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Construction of ^{1}H-^{15}N Double Resonance Solid-State NMR Probe for Membrane Proteins in Aligned Bicelles

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^{1}H-^{15}N heteronuclear dipolar coupling solid-state NMR experiments on lipid bilayer or bicelle samples are very useful for the structural studies of membrane proteins. However, to study these biological samples using solid-state NMR, a specific probe with high efficiency and high capability is required. In this paper, we describe the optimized design, construction, and efficiency of a 400 MHz wide-bore ^{1}H-^{15}N solid-state NMR probe with 5-mm solenoidal rf coil for high power, multi-pulse sequence experiments, such as 2D PISEMA or 2D SAMMY.

Key Words: Solid-state NMR, Home-built probe, Membrane proteins, Bicelles, Bilayers

Introduction

X-ray crystallography and conventional solution NMR spectroscopy techniques are difficult to use for the study of membrane protein structures, because the lipids required for the structural integrity and functionality of the membrane proteins prevent the crystallization of the proteins as well as reducing their rate of overall reorientation in solution.[1,2] A lipid bilayer provides the protein with a physical and chemical environment more similar to a natural membrane, but its large size and slow tumbling impose the use of solid-state NMR methods.[1,2] Solid-state NMR experiments on lipid bilayers or bicelle samples are valuable for membrane proteins with a predominantly helical secondary structure, because these oriented samples take advantage of the spectral simplifications that result from their uniaxial orientation parallel to the direction of the applied magnetic field. The orientational restraints resulting from membrane proteins in a lipid environment can be examined by ^{1}H-^{15}N heteronuclear dipolar coupling solid-state NMR experiments, such as polarization inversion spin exchange at the magic angle (PISEMA)[2-6] or high resolution separated local field spectroscopy based on magic-sandwich pulses (SAMMY).[6-8] Magnetically aligned bicelle samples in an NMR tube[9-13] or planar lipid bilayer samples which are mechanically aligned and supported on glass slides are often used for these experiments.[14-18] Especially, the sample preparation of bicelles is easier and bicelle samples are more stable than mechanically oriented lipid bilayer samples between glass plates and they can even be kept for more than a year. Therefore, in recent years, magnetically aligned bicelle samples have frequently been used to determine the structure of membrane proteins by solid-state NMR spectroscopy. Most of these biological samples have high dielectric properties, due to their containing large amounts of phospholipids and salts. These electrical properties cause a loss of efficiency of the probe by severely reducing its Q-factor (quality factor) and significantly down-shifting the tuned frequency. Moreover, these biological samples are readily heated and destabilized during radio frequency (rf) irradiation.[19,20] For these reasons, a specific probe with high efficiency and high capability is required to study these biological samples using solid-state NMR, and several probe designs have been developed in recent studies to improve their efficiency.[21,22]

In this paper, we present the optimized design, construction, and efficiency of a 400 MHz wide-bore (WB) ^{1}H-^{15}N solid-state NMR probe with 5-mm solenoidal rf coil for high power, multi-pulse sequence experiments, such as 2D PISEMA or 2D SAMMY. This probe provides short pulses, high power capability, and good rf homogeneity. The ^{1}H-^{15}N 2D SAMMY spectra from a single crystal and membrane protein in oriented phospholipid bicelles were successfully obtained using this home-built solid-state NMR probe at 400 MHz.

Experimental Methods

Design of home-built NMR probe. We designed and built a double resonance solid-state NMR probe for 400 MHz static round coil probe. The probe body was constructed from an aluminum 6061 pipe with an outer diameter of 71.9 mm. The 5-turn round solenoidal sample coil was made from gold-plated flat wire. The sample coil has an inner diameter of 5 mm and length of 12 mm in order to maximize the filling factor for the bicelle samples of membrane proteins in a glass tube (OD = 5 mm, L = 15 mm, T = 0.4 mm) with a flat bottom. The coil was placed at the center of a polyoxymethylene (POM) plate with a lower pipe (ID = 3 mm, OD = 5 mm, L = 52 mm) to provide a cooling airflow for the sample, as shown in Fig. 1. To control the temperature of the sample, the cap design at the top of the probe-head was connected to the Varian solid VT upper stack.

