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

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Atomic-level structure and dynamics are essential for understanding the function of membrane proteins and their roles in biological processes and for the development of compounds to treat related diseases. While these are challenging tasks for most techniques, recent studies have demonstrated that solid-state NMR spectroscopy is promising, as it neither requires a single crystal nor imposes a restriction on the molecular size to be investigated. Membrane proteins reconstituted in lipid bilayers are commonly used in solid-state NMR studies.[1] The key difficulties that limit the high-throughput application of solid-state NMR spectroscopy are (1) the requirement of a large amount of sample and/or a long data collection time to enhance the signal-to-noise ratio (S/N), (2) radio frequency (RF)-induced sample heating (due mainly to the long measurement time) that may denature expensive membrane proteins labeled with isotopes, (3) the need for stable NMR probes and electronics, and (4) the demand for the continuous availability of a spectrometer. Therefore, the development of new approaches to speed up solid-state NMR measurements is essential. Previous studies successfully utilized copper salts and Cu−EDTA in crystalline samples at lower temperature[2] and Gd−DTPA in glycosphingolipid bilayer samples.[3] However, the presence of excess water and molecular mobilities in fluid lamellar-phase membranes complicates the direct utilization of paramagnetic ions. In this study, we overcome this limitation by using a copper-chelated lipid to significantly enhance the sensitivity of an NMR experiment on lipid bilayers under either aligned static or unaligned magic-angle spinning (MAS) conditions.

Magnetically aligned bicelles[6] composed of a lipid [1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)], a detergent [1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC)], and subtilosin A[4] (Figure S1 in the Supporting Information) were used to demonstrate the paramagnetic effect of Cu^{2+} in reducing the spin−lattice relaxation time (T_1) and the recycle delay of NMR experiments in membranes. Cu^{2+} ions were introduced in bicelles either by adding Cu−EDTA or a copper-chelated lipid (Figure 1A and Figures S2−S4). ^{31}P NMR experiments were performed to optimize the alignment of the bicelles (Figure S5). ^{15}N spectra of uniformly ^{15}N-labeled subtilosin A embedded in bicelles are shown in Figure 1B. T_1 values of ^{1}H and ^{13}C nuclei in DMPC and ^{15}N nuclei in subtilosin A were also measured (Figure 2). These results suggested that a 2 s recycle delay provided the maximum S/N for the sample in the absence of Cu^{2+}. On the other hand, the presence of 2.56 mM copper-chelated lipid in bicelles reduced the T_1 of protons by a factor of 10, and therefore, ∼0.2 s recycle delay would have been sufficient. However, RF heating and dehydration or denaturing effects (including degradation of the magnetic alignment and protein stability) due to a very short recycle delay forced us to use a longer recycle delay. Nevertheless, it was found that a 1 s delay was sufficient to avoid RF heating, thus allowing 2-fold faster data collection. The experimental results given in Figure 1B suggest a ∼2.7-fold increase in the S/N due to the use of the copper-chelated lipid in bicelles.

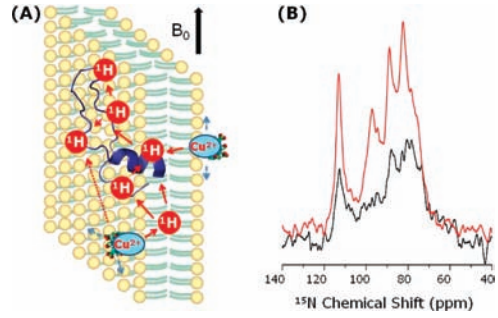

Figure 1. (A) Representation of lipid bilayers containing a paramagnetic copper-chelated lipid and subtilosin A. Subtilosin A is a 35-residue cyclic antimicrobial peptide (Figure S1) that has been shown to interact with lipid bilayers with the membrane orientation depicted in (A).[4] (B) ^{15}N spectra of aligned 7:3 DMPC/DHPC bicelles containing 12−14% uniformly ^{15}N-labeled (only 70−82 nmol) subtilosin A (red) with and (black) without the 2.56 mM copper-chelated lipid. The spectra were obtained on a 400 MHz Varian NMR spectrometer using a ramped-amplitude cross polarization (ramp-CP) sequence[5] with a contact time of 0.8 ms under static conditions at 37 °C. The S/N dependence on the contact time and the recycle delay were optimized (Figures S6 and S7). While the total data collection time was 8 h for both spectra, the recycle delay was different for samples without (2 s) and with (1 s) the copper-chelated lipid. The transfer of the paramagnetic effect in T_1 reduction for nuclei in the membrane via proton spin diffusion is also indicated in (A).

