Use of a Copper-Chelated-Lipid Speeds Up NMR Measurements From Membrane Proteins

*Kazutoshi Yamamoto[1], Jiadi Xu,[1,2] Karen E. Kawulka,[3] John C. Vederas[3], and Ayyalusamy Ramamoorthy,[1,2]**

[1]Department of Chemistry and [2]Biophysics, University of Michigan, Ann Arbor, MI 48109-1055, USA[1]

[3]Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada

Supporting information

Protein expression protocol :

In this study, we used an antimicrobial peptide subtilosin A embedded in bicelles as a test sample to demonstrate the efficiency of paramagnetic-doping to speed up solid-state NMR measurements. Subtilosin A is a 35-residues (a molecular weight of 3398.9 Da) cyclic antimicrobial peptide produced by *Bacillus subtilis* bacteria. As shown in Figure S1, this cyclic peptide has 3 unusual chemical bonds (three cross-links between sulphurs of cysteine and the a-positions of amino acids are linked by a chemical bond between residues (bonds in blue color): Cys-4 and Phe-31, Cys-7 and Thr-28, and Cys-13 and Phe-22) that render rigidity to the structure of the peptide. Our previous solid-state NMR studies have reported the mechanism of action of subtilosin A.[4]

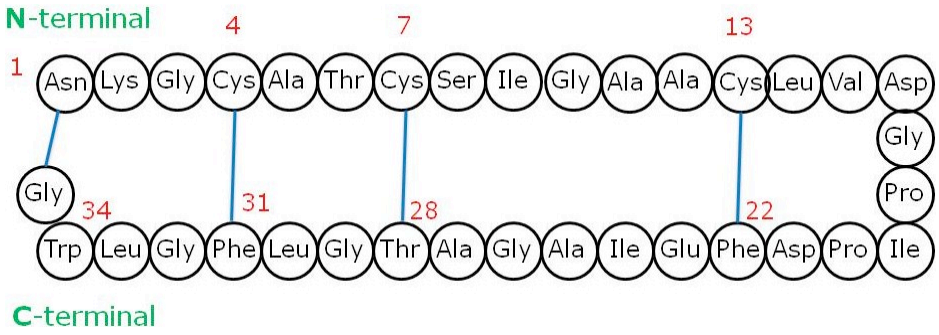

Figure S1. An amino acid sequence of a cyclic antimicrobial peptide, subtilosin A.

For this study, 12 to 14 % ^{15}N isotope labeled Subtilosin A was produced from fermentations of *Bacillus subtilis*, isolated and purified as reported elsewhere.[1,2,3] A filter-sterilized 10 mL of 0.15 M [^{15}N] NH_4NO_3 in 10 mL water and 0.5 mL of 1 M $CaCl_2 \cdot H_2O$ were used instead of 0.5 mL of 1 M $Ca(NO_3)_2 \cdot 4H_2O$ in the original procedure. MALDI TOF mass spectroscopy was applied to analyze the percentage of ^{15}N incorporation in subtilosin A as described previously.[2,3] Labeled [^{15}N] NH_4NO_3 was purchased from Cambridge Isotope Laboratories (Andover, MA).

Preparation of bicelles for solid-state NMR experiments

In this study, to demonstrate the efficiency of paramagnetic effects in reducing the spin-lattice relaxation (T_1) of protons, we used magnetically-aligned bicelles as a model membrane. As mentioned in the main text of the manuscript, the use of copper-EDTA resulted in sample heating that interfered with the alignment of bicells as observed from the broadening of ^{31}P spectral lines. Therefore, we decided to develop alternate approaches to optimize the sample