Fig. 2 shows the double-tuned rf circuit employed in our 400 MHz ^{1}H-^{15}N probe. In Fig. 2, L1 designates the sample coil and C designates the capacitors. Capacitors C3, C4, and C7 are utilized along with the $\lambda/4$ coaxial wavelength cable to tune and match the high frequency decoupled channel (^{1}H). The length of the $\lambda/4$ coaxial wavelength cable was calculated as follows:

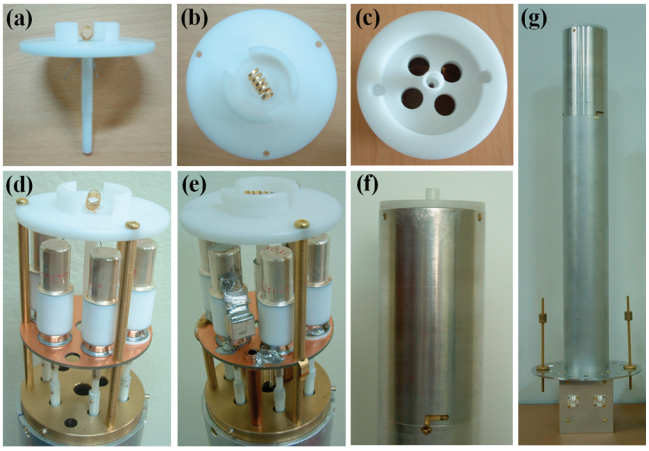

Figure 1. Pictures of the 400 MHz WB home-built solid-state NMR probe with a round solenoidal sample coil designed for ^{1}H and ^{15}N double resonance experiments. (a) side view and (b) top view of solenoidal coil at the center of a polyoxymethylene (POM) plate with lower pipe providing a cooling airflow for the sample, (c) top view and (f) side view of probe-head cap with a hole for interfacing with the Varian solid VT upper stack, (d) probe-head view before soldering, (e) probe-head view after soldering, (g) external appearance of whole probe.

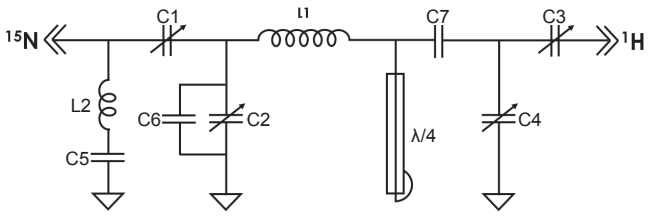

Figure 2. Schematic circuit diagram of the 400 MHz WB ^{1}H and ^{15}N double resonance probe with a 5 mm round solenoidal coil. Capacitors C3 and C4 provide tuning and matching for the decoupled frequency channel (400 MHz), respectively. Capacitors C1 and C2 provide tuning and matching for the observed frequency channel (40 MHz), respectively. Capacitors C1, C2, C3, and C4 are variable capacitors with a range of 1-10 pF. The values of the fixed capacitors are: C5 = 22 pF, C6 = 95 pF, C7 = 3.9 pF. L1 represents the five turn 5 mm round solenoidal coil. λ/4 is a quarter lambda coaxial wave length cable whose length is 12.5 cm. The resonator is impedance matched to 50 Ω.

$$\text{Length of } \lambda/4 \text{ cable} = \frac{c \cdot \varepsilon}{4 \cdot \nu}$$

$$= \frac{3\times10^{10}(\text{cm/sec})\times0.667}{4\times400\times10^{6}(\text{sec}^{-1})}$$