Our results suggest that while the use of Cu−EDTA also decreased the T_1 values effectively (as shown in Figure 2B), it was ineffective in enhancing the S/N in comparison with the immobilized Cu-chelated DMPE lipid (Figure 2F−H) and also resulted in more RF heating (Figure 2E) due to the presence of mobile Cu^{2+} ions; the heating effects were also observed in the ^{31}P spectra (Figures S8 and S9). The use of ∼15-fold less [Cu^{2+}] in the chelated form is of considerable advantage for NMR studies.

Since magnetic alignment of the bicelles is not needed and the sample temperature can be lowered in MAS experiments, the use of Cu-DMPE-DTPA-containing bicelles can be extended for MAS experiments. Two-dimensional (2D) MAS experiments were performed on such bicelles (Figure 3). An RF-driven dipolar recoupling (RFDR) technique was used during the mixing period to recouple the ^{1}H−^{1}H dipolar couplings.[7] Cross-peaks connecting dipolar-coupled protons can be seen in the spectra. While the spectra of bicelles without (Figure 3A) and with (Figure 3B) the copper-chelated lipid have similar overall S/N, the intensities of some of

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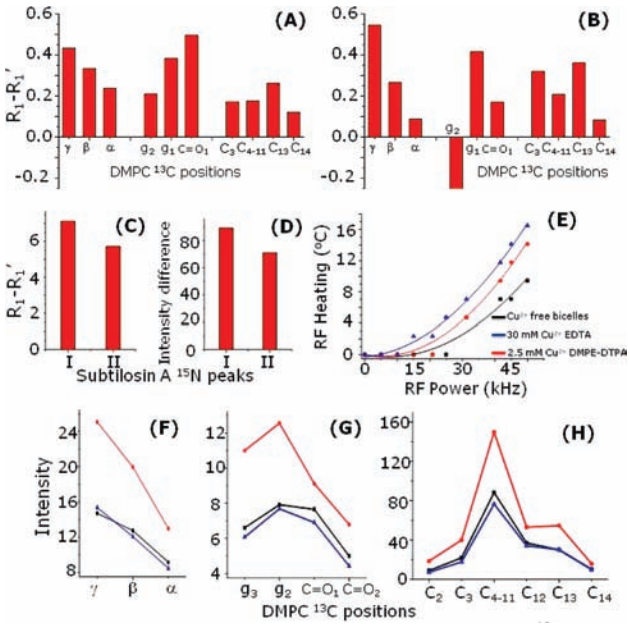

Figure 2. NMR relaxation measurements on bicelles and ^{15}N-labeled subtilosin A for the evaluation of the paramagnetic doping effect with (A) 2.56 mM copper-chelated lipid and (B) 30 mM Cu−EDTA. (C) Difference in spin−lattice relaxation rates with (R_1) and without (R_1') copper-chelated lipid. Labels I and II correspond to ^{15}N signals from the rigid transmembrane (78.3 ppm) and flexible membrane−surface (112.6 ppm) regions, respectively. (D) Difference in signal intensities obtained from bicelles with and without copper-chelated lipid. (E) Data showing RF-induced heating of bicelles measured as explained in Figure S8. (F−H) Comparison of ^{13}C CP signal intensities measured for bicelles with (E) no copper, (blue) 30 mM Cu−EDTA, and (red) 2.5 mM Cu−DMPE-DTPA.