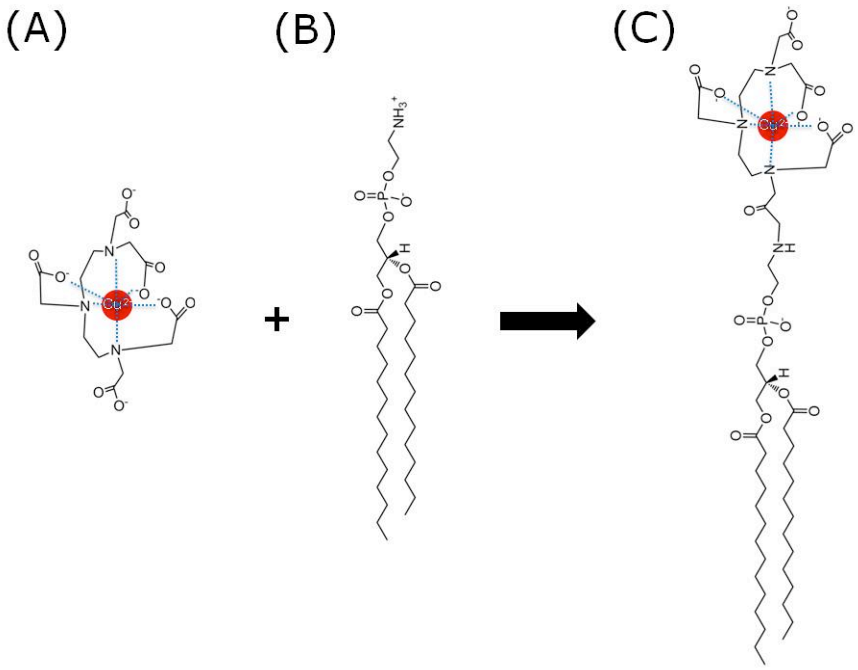

Figure S2. (A) The structure of DTPA that is commonly used as chelating metal ions. (B) The structure of DMPE (1,2-ditetradecanoyl-*sn*-glycero-3-phosphoethanolamine) lipid. (C) The structure of DMPE-DTPA, which is the combination of DMPE and DTPA.

preparation conditions that suit for solid-state NMR measurements. We found that the use of a copper-chelated phospholipid (DMPE-DTPA : 1,2-ditetradecanoyl-*sn*-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid) in magnetically-aligned bicelles (Figures S2 and S3) offer several advantages: (a) since copper is chelated to a lipid (Figure S2) that mixes well with other components of bicelles without altering the properties of bicelles, about 10 times lower concentration of copper was sufficient for our studies; (b) devoid of free copper ions in the sample considerably reduced sample heating and enabled fast data collection from aligned bicelles. Bicelles composed of a phospholipid (DMPC : 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) and a detergent (DHPC : 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine) with and without a chelated lipid were prepared as outlined below. DMPC, DHPC and DMPE-DTPA were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). All other chemicals are purchased from Sigma-Aldrich (St. Louis, MO).

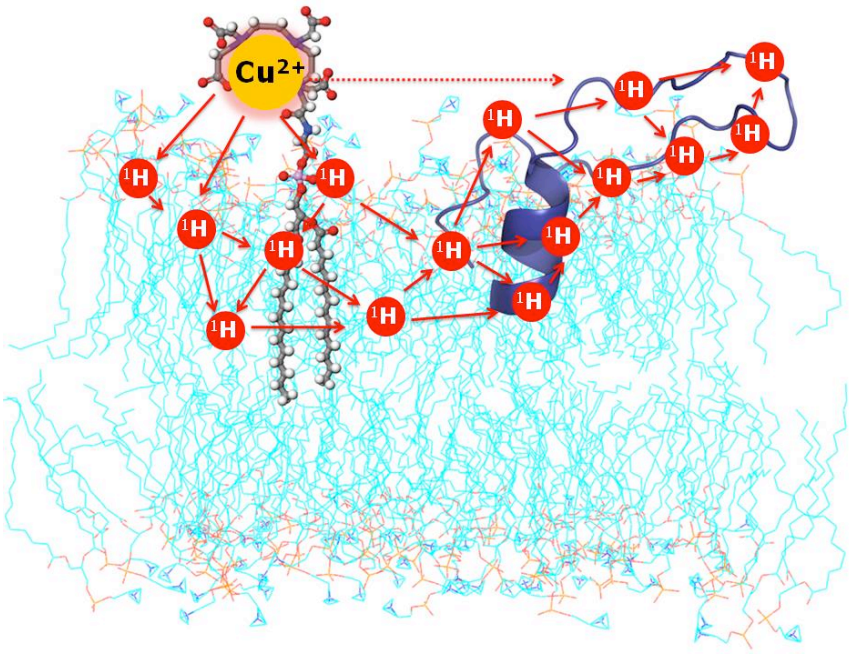

Figure S3. A model depicting lipid bilayers with copper-chelated DMPE-DTPA and a cyclic antimicrobial peptide Subtilosin A.