$$= 12.5 \text{ (cm)}$$

where c is the speed of light, ν is the frequency of the proton channel, and ε is the dielectric constant of the coaxial cable. Capacitors C1, C2, and C6 are used to tune and match the low frequency observed channel (^{15}N). The inductor L2 and fixed capacitor C5 connected in series are used as the high frequency reflection trap. The inductor L2 is made of 2.5 mm copper wire with a 1/2 turn. The L2-C5 trap allows for the effective tuning of the observed channel and provides better isolation between the decoupled frequency channel and observed channel. Capacitors C1 and C2 used to tune and match the nitrogen channel and capacitors C3 and C4 used to tune and match the proton channel are nonmagnetic, high voltage variable capacitors (Polyflon), which have capacitances ranging from 1 to 10 pF. C5 (22 pF), C6 (95 pF), and C7 (3.9 pF) are fixed capacitors with different sizes from American Technical Ceramics. All rf components were soldered on a grounded printed circuit board. A semirigid coaxial cable with PTFE dielectric was used for the transmitting and receiving paths between the probe circuit and probe port, because of its higher power handling capabilities. To provide a reliable ground between the rf components and probe cap, copper fingers were used. The low power tuning and matching measurements of the resonance circuit were first done with a network analyzer (Hewlett Packard 3753C). After the probe was inserted into the magnet, the observation of the resonance of the probe circuit was performed with the built-in tuning function of the Varian spectrometer software (VNMRJ 2.1B).

Sample preparation. hAPP-TM peptide from the transmembrane domain of human amyloid precursor protein was recombinantly expressed and purified to perform the NMR analysis. Briefly, the gene for the 39-residue sequence of hAPP-TM was cloned downstream of a 125 amino acid ketosteroid isomerase gene and upstream of a hexa-histidine tag sequence in pET-31b vector and transformed into *E. coli*. Uniformly ^{15}N-labeled hAPP-TM peptide was expressed in the form of inclusion bodies, purified by immobilized nickel affinity chromatography, and chemically cleaved by cyanogen bromide. The final purification of hAPP-TM was achieved by preparative reversed-phase high performance liquid chromatography.

The purified peptide was dissolved in 1 mL of trifluoroethanol/chloroform 1/3 (v/v). The organic solvents were removed by a stream of nitrogen gas, followed by drying under high vacuum overnight. 3 mg of the dried peptides were dissolved in 9.5 mg of 1,2-di-*O*-hexyl-sn-glycero-3-phosphocholine (6-*O*-PC) and then the micelle solution was added to 45.6 mg of 1,2-di-*O*-tetradecyl-sn-glycero-3-phosphocholine (14-*O*-PC). The opaque mixture solution became transparent after several cycles of freezing and thawing, indicating that bicelles were formed. The final molar ratio of long chain to short chain lipids (q) was 3.2 and the lipid concentration was 28% (w/v) in a volume of 200 μL. The final peptide concentration was 3 mM with pH 5.0. A short, flat-bottomed tube with an outer diameter of 5 mm was filled with 160 μL of the bicelle solution.

Solid-state NMR experiments. The All NMR experiments were carried out using a 400 MHz (9.4 T) WB magnet, a Varian unityINOVA Spectrometer, and a home-built probe. In some cases, comparison measurements were performed with a conventional NMR probe (Varian 4-mm T3 CP/MAS probe). The home-built probe was positioned in the central sweet spot of the magnet by observing the proton resonance of 50 mM $CuSO_4$-doped water. The heterogeneity of the magnetic field was minimized by preshimming on the proton signal of 50 mM $CuSO_4$-doped water. The rf power input to the probe was measured using a Tektronix TDS3052B Digital Phosphor Oscilloscope. In order to characterize the circuit efficiency and B_1 rf field homogeneity,

nutation experiments were performed on 50 mM $CuSO_4$-doped water and ^{15}N labeled glycine for both the ^1H and ^{15}N channels. The one-dimensional ^{15}N NMR spectra of a single crystal of ^{15}N-acetyl-leucine (NAL) and bicelle sample were obtained by a 1.0 ms cross polarization with SPINAL-64 ^1H decoupling.[23] The two-dimensional ^1H-^{15}N heteronuclear dipolar coupling solid-state NMR spectra of NAL and the bicelle sample were obtained using a SAMMY pulse sequence. All ^{15}N chemical shifts were set relative to the ^{15}N resonance of ammonium sulfate at 26.8 ppm.