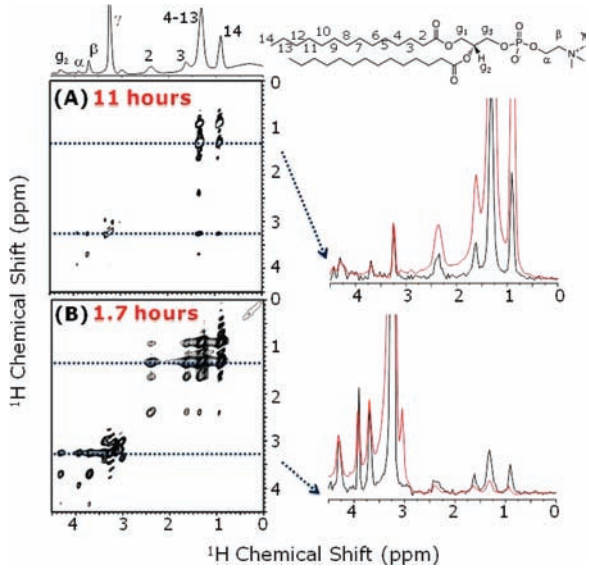

Figure 3. ^{1}H/^{1}H 2D chemical shift correlation spectra of bicelles without (A) and with (B) 2.56 mM copper-chelated lipid obtained under 5 kHz MAS with total data collection times of 11 and 1.77 h, respectively. A 6.2-fold reduction in data collection time with a similar S/N ratio was made possible by the use of the copper-chelated lipid, as can be seen from the 1D spectral slices taken at (top) 1.34 and (bottom) 3.25 ppm with (red) and without (black) the copper-chelating lipid. An RFDR[7] sequence with a 100 ms mixing time and a 100 ms low-power pulse for water saturation at 35 °C was used; 512 t_1 experiments with 32 scans were used, with recycle delays of 0.2 s (with copper-chelating lipid) and 2 s (without).

the peaks are different. The proximity of the Cu^{2+} ion broadens peaks associated with the lipid headgroup, whereas the intensities of peaks from the hydrophobic acyl chains are enhanced. Since no decoupling was used in these experiments, the much reduced RF-induced heating of the bicelles enabled a further reduction in the recycle delay period to 0.2 s, unlike in the static experiment of Figure 1. Therefore, a 6.2-fold overall reduction in the 2D experiment time was achieved.

In this study, we have shown that the inclusion of Cu^{2+} ions in bicelles results in a 10-fold T_1 reduction. It has also been demonstrated that the challenges posed by the molecular mobilities and presence of bulk water can be overcome by using a copper-chelated lipid. About 2.7-fold increase in S/N of spectra for static experiments and 6.2-fold decrease in MAS experimental time were achieved. While this enhancement is not as dramatic as that reported for crystalline solids,[2] given the tremendous challenges in the investigation of fluid membranes, this S/N enhancement is significant and will be of considerable use in structural studies of membrane proteins. For example, satisfactory completion of a typical 2D experiment used to study aligned lipid bilayers containing an ^{15}N-labeled membrane protein takes ~3 days time. The use of Cu−DMPE, on the other hand, could enable the completion of such experiments in <1.5 days. There are a number of membrane disrupting/permeating systems (such as antimicrobial peptides, toxins, amyloid peptides, and fusion peptides) whose concentration cannot be increased, as they result in nonlamellar conditions; such systems tremendously benefit from the use of the approach proposed in this study to reduce the amount of peptide/protein used in the experiment.

Interestingly, further reduction in the measurement time would be possible in the following cases: (a) lowering the sample temperature to avoid heating, as in MAS experiments on frozen samples; (b) use of efficient electric-field-free probes;[8] (c) use of bicelles that align at low temperatures (as shown in Figure S10); and (d) use of cryoprotectants. Therefore, we believe that the approach presented in this study will have a broad impact on structural studies of a variety of membrane-associated peptides and proteins that are scarcely available, quite unstable, and/or for which production could be very expensive.

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Supporting Information Available: ^{31}P and ^{15}N NMR spectra of bicelles and spectra related to relaxation measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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