DMPC/DHPC bicelles were prepared as follows (Figure S4). 40 mg DMPC and 7.6 mg DHPC corresponding to a q ratio, q = [DMPC]/[DHPC], 3.5 were cosolubilized in chloroform. Chloroform was removed under a stream of N_2 gas to form a lipid film on the

walls of a glass tube, which was kept under vacuum overnight to remove all residual solvent. In the glass tube, 111.9 µl of 100 mM HEPES buffer, pH 7.0 was added to the lipids. The resulting viscous mixture was homogenized by gently sonication in an ice bath for 30 minutes, and >4 freeze/heat cycles between liquid nitrogen and 40 °C of water. The resulting turbid gel is still viscous, but the viscosity was slightly reduced at 5 °C. Once a satisfactory mixing of the components was reached, the sample was transferred to a 5 mm NMR glass tube. It was sealed using a Teflon tape and was capped. The degree of alignment was measured using ^{31}P-NMR and the alignment was found to be stable after 1 h of equilibration. A previous study showed that a ytterbium-chelated phospholipid (DMPE-DTPA:Yb^{3+}) can be incorporated into DMPC/DHPC bicelles and aligned under an external magnetic field such that the average bilayer normal is parallel to the magnetic field due to the magnetic susceptibility of the chelated lanthanide ions.[7] In this study on bicelles with copper-chelated phospholipids, the spontaneous magnetic alignment was achieved at 37 ^{o}C and the average bilayer normal of bicelles is perpendicular to the magnetic field.

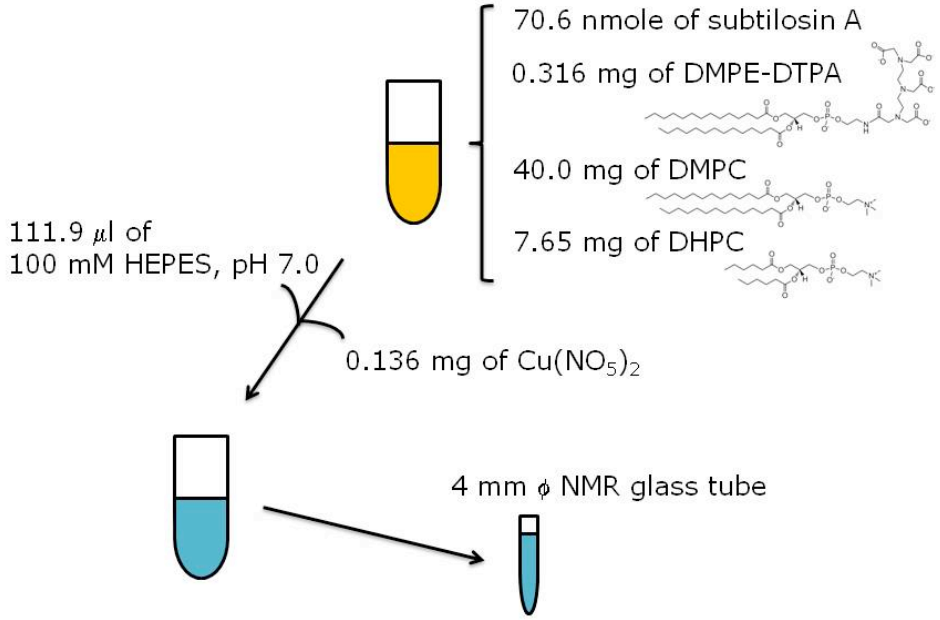

Figure S4. Preparation of DMPC:DHPC bicelles containing ^{15}N-labeled Subtilosin A and copper-chelated lipids for solid-state NMR experiments.

Bicelles containing ^{15}N-labeled Subtilosin A were prepared by mixing 48 mg of lipids (0.316 mg of DMPE-DTPA, 40 mg of DMPC and 7.65 mg of DHPC) with 2 mg of 12-14% ^{15}N-labeled Subtilosin A. The bicelles/peptide mixture sample making procedure was the same as that of pure bicelles except that 0.136 mg of $Cu(NO_5)_2$ was added in the bicelle/peptide mixture before adding 100 mM HEPES buffer (pH 7.0) as shown in Figure S4. DMPC/DHPC bicelles with 30 mM Cu-EDTA samples were prepared in the same manner except that Cu-EDTA was added to DMPC/DHPC bicelles before adding 100 mM HEPES buffer, pH 7.0.

