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

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## Abstract

Recent studies have demonstrated the abilities of solid-state NMR techniques to solve atomic-level resolution structures and dynamics of membrane-associated proteins and peptides. However, high-throughput applications of solid-state NMR spectroscopy are hampered by long acquisition times due to the low sensitivity of the technique. In this study, we demonstrate the use of a paramagnetic copper-chelated-lipid to enhance the spin-lattice relaxation and therefore to speed-up solid-state NMR measurements. Fluid lamellar phase bicelles composed of a lipid, detergent, copper-chelated-lipid, and containing a uniformly-$^{15}$N-labeled antimicrobial peptide, subtilosin A, are used at the room temperature. The use of a chelating lipid reduces the concentration of free copper and limits RF-induced heating, a major problem for fluid samples. Our results demonstrate 6.2 times faster and 2.7 folds improvement of S/N for solid-state NMR experiments under MAS and static conditions respectively. Further, solid-state NMR measurements are shown to be feasible even on nanomole concentration of a membrane-associated peptide.

Atomic-level structure and dynamics are essential to understand the function of membrane proteins, their roles in biological processes, and for the development of compounds to treat related diseases. While these are challenging tasks to 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 for solid-state NMR studies.1 The key difficulties that limit the high-throughput applications of solid-state NMR spectroscopy are: the requirement of a large amount of sample and/or a long data collection to enhance the signal-to-noise (S/N) ratio, radio frequency (RF) induced sample heating mainly due to a long experimental measurement that may denature expensive membrane proteins labeled with isotopes, need for stable NMR probes and electronics, and the demand on 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 temperature2 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 the use of 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 DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine), a detergent DHPC (1,2-dihexanoyl-sn-glycero-3-phosphocholine), and subtilosin A[4] (Fig.S1) were used to demonstrate the paramagnetic effect of $Cu^{2+}$ in reducing the spin-lattice relaxation $(T_1)$ 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 (Figs. 1A, S2-S4). $^{31}P$ NMR experiments were performed to optimize the alignment of bicelles (Fig. S5). $^{15}N$ spectra of uniformly-$^{15}N$-subtilosin-A embedded in bicelles are shown in Fig. 1B. $T_1$ values of $^1H$ and $^{13}C$ nuclei from DMPC and $^{15}N$ of subtilosin A were also measured (Fig. 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 a 2.56 mM copper-chelated-lipid in bicelles reduced $T_1$ 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 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 and thus allowing 2 times faster data collection. The experimental results given in Fig. 1B suggest a ~2.7-fold increase in the S/N due to the use of copper-chelated-lipid in bicelles.

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

Since the magnetic-alignment of bicelles is not needed and the sample temperature can be lowered in MAS experiments, the use of $Cu^{2+-}$DMPE-containing bicelles can be extended for MAS experiments. 2D MAS experiments were performed on such bicelles (Fig. 3). An RFDR (radio frequency driven dipolar recoupling) technique was used in the mixing period to recouple $^1H$-$^1H$ dipolar couplings.[7] Cross peaks connecting dipolar coupled protons can be seen in the spectra. While the spectra of bicelles without (Fig. 3A) and with (Fig. 3B) the copper-chelated-lipid are similar in overall S/N, the intensities of some of the peaks are different. The proximity of $Cu^{2+}$ ion broadens peaks associated with the lipid head group 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 on bicelles enabled a further reduction in the recycle delay period to 0.2 s, unlike in the static experiment of Fig. 1. Therefore, a 6.2-fold overall reduction in the 2D experimental 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 is also demonstrated that the challenges posed by the molecular mobilities and presence of bulk water can be overcome by using a copper-chelated-lipid. About 2.5 and 6.2 folds increase in S/N of spectra obtained from static and MAS experiments (6.2 times faster experiments for MAS) respectively. 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 the structural studies of membrane proteins. For example, a satisfactory completion of 2D experiments typically used on aligned lipid bilayers containing a $^{15}N$-labeled membrane protein takes about 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 non-lamellar 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 like 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 Fig. S10), and (d) use of cryoprotectants. Therefore, we believe that the approach presented in this study will have broad impacts on the structural studies of a variety of membrane-associated peptides and proteins that are scarcely available and/or their production could be very expensive and are quite unstable.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

## References

(1). (a) Lange A, Giller K, Hornig S, Martin-Eauclaire MF, Pongs O, Becker S, Baldus M. Nature 2006;440:959–962. [PubMed: 16612389] (b) Dürr UH, Waskell L, Ramamoorthy A. Biochim. Biophys. Acta 2007;1768:3235–3259. [PubMed: 17945183] (c) Lorieau JK, Day LA, McDermott AE. Proc. Natl. Acad. Sci 2008;105:10366. [PubMed: 18653759] Xu JD, Dürr UH, Im SC, Gan ZH, Waskell L, Ramamoorthy A. Angew. Chem. Intl. Ed 2008;47:7864–7867.

