

Sensitivity and resolution enhancement in solid-state NMR spectroscopy of bicelles

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Abstract

Magnetically aligned bicelles are becoming attractive model membranes to investigate the structure, dynamics, geometry, and interaction of membrane-associated peptides and proteins using solution- and solid-state NMR experiments. Recent studies have shown that bicelles are more suitable than mechanically aligned bilayers for multidimensional solid-state NMR experiments. In this work, we describe experimental aspects of the natural abundance ¹³C and ¹⁴N NMR spectroscopy of DMPC/DHPC bicelles. In particular, approaches to enhance the sensitivity and resolution and to quantify radio-frequency heating effects are presented. Sensitivity of ¹³C detection using single pulse excitation, conventional cross-polarization (CP), ramp-CP, and NOE techniques are compared. Our results suggest that the proton decoupling efficiency of the FLOPSY pulse sequence is better than that of continuous wave decoupling, TPPM, SPINAL, and WALTZ sequences. A simple method of monitoring the water proton chemical shift is demonstrated for the measurement of sample temperature and calibration of the radio-frequency-induced heating in the sample. The possibility of using ¹⁴N experiments on bicelles is also discussed.

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1. Introduction

The molecular aggregates formed by phospholipids in aqueous solution, which usually exist as bilayers in the form of vesicles, gels, or lamellae, are often used as models for cell membranes. Although the disordered multilamellar vesicles have extensively been used in NMR studies, the macroscopically oriented phases have the advantage of preserving anisotropic spin interactions that offer a wealth of information about the molecular geometry and dynamics [1]. Therefore, considerable efforts have been devoted to develop magnetically oriented phospholipid bilayers [2–9].

While several studies have reported on the preparation, characterization, and applications of bicelles [4–6,8], a systematic optimization of solid-state NMR experiments on bicelles is lacking.

Since molecules embedded in bicelles are dynamic, the molecular motion could degrade the efficiency of solid-state NMR pulse sequences. For example, it is difficult to establish an experimental condition that efficiently transfers magnetization from protons to carbons (or other low sensitive nuclei) at all sites of a molecule, and also for all molecules, in a bicelle sample. A short contact time cross-polarization (CP) [10] sequence is more efficient for rigid parts of a molecule than for mobile regions, as heteronuclear dipolar couplings in the latter case are averaged by the motion [11,12]. On the other hand, a long contact time or the NOE (nuclear Overhauser effect)-type magnetization transfer may be better for mobile sites of a molecule [13].

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In this study, we have compared the efficiencies of different methods to enhance the sensitivity of ^{13}C signal from bicelles.

The use of high RF (radio frequency) decoupling field strengths is necessary to achieve line-narrowing in most solid-state NMR applications [14,15]. Proton decoupling in the form of multiple pulse sequences is commonly used to record signals of less sensitive nuclei (such as ^{13}C and ^{15}N). An actual RF power required to accomplish an efficient proton decoupling depends on the design of a decoupling pulse sequence. The use of high RF field strengths, which becomes important for experiments at high magnetic fields, however, generates sample heating because of the RF power dissipation within the sample. A majority of biological samples are very susceptible to RF-induced heating, because of the interaction of fast oscillating fields with ions in salty samples and/or the electric dipolar moment of the molecules in dielectrics [16–19]. These effects are a major concern in the application of multidimensional SLF (separated-local-field) [20,21] experiments to static samples such as bicelles. Since most physicochemical properties of biological molecules depend on the sample temperature, an adequate temperature control during NMR experiments is, therefore, important. The RF heating effects are not typically measurable from the readings of a variable-temperature control unit of an NMR spectrometer, and therefore they must be properly calibrated and corrected prior to an experiment. In this study, we propose a simple method to measure the sample temperature during solid-state NMR experiments on bicelles. The efficiency of various proton-decoupling sequences and the requirement of the RF field strength are also analyzed in this study.

2. Results and discussion

2.1. Sample heating due to an RF irradiation

To assess the sample heating induced by the ^1H decoupling, we used the high sensitivity of the water proton chemical shift to the sample temperature [22,23]. Fig. 1a shows the temperature dependence of the water proton chemical shift. The slope is 0.010 ± 0.001 ppm/ $^{\circ}\text{C}$ (or 4.0 ± 0.4 Hz/ $^{\circ}\text{C}$ at a magnetic field of 9.4 T), which is similar to the value reported for the pure water [22]. The experimental setup used in this study resulted in a line width of 23 Hz, thus providing an accuracy of about 0.6 $^{\circ}\text{C}$ in the sample temperature measurement. To measure the RF-induced sample heating, an RF irradiation emulating the heteronuclear decoupling sequence was applied at the proton resonance frequency and, after a short magnetization recovery period, the proton FID signal was recorded following a 90° pulse. A 40 ms RF irradiation time corresponding to the ^1H decoupling time in ^{13}C experiments was used. A delay of 200 ms after the decoupling pulse required for the magnetization recovery was chosen sufficiently short to avoid significant heat dissipation in the sample before acquiring the spectrum. For each measure-