Magnetic-alignment of bicelles measured using ^{31}P NMR experiments

Proton-decoupled ^{31}P-chemical shift spectra of 3.5:1 DMPC/DHPC bicelles containing different concentrations of copper-chelated phospholipids were obtained to examine the magnetic-alignment of bicelles (Figure S5). Pure bicelles without Cu DMPE-DTPA (spectrum D in Figure S5) show a high degree of alignment as suggested by the narrow lines for DMPC (a peak observed in the high field region) and DHPC (a peak observed in the low field region), while the addition of 3.85 mM Cu DMPE-DTPA (spectrum A in Figure S5) increased the line width due to the paramagnetic quenching of relaxation as the copper ions are located close to ^{31}P nuclei. Our results shown in Figure S5 suggest that this paramagnetic quenching effect decreased when the concentration of added copper was lowered as can be seen for 2.56 mM Cu DMPE-DTPA (spectrum B in Figure S5) and 1.92 mM Cu DMPE-DTPA (spectrum C in Figure S5). In spite of the observed ^{31}P line broadening, a stable macroscopic alignment was achieved as indicated by the chemical shift values. This was further confirmed by the observation of resolved ^{15}N spectral lines (Figure 1 in the main text, Figure S6 and S7).

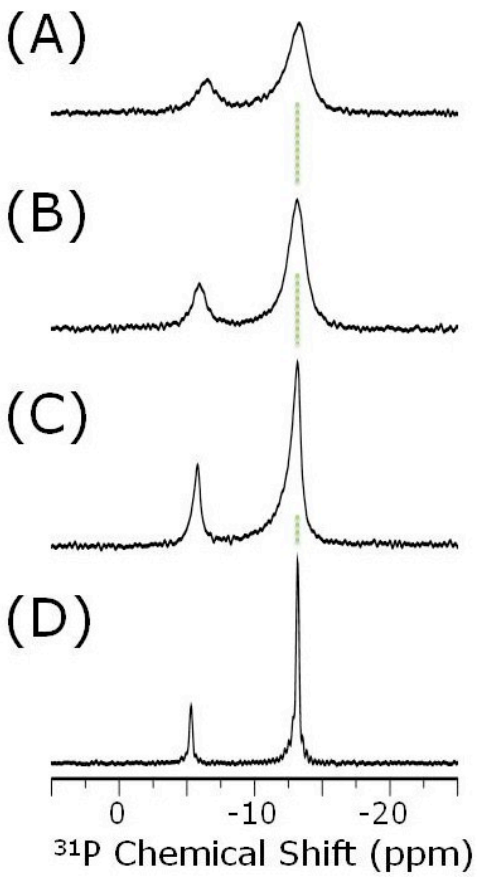

Figure S5. ^{31}P NMR spectra of q = 3.5 DMPC/DHPC bicelles with (A) 3.85 mM Cu DMPE-DTPA, (B) 2.56 mM Cu DMPE-DTPA, and (C) 1.92 mM Cu DMPE-DTPA. (D) Copper-free DMPC/DHPC bicelles. ^{31}P spectra were acquired after a 90^{o} RF pulse by decoupling protons using TPPM pulse sequence under static condition at 37 ^{o}C.

^{1}H spin-lattice (T_1) relaxation time measurements

In order to evaluate the paramagnetic relaxation effect on ^{15}N-labeled subtilosin A from copper-chelated phospholipid, ^{1}H inversion recovery experiemnts were performed. ^{1}H inversion recovery time dependance of ^{15}N spectra with and without 2.56 mM copper DMPE-DTPA are shown in Figure S6. ^{1}H spin-lattice relaxation (T_1) times without paramagnetic copper ions were 1.21 s for the mobile soluble domain of subtilosin A and 1.95 s for the immobile domain of subtilosin A. On the other hand, ^{1}H T_1 times with 2.56 mM copper DMPE-DTPA were 153 ms for the mobile soluble domain of subtilosin A and 131 ms for the immobile domain of subtilosin A. The relaxation times between samples with and without copper-chelated phospholipid differ by a factor of ~10.