(2). (a) Ganapathy S, Naito A, McDowell CA. J. Am. Chem. Soc 1981;103:6011–6015.Wickramasinghe NP, Kotecha M, Samoson A, Past J, Ishii Y. J. Magn. Reson 2007;184:350–356. [PubMed: 17126048] (c) Wickramasinghe NP, Parthasarathy S, Jones CR, Bhardwaj C, Long F, Kotecha M, Mehboob S, Fung LW, Past J, Samoson A, Ishii Y. Nat. Methods 2009;6:215–218. [PubMed: 19198596] (d) Wickramasinghe NP, Shaibat M, Ishii Y. J. Am. Chem. Soc 2005;127:5796–5797. [PubMed: 15839671] (e) Weliky DP, Bennett AE, Zvi A, Anglister J, Steinbach PJ, Tycko R. Nat. Struct. Biol 1999;6:141–145. [PubMed: 10048925] (f) Linser R, Chevelkov V, Diehl A, Reif BJ. J. Magn. Reson 2007;189:209–216. [PubMed: 17923428] (g) Cai S, Seu C, Kovacs Z, Sherry AD, Chen Y. J. Am. Chem. Soc 2006;128:13474–8. [PubMed: 17031960]

(3). DeMarco ML, Woods RJ, Prestegard JH, Tian F. J. Am. Chem. Soc 2010;132:1334–1338. [PubMed: 20058858]

(4). (a) Kawulka K, Sprules T, McKay RT, Mercier P, Diaper CM, Zuber P, Vederas JC. J. Am. Chem. Soc 2003;125:4726–4727. [PubMed: 12696888] (b) Thennarasu S, Lee DK, Poon A, Kawulka KE, Vederas JC, Ramamoorthy A. Chem. Phys. Lipids 2005;137:38–51. [PubMed: 16095584]

(5). Metz G, Wu X, Smith SO. J. Magn. Reson. A 1994;110:219–227.

(6). (a) Sanders CR, Hare BJ, Howard KP, Prestegard JH. Prog. Nucl. Magn. Reson. Spec 1994;26:421–444. (b) Prosser RS, Evanics F, Kitevski JL, Al-Abdul-Wahid MS. Biochemistry 2006;45:8453–8465. [PubMed: 16834319] (c) Inbaraj JJ, Cardon TB, Laryukhin M, Grosser SM, Lorigan GA. J. Am. Chem. Soc 2006;128:9549–9554. [PubMed: 16848493] (d) Yamamoto K, Soong R, Ramamoorthy A. Langmuir 2009;25:7010–7018. [PubMed: 19397253] (e) Prosser SR, Volkov VB, Shiyanovskaya IV. Biophys. J 1998;75:2163–2169. [PubMed: 9788910] (f) Dvinskikh SV, Yamamoto K, Dürr UHN, Ramamoorthy A. J. Magn. Reson 2007;184:228–235. [PubMed: 17084096]

(7). Bennett AE, Ok JH, Griffin RG, Vega S. J. Chem. Phys 1992;96:8624–8627.

(8). (a) McNeill SA, Gor'kov PL, Shetty K, Brey WW, Long JR. J. Magn. Reson 2009;197:135–144. [PubMed: 19138870] (b) Gor'kov PL, Chekmenev EY, Li C, Cotten M, Buffy JJ, Traaseth NJ, Veglia G, Brey WW. J. Magn. Reson 2007;185:77–93. [PubMed: 17174130] (c) Stringer JA,

