange at the magic angle (*89*, *90*) or SAMMY homonuclear decoupling schemes (*80*, *91*).

Figure 4 reveals the essential features of the ^{1}H−^{15}N dipole coupling/^{15}N chemical shift experiment, wherein an ideal helix is considered (Figure 4A) in which the principal axis of the chemical shift tensor is assumed to be within 17° of the N−H bond axis. As shown in Figure 4B, a dramatic profile of so-called PISA wheels is observed as a range of helix orientations, τ, is considered (*92*, *93*). As shown by the actual PISEMA data in Figure 4C for the transmembrane segment (residues 22−46) of the M2 protein from the

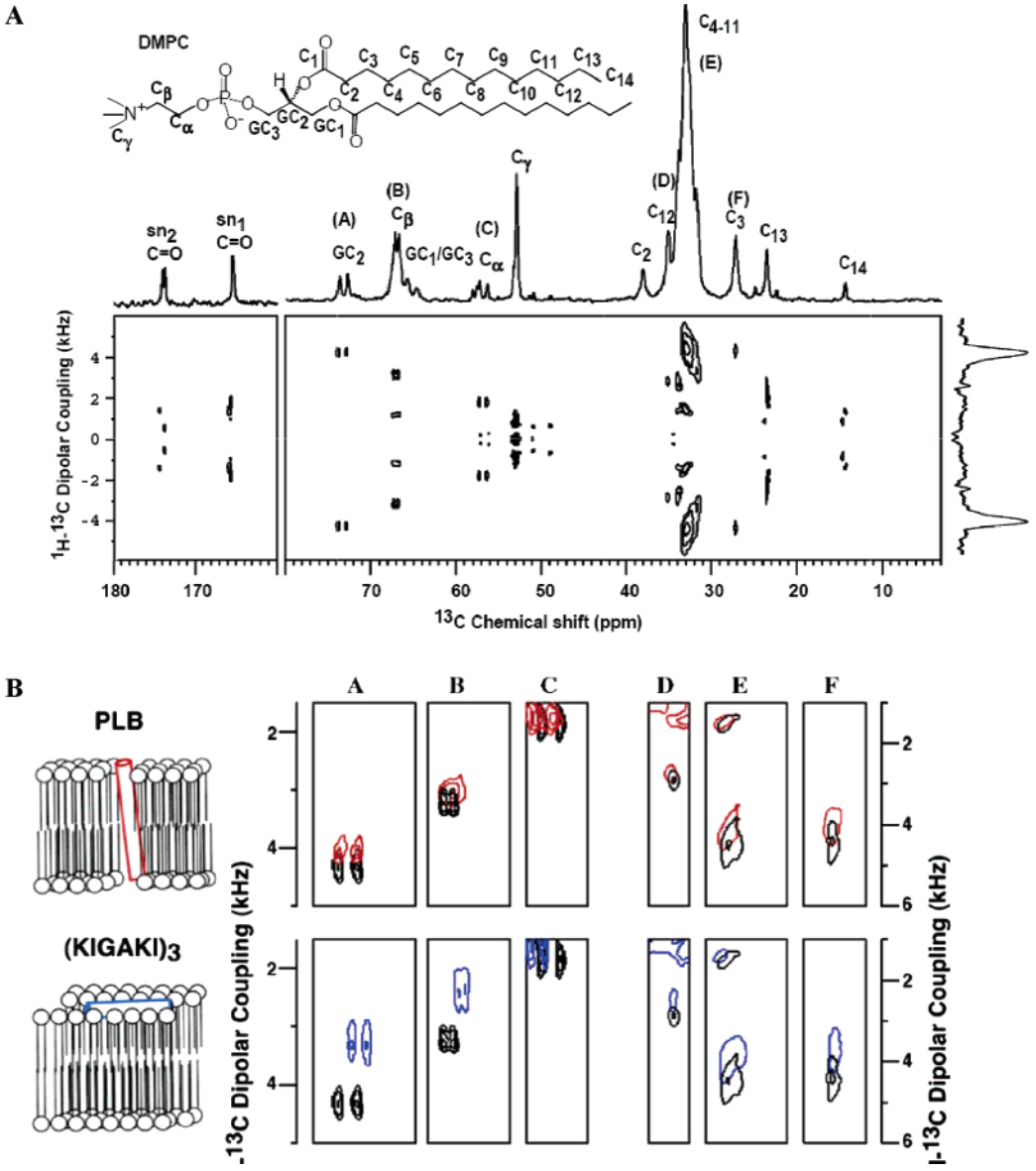

FIGURE 3: (A) Two-dimensional $^1H-^{13}C$ dipolar coupling/^{13}C chemical shift spectrum of a $q = 3.5$ DMPC/DHPC bicelle at 37 °C, utilizing the SAMMY pulse sequence on a Bruker 500 MHz narrow bore NMR spectrometer. The Hartmann−Hahn contact time was 1 ms; the ^1H 90 pulse was 10.1 μs, and the recycle delay was 3 s. The spectrum was collected with 200 t_1 increments and 512 scans per increment. The carbon position labeling is defined in the inset with the DMPC structure. The slice on the dipolar dimension represents the $^1H-^{13}C$ dipolar coupling taken from the GC_2 group. Labels A−F were used to indicate specific spectral regions in the lipid. (B) Position of labels A−F in the $^1H-^{13}C$ dipolar coupling/^{13}C chemical shift spectrum in the pure DMPC/DHPC bicelle (black) and the identical bilayer enriched with either the transmembrane segment of PLB (red) or an antimicrobial $(KIGAKI)_3$ peptide (blue). The left two bilayer models show the proposed modes of interaction between the lipid bilayer and the added biological molecules. Reproduced with permission from ref *79*. Copyright 2006 Elsevier Inc.