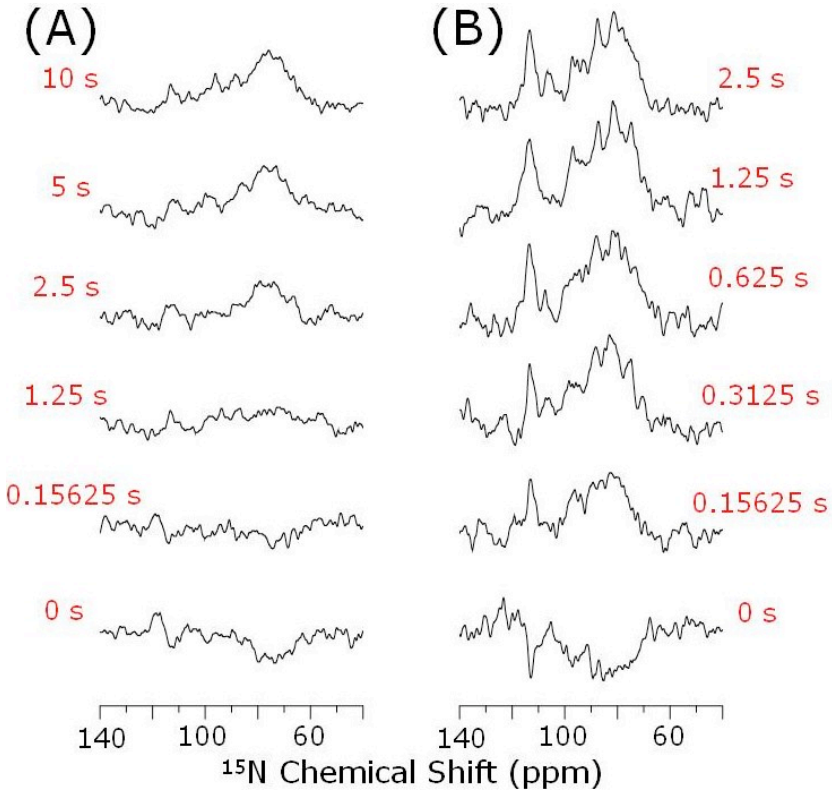

Figure S6. ^{1}H inversion recovery experiments of ^{15}N-labeled Subtilosin A in q = 3.5 DMPC/DHPC bicelles: (A) without and (B) with 2.56 mM Cu DMPE-DTPA at 37 ^{o}C. Inversion recovery times are indicated for each spectrum.

^{15}N cross-polarization experiments to optimize the recycle delay

A ramped cross-polarization sequence with a contact time of 0.8 ms was used to record the ^{15}N spectra under 31.25 kHz TPPM decoupling with a 25 ms of acquisition time at 37 ^{o}C. The recycle delays of ^{15}N CP experiments were optimized. The experiment time was kept as a constant while the recycle delay was changed to determine the optimum time that result in a high signal-to-noise ratio (Figure S7).

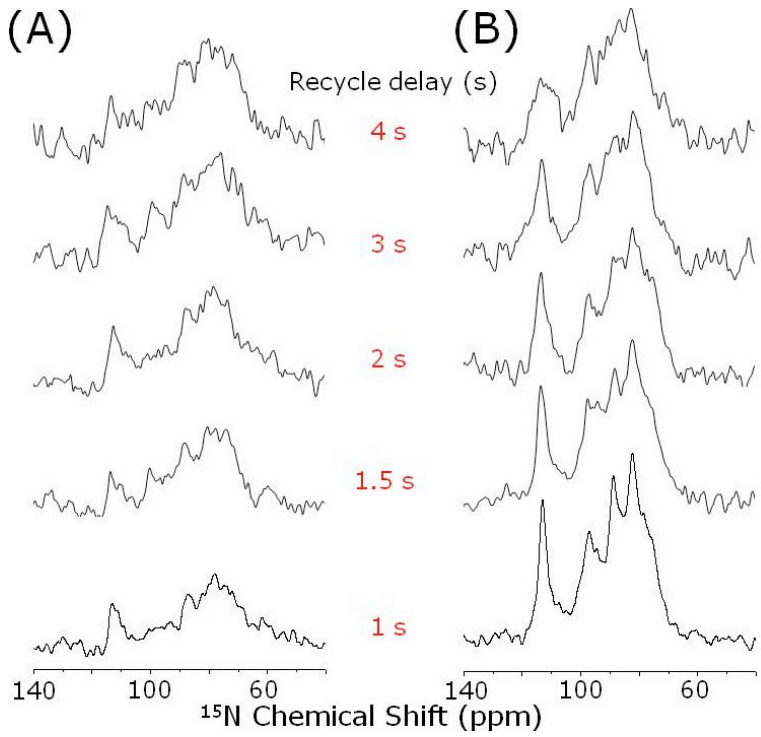

Figure S7. The recycle delay dependence of U-^{15}N Subtilosin A in q = 3.5 the DMPC/DHPC bicelles (A) without and (B) with 2.56 mM Cu DMPE-DTPA at 37 ^{o}C. The recycle delay times in the experiments are indicated in the figure. The total experimental time for each spectrum was 8 hours.