Bronnimann CE, Mullen CG, Zhou DH, Stellfox SA, Li Y, Williams EH, Rienstra CM. J. Magn. Reson 2005;173:40–48. [PubMed: 15705511]
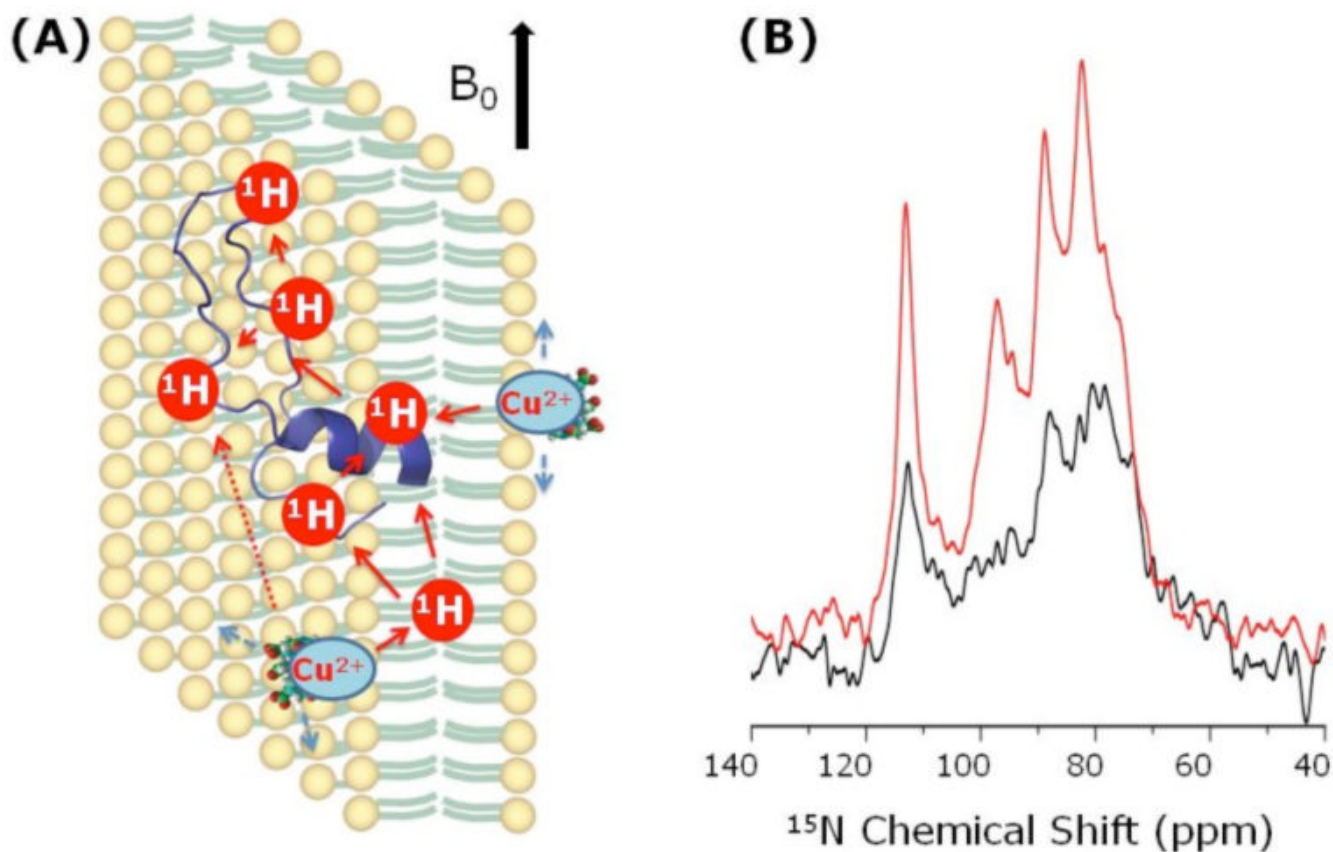
**Fig. 1.**
(A) A representation of lipid bilayers containing a paramagnetic copper-chelated lipid and subtilosin A. Subtilosin A is a 35-residue cyclic antimicrobial peptide (Fig. S1) that has been shown to interact with lipid bilayers with a membrane orientation as given in (A)[4]. (B) $^{15}$N spectra of aligned 7:3 DMPC:DHPC bicelles containing a 12-14% of uniformly-$^{15}$N-labeled (only 70-82 nmoles) subtilosin A and with (red) and without (black) the 2.56 mM copper-chelated-lipid. The spectra were obtained from a 400 MHz Varian NMR spectrometer using a ramp-CP sequence[5] with a 0.8 ms contact time under static conditions at 37 °C. The S/N dependence on