Results and Discussion

Before mounting the home-built solid-state NMR probe into the magnet, the tuning, matching, and electrical measurements of the probe circuit were performed using a network analyzer. The Q-factors for both the ^1H and ^{15}N channels of the home-built probe were measured from a graph of the reflected power versus frequency as follows:

$$\text{Q-factor} = \frac{\text{Resonant frequency}}{\text{Bandwidth}} = \frac{f_0}{|f_1 - f_2|}$$

where f_0 is the resonant frequency of the respective channel, and f_1 and f_2 are the frequencies at -3 dB from the baseline of total reflection of the respective resonant frequency. Both channels of the home-built probe show high Q-factors of more than 250. The isolation between the ^1H and ^{15}N channels was measured to be 26 dB from high to low frequency and 25 dB from low to high frequency. This high isolation factor and high Q-factor are good enough to measure high resolution solid-state NMR spectra. After mounting the home-built probe into the magnet, final tuning and matching were achieved using Varian QTUNE software.

The rf performance of the 400 MHz ^1H-^{15}N round coil probe was tested by delivering various pulse widths with different input powers in 50 mM $CuSO_4$-doped water and ^{15}N labeled glycine. A B_1 rf field strength of 114 kHz was achieved at an input power of 300 W in the ^1H frequency channel (399.9 MHz) and a

Table 1. Summary of the performance of the home-built probe.

Channel	^1H	^{15}N
Q-factor	> 250	> 250
Isolation	$^1H \rightarrow {}^{15}N$; 26 dB	$^{15}N \rightarrow {}^1H$; 25 dB
Nutation frequency, B_1	114 kHz	83 kHz
Power	300 Watts	850 Watts
B_1 homogeneity ($A810^o/A90^o$) × 100%	71%	81%

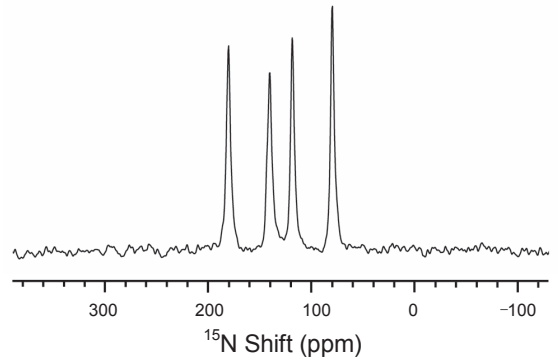

Figure 4. Experimental one-dimensional ^{15}N chemical shift NMR spectrum of a single crystal of NAL. The spectrum was obtained at 298 K using a 9.4 T magnet with an ^1H resonance frequency of 400 MHz and the home-built probe with a 5-mm double-tuned solenoidal round rf coil. 1024 data points were acquired using cross-polarization with B_1 fields of 62 kHz, a mixing time of 1 ms and a recycle delay of 5 s. The acquisition time was 25.3 ms with 128 accumulations.

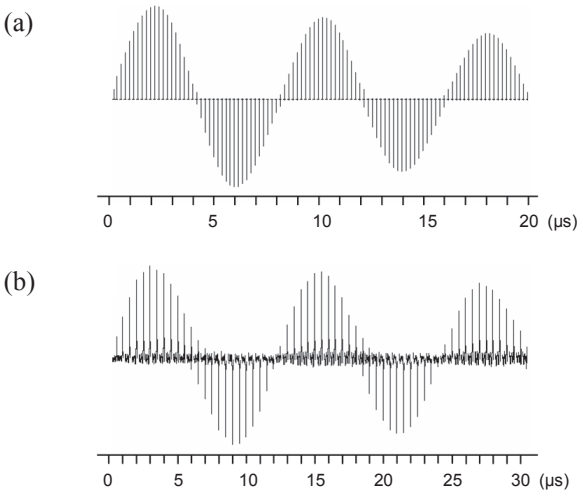

Figure 3. ^1H and ^{15}N B_1 nutation profiles for 50 mM $CuSO_4$-doped water and ^{15}N labeled glycine. (a) rf homogeneity for ^1H channel: Starting at 0.2 μs with intervals of 0.2 μs. A 90° pulse length of 2.2 μs at 300 W is achieved. $A_{810°}/A_{90°} = 71\%$, (b) rf homogeneity for ^{15}N channel: Starting at 0.5 μs with intervals of 0.5 μs. A 90° pulse length of 3.0 μs at 850 W is achieved. $A_{810°}/A_{90°} = 81\%$.