The optimized conditions were determined to be 2 s (^{1}H T_1 was about 1.9 s) for bicelles containing no copper-chelated phospholipid and 1 s (^{1}H T_1 was about 153 ms) for bicelles containing a 2.56 mM Cu DMPE-DTPA. Theoretically, the recycle delay for with 2.56 mM Cu DMPE-DTPA can be around 0.2 s, however, in order to keep the spontaneous magnetic alignment by avoiding RF-induced sample heating (Figure S8) the minimum recycle delay was determined to be around 1 s experimentally.

Calibration of radio frequency induced sample heating

In order to evaluate the RF-induced sample heating by high frequency ^{1}H irradiation during ^{15}N (or ^{13}C) ssNMR experiments, the water proton chemical shift was used because the proton chemical shift is highly sensitive to the sample temperature. Therefore, the water proton signals was used to calibrate the sample temperatures in our experiments as shown in Figure S8 (A) using the pulse sequence shown in Figure S8 (B). The ^{1}H continuous-wave (CW) irradiation of duration in this pulse sequence emulates high frequency ^{1}H heteronuclear decoupling during ^{15}N (or ^{13}C) signal acquisition. After a short period time τ_2, a 90^{o} pulse is applied to measure the water-proton signal. τ_2 was optimized to be sufficiently short to avoid significant sample cooling before the signal acquisition. And the recycle time was also optimized to avoid accumulated sample heating during experiments.[5]

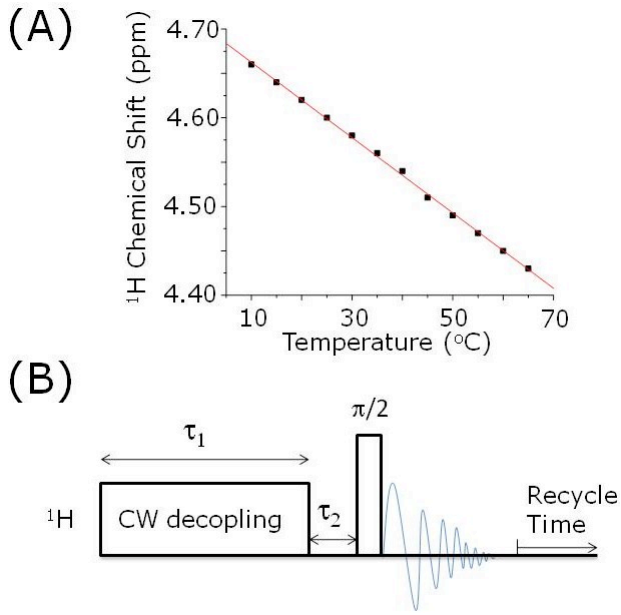

Figure S8. (A) Variation of the proton chemical shift of water as a function of sample temperature measured using the pulse sequence given in (B). The proton chemical shift of water was set at 4.6 ppm at 25 ^{o}C. (B) A ^{1}H pulse sequence for evaluating RF-induced sample heating in well hydrated samples. τ_1 = 20 ms, τ_2 = 200 ms, recycle time = 4 s, 20 dummy scans, and 20 scans were used to generate each data point in (A).

Sample heating dependence on the recycle delay of an NMR experiment

In order to obtain a highly resolved ^{15}N NMR spectrum of membrane associated protein embedded in static magnetically-aligned bicelles, it is essential to get high degree of alignment of bicelles in the presence of an external magnetic field. To assess the alignment stability of bicelles against accumulative sample heating, proton-decouplied ^{31}P chemical shift spectra were obitained for different recycle delay times as shown in Figure S9. The ^{31}P peak position (-11.4 ppm) and a narrow line width (100 Hz) of DMPC signal indicate a high degree of alignment with the bilayer normal perpendicular to the external magnetic field of the spectrometer for all recycle times as shown in Figure S9. However, the ^{31}P spectra obtained using a shorter recycle time (<1 s) show poor magnetic-alignment of bicelles due to accumulated sample heating.