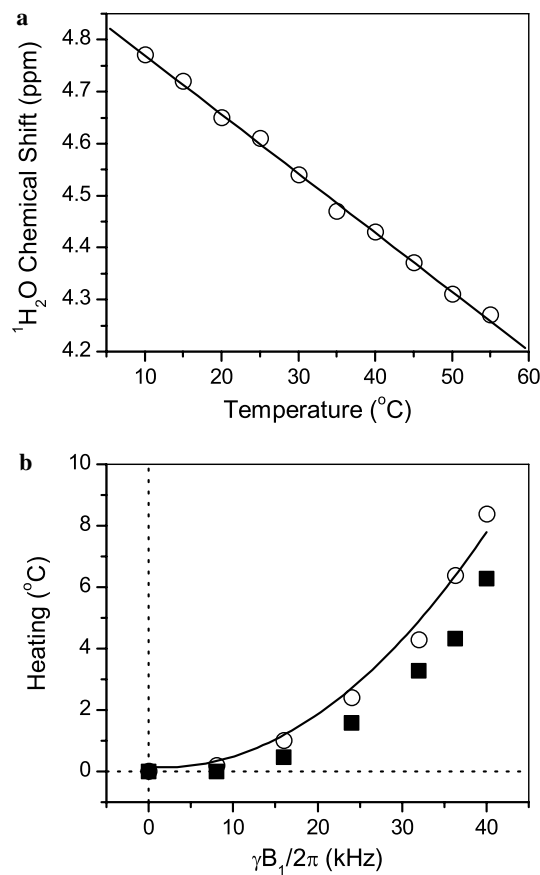


Fig. 1. (a) Variation of the chemical shift of water protons in a bicelle sample with the temperature. The proton chemical shift of water was set at 4.6 ppm at 25 $^{\circ}\text{C}$. (b) Open symbols represent the heating effect versus the RF field strength. The line is the fit to a second power dependence. Solid symbols represent the temperature gradient in the sample volume induced by the RF irradiation.

ment, 16 transients with a delay of 5 s were accumulated preceded by 16 dummy scans to ensure a steady state sample temperature. The heating effect in the bicelle sample versus the RF field strength is presented in Fig. 1b. In agreement with theory [24,25], a second power dependence of the heating effect on the RF power was observed. The temperature gradient in the sample volume induced by the RF irradiation was estimated from the increase in the linewidth of the $^1\text{H}_2\text{O}$ peak. The results are also included in Fig. 1b. In principle, the average temperature shift can be corrected by adjusting the setting on the temperature control unit. On the other hand, the temperature gradient within the sample and the heating during the decoupling pulse cannot be dealt with in that way.

In the ^{13}C experiments presented below, a 20 kHz proton-decoupling field was typically used. This resulted in an increase of the average sample temperature by about 2 $^{\circ}\text{C}$. A comparable effect is also expected due to the RF irradiation of protons during the indirect period (t_1) of a 2D experiment. For example, the popular PISEMA (polarization inversion spin exchange at the magic angle) experiment [13,26–28] and other rotating-frame SLF experiments

[29–35] for the measurement of the heteronuclear dipolar coupling utilize a high RF power in the t_1 period. On the other hand, the effect of the RF irradiation on the ^{13}C channel (for example, as applied in a cross-polarization sequence, and also during the evolution period of rotating-frame experiments) was found to be negligible. This stems from less power losses at a lower RF frequency [24]. Generally, experiments at a higher magnetic field lead to more significant sample heating due to a higher resonance frequency [24] and also due to wider signal dispersion by the chemical shift which requires a stronger RF field for effective decoupling of protons. The amount of heating may also depend on the sample size and geometry [36,37], sample composition [17,19,38], RF coil design [37,39,40], and the flow-rate of the heating/cooling gas [24]. Therefore, the calibration of the RF heating effect on each sample is required for an experimental set-up. Finally, we note that the recent developments in the probe and coil design minimizing the electric field inside the sample provide a significant reduction of sample heating [40].