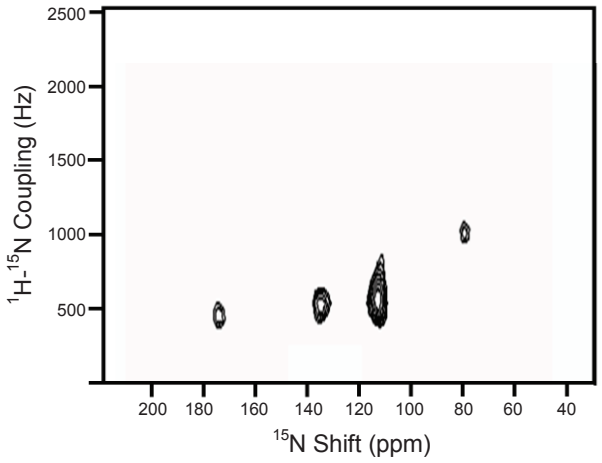

Figure 5. Experimental two-dimensional ^1H-^{15}N heteronuclear dipolar coupling/^{15}N chemical shift SAMMY spectrum of a single crystal of NAL. The spectrum was obtained at 298 K using a 9.4 T magnet with an ^1H resonance frequency of 400 MHz and the home-built probe with a 5-mm double-tuned solenoidal round rf coil. The acquisition parameters were B_1 fields of 62 kHz, 8 scans, a cross-polarization mixing time of 1 ms, recycle delay of 5 s, and acquisition time of 25.3 ms using SPINAL-64 for heteronuclear decoupling.