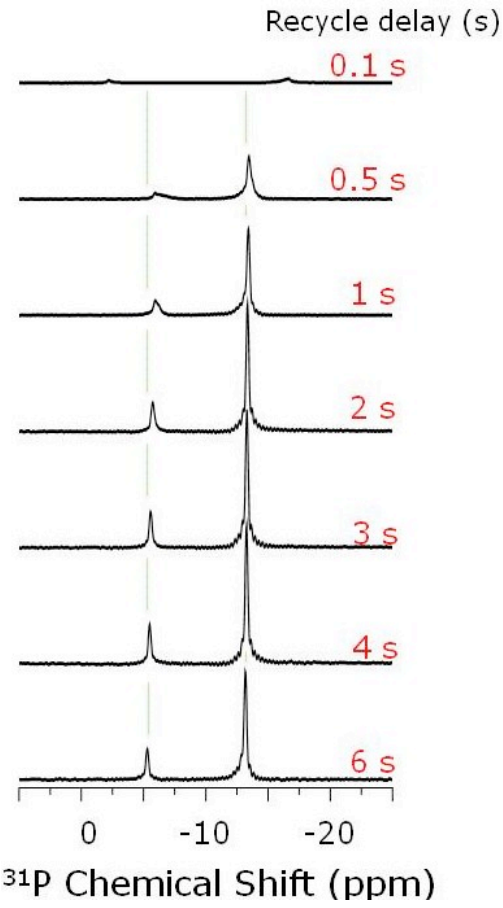

Figure S9. The recycle delay dependence of ^{31}P NMR spectra of q = 3.5 DMPC/DHPC bicelles at 37 ^{o}C. The recycle delay times in the experiments are indicated in the figure. The total experimental time for each spectrum was 24 s.

Use of low temperature aligned bicelles

In order to overcome the sample heating, bicelles that can align at a lower temperature were made. These DMPC/POPC/DHPC bicelles can be aligned at 16 ^{o}C and also exhibit a high degree of alignment at 20 ^{o}C even for a recycle time of 0.5 s (Figure S9 (A)).

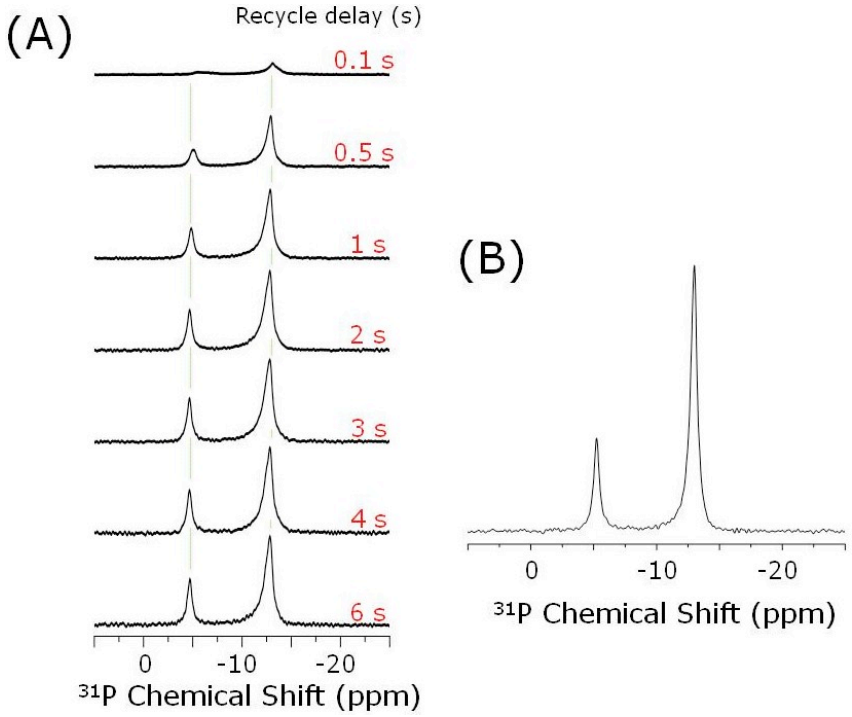

Figure S10. (A) The recycle delay dependence of ^{31}P NMR spectra of magnetically-aligned DMPC/POPC/DHPC bicelles at 20 ^{o}C. The recycle delay times are indicated. (B) ^{31}P NMR spectrum of DMPC/POPC/DMPE-DTPA/DHPC bicelles at 20 ^{o}C. The total experimental time for each spectrum was 24 s for spectra in Figure S9 (A). On the other hand, 10 transients were accumulated with a 6 s recycling delay for spectra in Figure S9 (B).

Comparison of ^{13}C chemical shift spectra of magnetically-aligned bicelles

^{13}C chemical shift spectra of bicelles with different compositions obtained at 37 ^{o}C are shown in Figure S11. A ramp-CP scheme with a contact time of 5 ms was used to record ^{13}C spectra under 31.25 kHz FLOPSY8 decoupling during 14.8 ms acquisition time.[6] 1200 transients were accumulated with a 2 s recycling delay. The peak height comparison is used Figure 2 of the main text based on these ^{13}C NMR spectra.