the contact time and the recycle delay were optimized (Figs. S6 & S7). While the total data collection time was 8 hours for both spectra, the recycle delay was different for samples without (2s) and with (1s) a copper-chelated-lipid. The transfer of paramagnetic effect in $T_1$ reduction for nuclei in the membrane via the proton spin diffusion is also indicated in (A).
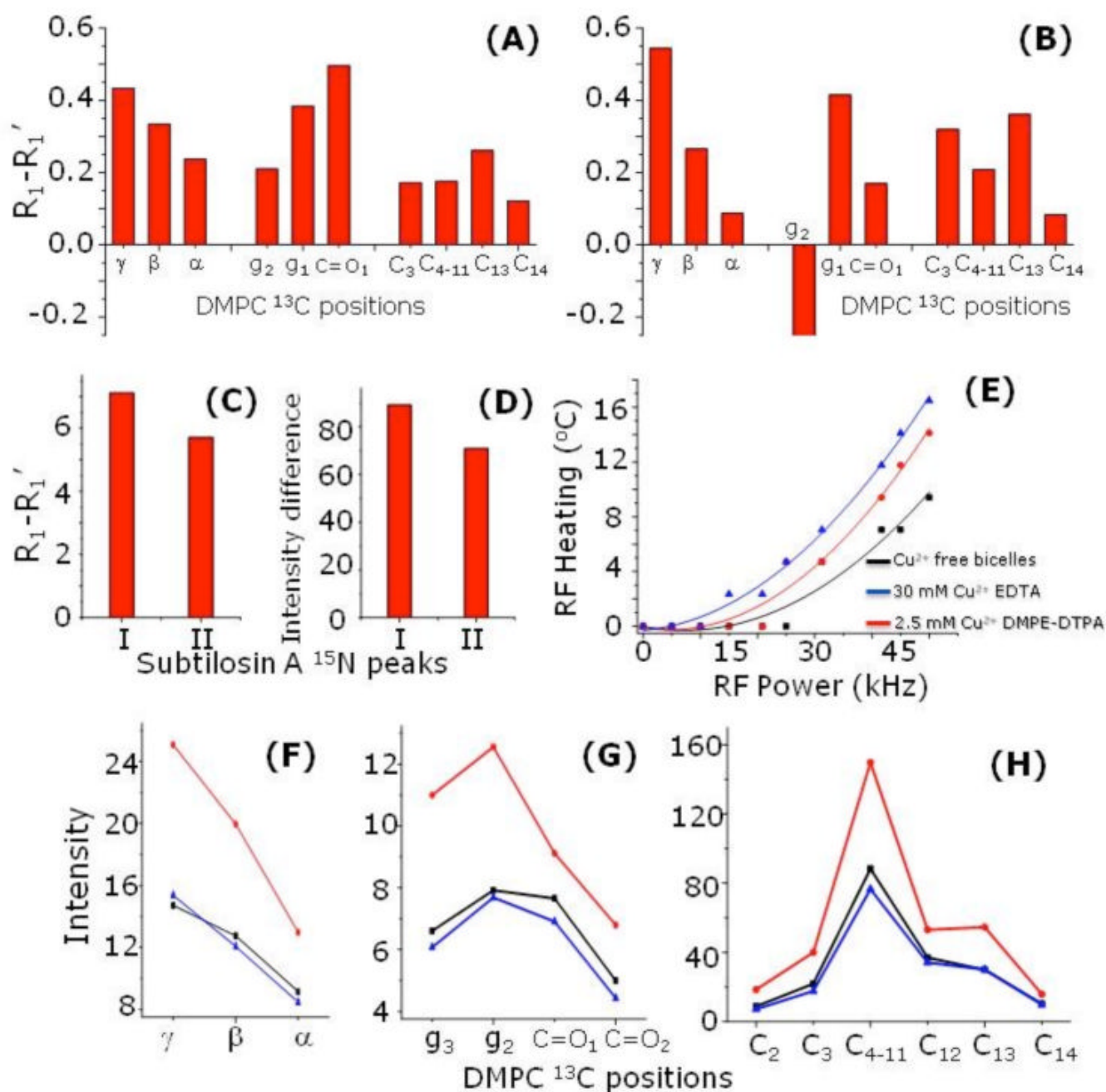
**Fig. 2.**
NMR relaxation measurements on bicelles and $^{15}$N-labeled subtilosin A for the evaluation of paramagnetic doping effect: (A) with 2.56 mM copper-chelated lipid; (B) with 30 mM copper-EDTA. (C) Difference in spin-lattice relaxation rates with ($R_1$) and without ($R_1'$) copper-chelated lipid; labels I and II correspond to $^{15}$N signal 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 the RF-induced heating