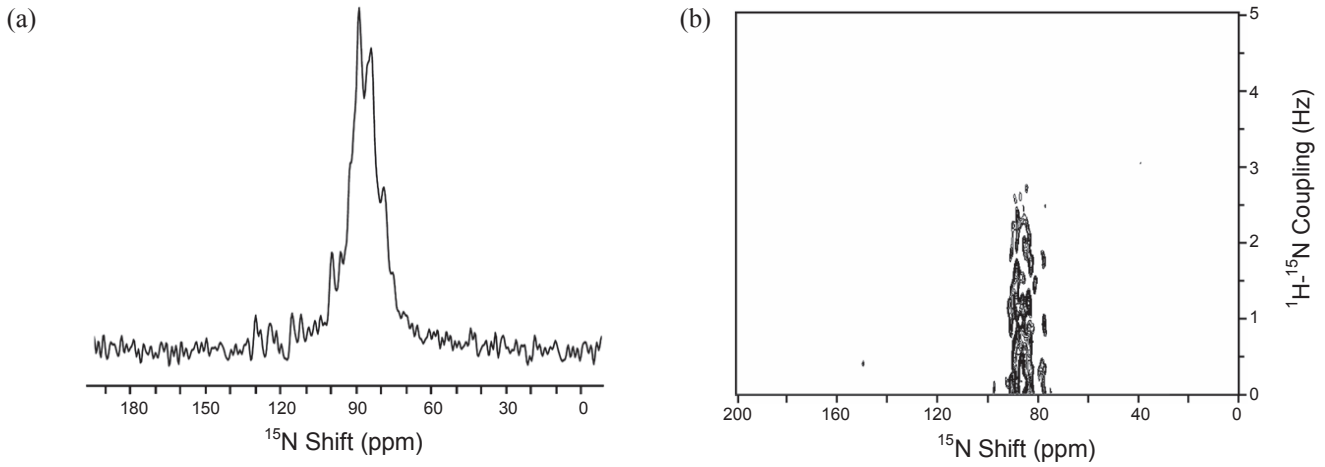

Figure 6. Experimental NMR spectra of hAPP-TM peptide using the home-built probe. (a) One-dimensional ^{15}N chemical shift spectrum and (b) two-dimensional ^{1}H-^{15}N SAMMY spectrum of hAPP-TM peptide in 14-*O*-PC/6-*O*-PC (q = 3.2) bicelles aligned magnetically with their normals perpendicular to the magnetic field. 3 mg of uniformly ^{15}N labeled hAPP-TM peptide were used and the sample temperature was 315 K with pH 4.5. Experimental parameters: (a) 4096 transients with recycle delay of 5 s, B_1 ^{1}H SPINAL decoupling = 62.5 kHz, B_1 cross-polarization = 62.5 kHz, (b) 184 t1 increments with 256 transients each, recycle delay of 5 s, B_1 ^{1}H SPINAL decoupling = 62.5 kHz, B_1 cross-polarization = 71.4 kHz, B_1 spinlock for magic-sandwich cycles = 71.4 kHz.

B_1 rf field strength of 83 kHz was achieved at an input power of 850 W in the ^{15}N frequency channel (40.5 MHz) without arcing. To characterize the homogeneity of the B_1 rf field of the probe coil, nutation experiments were performed on the same samples. The B_1 rf field homogeneity was measured as the ratio of the 810° to 90° pulse amplitudes for both the ^{1}H and ^{15}N channels. The ratios were found to be 71% and 81% in the ^{1}H and ^{15}N channels, respectively, indicating highly homogeneous rf fields that benefit the cross-polarization experiments. Figs. 3(a) and (b) show the B_1 nutation profiles obtained at both frequencies. The rf performance of the 400 MHz ^{1}H-^{15}N home-built round coil probe is summarized in Table 1.

Fig. 4 shows the one-dimensional ^{15}N chemical shift NMR spectrum of a NAL single crystal sample obtained with the home-built solid-state NMR probe. The acquisition time was 25.3 ms with 128 accumulations. Although each molecule has only one ^{1}H-^{15}N bond, the spectrum has four resonances, because there are four different conformational sites in the unit cell of the crystal.

The two-dimensional heteronuclear dipolar coupling spectrum shown in Fig. 5 was obtained with the SAMMY pulse sequence at a ^{1}H resonance frequency of 400 MHz. With 128 t_1 increments, 1024 t_2 complex points, 8 transients, and recycle delay of 5 s, the total acquisition time was 1 hour 26 minutes. The $\pi/2$ pulse was calibrated at 4.0 μs and a B_1 field strength of 62 kHz was used for the Hartmann-Hahn match during the CP contact period. The dipolar axis is adjusted to account for the scaling factor of 1.37. The spectrum shows an average linewidth of 6 ppm in the chemical shift and an average linewidth of 280 Hz in the dipolar dimension.

Fig. 6 shows the one- and two-dimensional solid-state NMR spectra of uniformly ^{15}N-labeled hAPP-TM peptide in 14-*O*-PC/6-*O*-PC (q = 3.2) bicelles aligned magnetically with their normals perpendicular to the magnetic field obtained with the home-built probe. The ^{15}N chemical shifts, which depend on the orientation of the transmembrane helix (70 - 100 ppm), are obtained from the 1D spectrum, as shown in Fig. 6(a). Fig. 6(b) shows the two-dimensional SAMMY spectrum of the aligned bicelle sample. Each amide resonance is characterized by the frequencies from the ^{15}N chemical shift and ^{1}H-^{15}N heteronuclear dipolar coupling interactions. The resonance frequencies suggest that the helical structure is in an orientation parallel to the normal of the bicelles. The detailed structural information will be discussed in another paper.

Conclusions

A 400 MHz WB ^{1}H-^{15}N home-built solid-state NMR probe with 5-mm solenoidal rf coil was designed and constructed for the structural studies of oriented biological samples, and preliminary NMR data were acquired to demonstrate its efficiency. The isolation between the high and low-frequency channels and probe Q factor were optimized. The RF circuit showed good B_1 rf field homogeneity during the nutation experiments. In addition, we successfully obtained the 1D and 2D NMR spectra of a magnetically aligned biological bicelle sample.

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