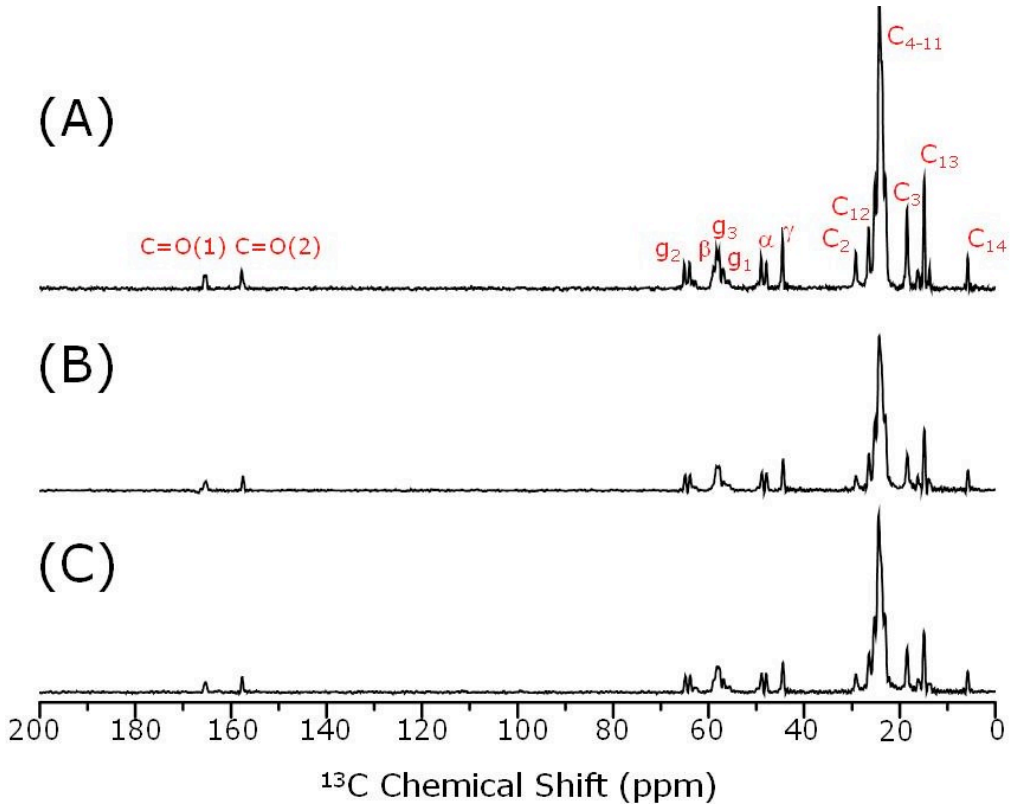

Figure S11. A comparison of ^{13}C spectra of DMPC/DHPC bicelles containing (A) 2.56 mM Cu DMPE-DTPA, (B) 30 mM Cu-EDTA, and (C) no copper at 37 ^{o}C.

References

1. Caplan, J. F.; Sutherland, A.; Vederas, J. C. *J. Chem. Soc. Perkin Trans. 1*, **2000**, 2227-2220.
2. Kawulka, K. E.; Sprules, T.; McKay, R. T.; Mercier, P.; Diaper, C. M.; Zuber, P.; Vederas, J. C. *J. Am. Chem. Soc.* **2003**, *125*, 4726-4727.
3. Kawulka, K. E.; Sprules, T.; Diaper, C. M.; Whittal, R. M.; McKay, R. T.; Mercier, P.; Zuber, P.; Vederas, J. C. *Biochemistry* **2004**, *43*, 3385-3395
4. Thennarasu, S.; Lee, DK.; Poon, A.; Kawulka, K. E.; Vederas, J. C.; Ramamoorthy, A. *Chem. Phys. Lipid* **2005**, *137*, 38-51
5. Dvinskikh, S. V.; Castro, V.; Sanderstrom, D. *Magn. Reson. Chem.* **2004**, *42*, 875-881.
6. Dvinskikh, S. V.; Durr, U. H.; Yamamoto, K.; Ramamoorthy, A. *J. Am. Chem. Soc.* **2007**, *129*, 794-802.
7. Prosser, S. R.; Volkov, V. B.; Shiyanovskaya, I. V. *Biophys. J.* **1998**, *75*, 2163-2169.