influenza A virus, the helix orientation can be defined extremely reliably. Moreover, as shown in Figure 5, assignment can be performed sequentially from such wheel-like patterns with knowledge of a single resonance. This procedure is made easier by mapping the observed NH dipole couplings to a sinusoid termed a dipolar wave. In the example shown in Figure 5, a PISA wheel and corresponding dipolar wave are shown for an ideal helix tilted by 20°. In this case, the authors assume that a single resonance associated with residue 28 has been assigned, for example, by isotopic labeling. Note that the position of the resonance and the corresponding phase of the dipolar wave are uniquely sensitive to the azimuthal rotation of the residue around the helix axis, as shown by two orientations (0° and 180°) in Figure 5. If a residue were present in multiple locations, assignment would be equally straightforward since the positions of the NH couplings on the dipolar wave and corresponding locations on the PISA wheel would be dictated by the orientational properties of the helix and the primary sequence. The dipolar waves have proven to be useful in studies of polytopic α-helical membrane proteins and in situations where a helix is interrupted by a kink or exhibits bending. The two-dimensional $^1H-^{15}N$ dipole coupling/^{15}N chemical shift experiment has been extended to three or more dimensions for the purposes of expanding the overall dispersion (*94*) and for the purposes of deriving alternative ways of making sequential assignments. Pulse sequence schemes have been implemented in which short-range

