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Solid-State NMR Spectroscopic Studies of an Integral Membrane Protein Inserted into Aligned Phospholipid Bilayer Nanotube Arrays

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Oriented phospholipid bilayers have been used to investigate the structural and dynamic properties of integral membrane proteins utilizing solid-state NMR spectroscopic techniques.[1−5] Aligned membrane systems provide unique structural information, when compared with unoriented phospholipid bilayers consisting of multilamellar vesicles.[1] For example, solid-state NMR studies of ^{2}H-labeled or ^{15}N-labeled site-specific proteins incorporated into oriented membrane systems can determine the topology of the protein with respect to the phospholipid bilayer and the static magnetic field (B_o).[1,6] Generally, these techniques have used phospholipid bilayers mechanically aligned on glass plates or magnetically aligned phospholipid bilayers (bicelles).[1,3,7,8] In this communication, we successfully demonstrate for the first time that an integral membrane peptide can be incorporated into aligned phospholipid bilayer nanotube arrays and investigated with solid-state NMR spectroscopy. The ^{2}H solid-state NMR data are compared with parallel work conducted on magnetically aligned 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine/1,2-dihexanoyl-*sn*-glycero-3-phophatidylcholine (DMPC/DHPC) phospholipid bilayers.[5]

The phospholipid bilayer nanotube arrays were prepared utilizing nanoporous anodic aluminum oxide (AAO) as the substrate.[9] The proposed structure of the phospholipid bilayer nanotube arrays and the AAO substrate are shown in Figure 1.[9]

The structure of the membrane was characterized using ^{2}H solid-state NMR spectroscopy. For comparison, Figure 2A displays the ^{2}H NMR spectrum of magnetically aligned DMPC/DHPC/DMPCd_{54} phospholipid bilayers. For the bicelles, the bilayer normal is oriented perpendicular to the static magnetic field. Figure 2B shows the ^{2}H NMR spectrum of DMPC/DMPCd_{54} phospholipid bilayer nanotube arrays. Clearly, the well-resolved peaks in the NMR spectrum indicate that the phospholipid bilayers are well-aligned with the bilayer normal oriented perpendicular with respect to B_o. The quadrupolar splittings of the peaks corresponding to the CD_3 and CD_2 groups on the acyl chains of DMPC indicate a slight increase in order when compared to the bicelle sample in Figure 2A. The ^{2}H quadrupolar splitting (24 kHz) of the plateau region for the nanotubes is approximately equal to DMPCd_{54} phospholipid bilayers mechanically aligned on glass plates with the membrane normal perpendicular to B_o.[10] For comparison, the ^{2}H quadrupolar splitting of the bicelle plateau region is 20 kHz. The unique aligned lipid bilayers are formed in the small nanopores of the substrate, as opposed to a series of several hundred macroscopic bilayer sheets formed on top of mechanically aligned glass plates.[9,11]

We have used a transmembrane peptide containing the amino-proximate TM-A domain of the integral membrane acetylenase CREP-1 (SSYYIVHDAIIAYIFYFLADKYI) to illustrate the feasibility of using phospholipid bilayer nanotube arrays for solid-state NMR studies on integral membrane proteins.[12] The procedure for synthesizing and purifying the peptide has been established in the literature.[12] CD studies on the integral membrane protein TM-A have indicated that the peptide is dominantly α-helical.[12] Figure 2C shows the solid-state ^{2}H NMR spectrum of ^{2}H-labeled CD_3 Ala-56 CREP-1 TM-A peptide inserted into bicelles. The bilayer normal is perpendicular to B_o, and the resultant quadrupolar splitting is 14.8 kHz. Addition of Yb^{3+} to the bicelle flips the bicelle 90°, such that the bilayer normal is now parallel with B_o. At this orientation, the quadrupolar splitting doubles to 30.0 kHz (data not shown). This additional orientation enables the helical tilt to be calculated more accurately.[13−17] Figure 2D represents the ^{2}H solid-state NMR spectrum of the same ^{2}H-labeled CD_3 Ala-56 TM-A peptide inserted into DMPC phospholipid bilayer nanotube arrays. The resultant quadrupolar splitting of 15.0 kHz is slightly larger than the splitting for the bicelle spectrum. The nanotube array ^{2}H line widths are marginally broader than the corresponding bicelle line widths. The increased line width may be due to a slight misalignment of the nanotubes or the more homogeneous B_o in

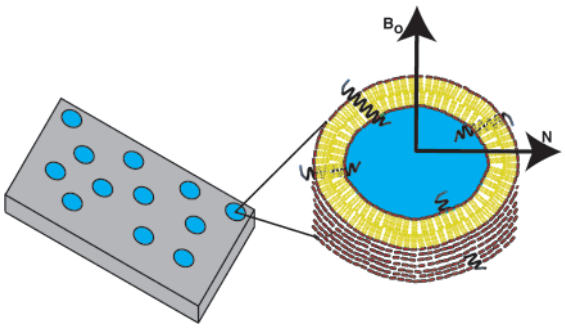

Figure 1. Illustration of phospholipid bilayer nanotube arrays. The AAO substrate is shown on the left. The pore size is shown in blue. On the right, a blowup of the water-filled phospholipid bilayer nanotube arrays contained within the AAO substrates is presented.