on bicelles measured as explained in Fig. S8. (F-H) A comparison of $^{13}$C CP signal intensities measured from bicelles with no copper (E), 30 mM Cu-EDTA (blue) and 2.5 mM Cu DMPE-DTPA (red).
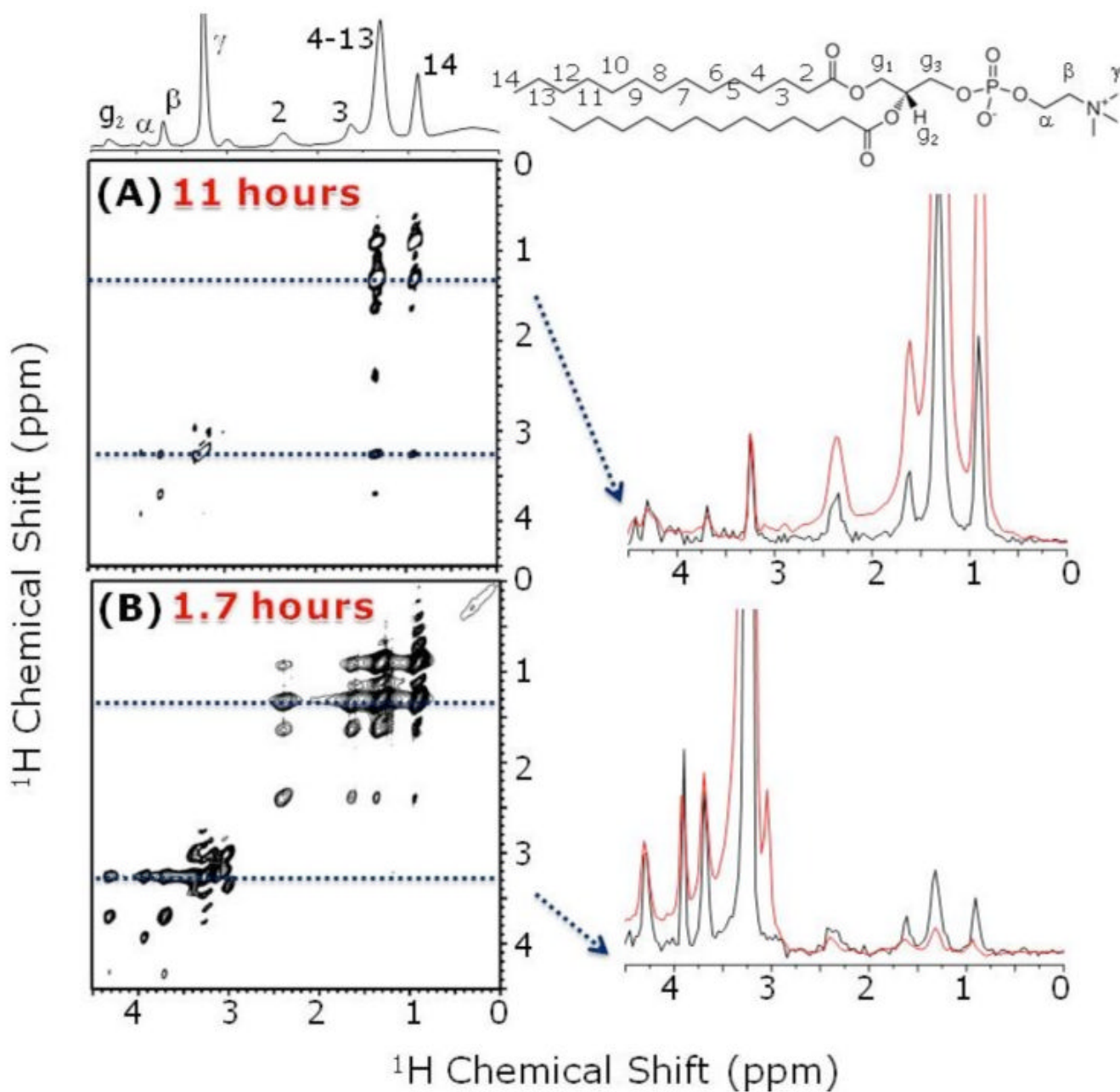
**Fig. 3.**
2D $^1$H/$^1$H chemical shift correlation spectra of bicelles without (A) and with (B) a 2.56 mM copper-chelated lipid obtained under 5 kHz MAS with a total data collection times of 1.77 and 11 hours respectively. A 6.2-fold reduction in data collection time was made possible due to the use of the copper-chelated lipid with a similar S/N ratio as can be seen from 1D spectral slices taken at 1.34 ppm (top) and 3.25 ppm (bottom) with (red) and without (black) a 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 a recycle delay of 0.2 s (with a copper-chelating lipid) and 2 s (without).