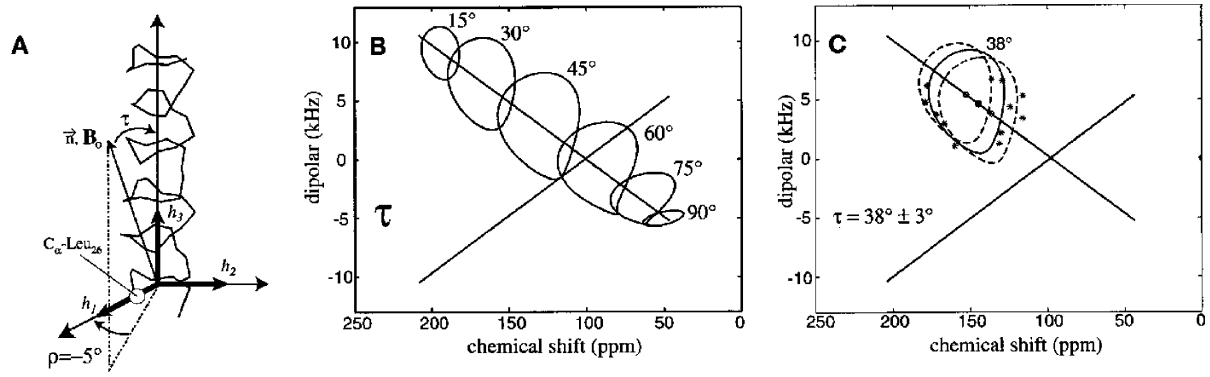

FIGURE 4: (A) Hypothetical α-helix defined by an orientation, τ, with respect to the magnetic field, and an azimuthal angle, ρ. (B) "Circles" drawn for one of the dipolar transitions using average values of tensor elements ($\sigma_{11} = 31.3$ ppm, $\sigma_{22} = 55.2$ ppm, and $\sigma_{33} = 201.8$ ppm) and the relative orientations of N−H bond and principal axis of a chemical shift tensor of 17°. (C) Characterization of the M2-TMP helix tilt from experimental PISEMA data which show a correspondence with a helix tilt of 38 ± 3°. Note that the center of the PISA wheels falls on a line that passes through the isotropic chemical shift (96 ppm) at 0 kHz on the dipolar scale. Reproduced with permission from ref *92*. Copyright 2000 Elsevier Inc.

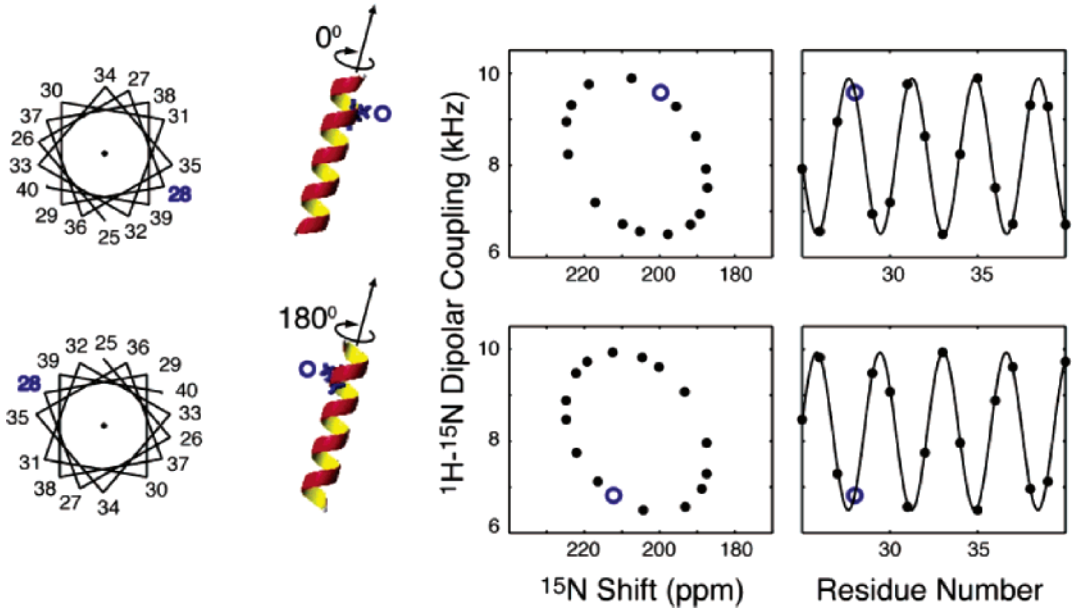

FIGURE 5: Ideal α-helix tilted at 20° relative to the director and corresponding PISA wheel patterns and NH dipolar waves, assuming two possible azimuthal orientations of the helix around its symmetry axis as shown. The position of a resonance from a particular residue in the wheel-like pattern is determined by its azimuthal orientation. Reproduced with permission from ref *13*. Copyright 2004 American Chemical Society.

homonuclear ^{1}H−^{1}H spin exchange can be used to sequentially connect ^{15}N−^{1}H pairs in a three-dimensional PISEMA experiment (*83*, *95*−*97*). Other triple-resonance experiments which connect the ^{1}H−^{15}N dipole coupling/^{15}N chemical shift to the adjacent ^{13}C′ (*82*) or ^{13}Cα species (*85*, *98*) have been carried out.