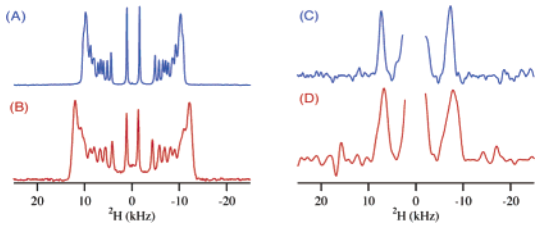

Figure 2. ^{2}H solid-state NMR spectra of bicelles (blue) and aligned phospholipid bilayer nanotube arrays (red). (A) DMPC/DMPC$_{d54}$/DHPC bicelles. (B) DMPC/DMPC$_{d54}$ phospholipid bilayer nanotube arrays. The quadrupolar splitting and well-resolved peaks indicate that the bilayer normal is perpendicular to B_o. For these spectra, 8 K scans were averaged. (C) ^{2}H-labeled CD_3 Ala-56 TM-A inserted into bicelles. (D) ^{2}H-labeled CD_3 Ala-56 TM-A inserted into aligned lipid nanotube arrays. For the TM-A spectra, 400 K scans were signal-averaged. All spectra were acquired using a standard quad echo pulse sequence with a 3.0 μs 90° pulse and a 300 ms recycle delay. The bicelle and bilayer nanotube array spectra were collected at 37 °C.

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the high-resolution CP-MAS probe used to collect the bicelle spectra. The peak centered at 0 kHz is due to residual D_2O from the ^{2}H-depleted water. The absence of any powder pattern components indicates that the TM-A peptide is well-oriented in the phospholipid bilayer nanotube arrays. ^{2}H NMR analysis of the aligned quadrupolar splittings indicate that the helical tilt of the TM-A CREP-1 peptide is $6° \pm 6°$ with respect to the bilayer normal.[13−17] Thus, phospholipid bilayer nanotube arrays are an excellent model membrane system for conducting oriented solid-state NMR experiments.

The bilayer nanotube arrays were prepared by mixing 20 mg of DMPC and 2 mg of $DMPCd_{54}$ in chloroform.[9] The solvent was removed with a rotary evaporator, and the flask was placed under high vacuum overnight to completely remove residual chloroform. For the TM-A sample (0.75 mg), TM-A was dissolved in trifluoroethanol (TFE) before being transferred to the DMPC. The mixture was then evaporated to remove the chloroform and TFE. The following day, Tris buffer (50 mM, pH 7.0) was prepared using deuterium-depleted water, and 190 μL of the buffer was added to the flask containing the lipid. The flask was then vortexed gently until a homogeneous suspension was obtained. Several freeze (77 K)/thaw cycles were made at 40 °C to remove all of the air bubbles. Typically, the total mass of the prepared sample was 190 mg. The DMPC/TM-A sample was deposited on nanoporous AAO disks via a Pasteur pipet. The AAO disks (Whatman) were 47 mm in diameter with a pore size of 200 nm. Approximately 20 AAO disks of dimensions 8.5×14 mm were used to deposit the sample. The AAO disks containing the lipids were incubated for 5 min in an oven at 40 °C. Deuterium-depleted water was deposited on the AAO disks containing the lipids, and any excess of the sample was carefully removed from the surface using Kimwipes disposable wipes. The fully hydrated disks were stacked on top of each other for the NMR studies. The sample was placed in a flat coil solid-state NMR probe that is typically used for experiments with phospholipid bilayers mechanically oriented on glass plates.

The ^{2}H bicelle samples were prepared using DMPC and DHPC phospholipids at a q ratio of 3.5. DMPC (72 mg) was placed into a pear-shaped flask, and DHPC (15 mg) was added to a second pear-shaped flask. ^{2}H-labeled Ala-56 TM-A (2 mg) was dissolved in TFE (0.2 mL) and added to the second flask. Both flasks were concentrated to dryness using a rotary evaporator and stored under vacuum overnight. HEPES buffer (270 μL, 100 mM, pH 7.0) made from deuterium-depleted water was added to the second flask. The flask was vortexed, sonicated, frozen, and thawed as necessary to homogenize the sample. The sample was transferred to a static 5-mm round coil Bruker solid-state NMR probe operating at a frequency of 76.7 MHz for ^{2}H.

Phospholipid bilayer nanotube arrays offer several unique advantages for solid-state NMR spectroscopic studies. First, in a standard flat-coil solid-state NMR probe, the membrane normal of the lipid bilayers in the nanotubes are aligned perpendicular to B_o. Conversely, using the same solid-state NMR probe with mechanically aligned glass plate substrates, the membrane sample is aligned such that the bilayer normal is parallel with the static magnetic field. Thus, the same probe can be used to study membrane samples aligned both parallel (glass plates) and perpendicular (nanotube arrays) with respect to the static magnetic field without changing coils or altering the orientation of the coil of the probe. It is advantageous for protein ^{2}H NMR studies to compare two unique orientations. Second, phospholipid bilayer nanotube arrays are more rigid when compared to bicelles. Thus, the ^{1}H magnetization transfer will be more efficient for studying low-abundance nuclei such as ^{13}C or ^{15}N under cross-polarization conditions. Also, the phospholipid bilayer nanotube arrays are defined by the rigid homogeneous size of the pores on the AAO substrate.[11] The pore size can be easily adjusted over a range from 4 to 200 nm.[9,11] Different pore sizes may be advantageous for aligned solid-state NMR experiments with lipids differing in acyl chain length. Finally, previous EPR studies on oriented phospholipid bilayer nanotubes have been conducted at low temperatures down to 150 K.[9] The phospholipid bilayer nanotubes formed in the rigid pores have retained alignment at low temperatures well into the gel phase. Thus, at low temperatures oriented phospholipid bilayer nanotubes may be beneficial for solid-state NMR experiments that suffer because of poor signal sensitivity.[18] At low temperatures, the NMR signal will be significantly enhanced because of the increase in magnetization from the Boltzmann factor.

The results in this communication indicate for the first time that phospholipid bilayer nanotube arrays are an excellent model membrane system for conducting solid-state NMR studies on oriented integral membrane protein systems.

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