New Trends in Studies of Membrane Proteins in Magnetically Aligned Bicelles. Figure 6 depicts a solid state NMR spectrum of a uniformly ^{15}N labeled G protein-coupled receptor, CXCR1, in $q = 3.2$ magnetically aligned DMPC/DHPC bicelles (*87*). The absence of a powder pattern suggests that the 350-residue membrane protein effectively undergoes fast axially symmetric diffusion, giving the equivalent of a spectrum resulting from a uniaxially aligned sample where the symmetry axis (average bilayer normal) is perpendicular to the magnetic field. The authors estimate that for this to be the case, the rotational diffusion rate of the protein must be on the order of $\geq 10^5$ Hz. The spectral quality and line widths exhibited in the two-dimensional PISEMA spectrum of CXCR1, labeled only with [^{15}N]Ile, in the identical bicelle medium, attest to the alignment and fast motional averaging. These preliminary spectra demonstrate that there are now tremendous possibilities for solid state NMR studies of large integral membrane proteins in bicelles. At this early stage, it is also not clear to what extent the bicelle medium can be further optimized for purposes of maximizing dispersion and minimizing line widths in solid state NMR studies such as PISEMA. For example, flipped bicelles (*22*) which are obtained by doping the bicelles with Yb^{3+} ions or lipid chelates (*23*, *24*) complexed with Yb^{3+} should give spectra with the greatest separation of resonances (by a factor of 2 over bicelles) and should provide spectra characteristic of a true uniaxially aligned system, regardless of its correlation time. What remains is to optimize lipid composition to improve homogeneous broadening in both bicelles and flipped bicelles. Figure 7 reveals a cross section of PISEMA spectra obtained in oriented samples, with a careful consideration of line width as a function of protein

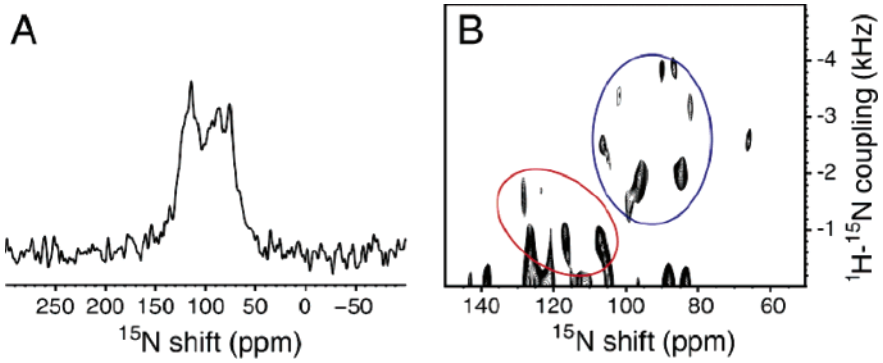

FIGURE 6: (A) Solid state NMR spectrum of uniformly ^{15}N labeled CXCR1 in a 28% (w/v) $q = 3.2$ DMPC/DHPC magnetically aligned bicelle medium, containing 0.6 mM protein. (B) Two-dimensional PISEMA spectrum of a [^{15}N]Ile-labeled sample collected with 24 t_1 increments and 1024 scans per increment. The spectra were obtained at 750 MHz on 150 μL samples at 40 °C. Reproduced with permission from ref *87*. Copyright 2006 American Chemical Society.

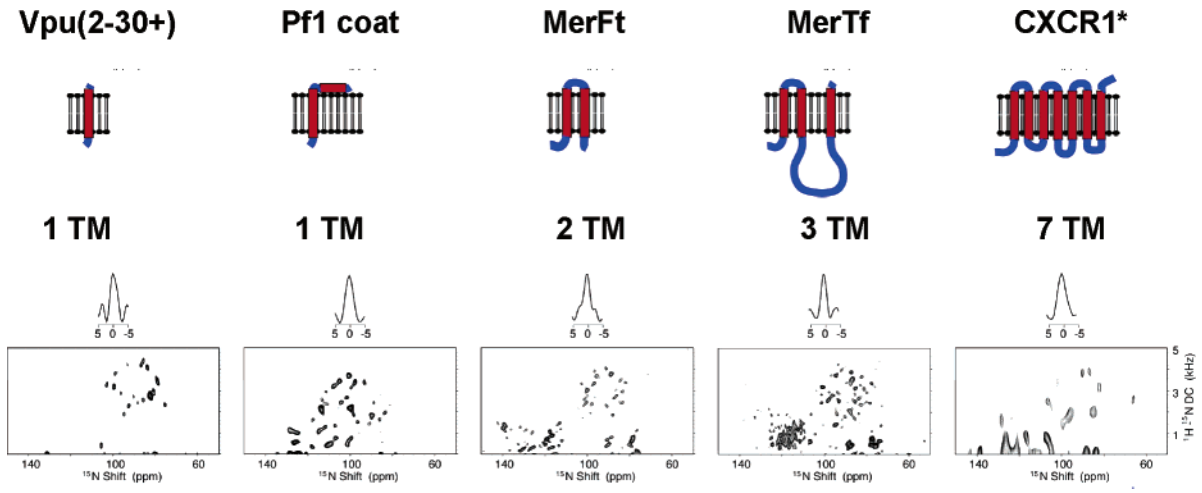

FIGURE 7: ^{15}N−^{1}H dipole/^{15}N chemical shift PISEMA spectra of uniformly ^{15}N labeled membrane proteins possessing one transmembrane domain [Vpu(1−30^{+}) (*101*) and Pf1 coat protein (*102*)], two transmembrane domains (MerFt) (*44*, *103*), three transmembrane domains (MerTf), and seven transmemrane domains (CXCR1). In the latter case, only the isoleucine residues were labeled. These spectra were recorded by A. A. DeAngelis. S. C. Howell, and S. H. Park in the group of S. J. Opella (University of California, San Diego, CA).

size. The remarkable feature is that all of the spectra are characteristic of fast axially symmetric motion, and thus, the corresponding systems are suitable for study with conventional bicelles. Recent measurements of the orientational dependence of ^{15}N line widths associated with the transmembrane helix of the Vpu protein from HIV-1 suggested the protein undergoes fast axially symmetric rotational diffusion at a rate of $\geq 10^4$ Hz (*99*). The associated line narrowing was observed in both mechanically aligned membranes and bicelles aligned with the bilayer symmetry axis both perpendicular and parallel to the magnetic field. A MAS NMR study of ^{13}C- and ^{15}N-enriched Vpu(1−40) recently confirmed this observation, in part on the basis of sideband intensities arising from modulation of the ^{13}CO CSA (*100*). The condition of fast axially symmetric diffusion in bicelles appears to be prevalent among the majority of ^{15}N-labeled membrane proteins of arbitrary size, as evidenced in Figure 7 by the ^{15}N line widths obtained from PISEMA spectra of an assortment of membrane proteins. Note that the spectra, which in the absence of fast axially symmetric diffusion would all appear as a powder pattern, are consistent with fast axially symmetric diffusion for ^{15}N-labeled membrane proteins possessing one transmembrane domain [Vpu(1−30^{+}) (*101*) and Pf1 coat protein (*102*)], two transmembrane domains (MerFt) (*44*, *103*), three transmembrane domains (MerTf), and seven transmemrane domains (CXCR1). Thus, major obstacles in terms of membrane protein expression, purification, and reconstitution in lipid bilayers and bicelles have been cleared. New homonuclear decoupling schemes and pulse sequences which improve overall dispersion and sequential assignment, in addition to probable improvements in bicelle recipes for studies of aligned membrane proteins, will provide for steady improvements in studies of integral membrane proteins, including GPCRs.

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