2.2. Signal enhancement

In NMR spectroscopy of low-gamma nuclei in liquids, the common approaches for sensitivity enhancement are J -coupling mediated polarization transfer using the INEPT (insensitive nuclei enhanced by polarization transfer) sequence [41] and heteronuclear cross-relaxation under the condition of saturating ^1H RF field using NOE [42]. In solids, on the other hand, CP in the rotating frame [10] is the most popular technique, which is also frequently used in anisotropic biological samples. However, the motional averaging in bicelles reduces C–H dipolar couplings, as compared to rigid solids, and results in an increased sensitivity to the Hartmann–Hahn mismatch [43] of RF fields during CP. Hence, a ramp-CP sequence [44] is advantageous to overcome the Hartmann–Hahn mismatch [10,43]. On the other hand, high molecular mobility may favour the NOE approach [45], which becomes efficient when the correlation time of the motion is small compared to the inverse of resonance frequency. The INEPT transfer is not practical in static anisotropic samples, since the J_{CH} couplings are unresolved in the presence of strong residual heteronuclear dipolar interactions. (INEPT, however, has been employed in lipid bilayer samples under the MAS condition, when dipolar couplings are suppressed by the sample spinning [45].)

In Fig. 2, ^{13}C chemical shift spectra obtained by single pulse excitation (SPE), conventional CP [10], ramp-CP [44], and NOE [45] techniques are compared. Overall, the ramp-CP [44] provides the largest enhancement factor (of about three with respect to SPE) in our sample for most of the sites. The optimal contact time in CP strongly differs for various sites in the molecule resulting in a non-uniform enhancement. NOE is superior only for the most mobile groups, the γ and C_{14} sites of a lipid molecule.

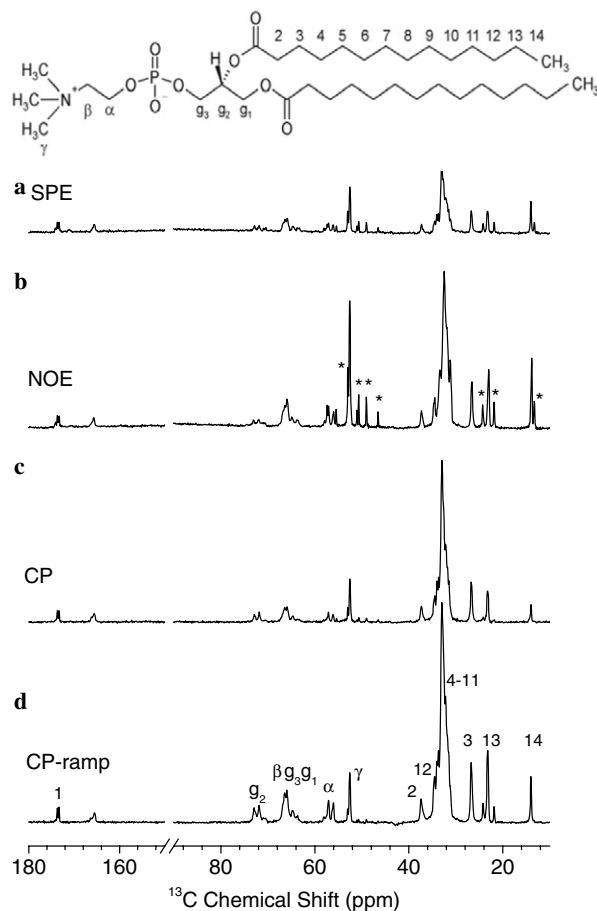


Fig. 2. Comparison of the ^{13}C spectra of DMPC/DHPC bicelles at 37 °C measured with different sensitivity enhancement techniques: (a) 90° single pulse excitation (SPE), (b) NOE, (c) constant amplitude CP, and (d) ramped amplitude CP. Signals from DHPC detergent, observed most clearly in NOE and SPE spectra, are indicated by asterisks. The molecular structure of DMPC is shown at the top.

2.3. Heteronuclear decoupling

Since RF heating is a problem for NMR studies on bicelles, it is important to effectively utilize the RF power of pulse sequences. A long data acquisition under proton decoupling, needed to obtain a high-resolution ^{13}C chemical shift spectrum of bicelles, is an obvious heat-generating part of an experiment and therefore needs to be optimized. It is known that the RF power needed to achieve efficient heteronuclear spin decoupling depends on the design of a decoupling scheme [46]. A careful choice of the decoupling scheme is, therefore, important to obtain highly resolved ^{13}C spectra at a limited RF power level. We examined the performances of decoupling sequences that are frequently used in studies on biological samples and liquid crystals. In particular, we investigated the efficiencies of TPPM (two pulse phase-modulation) [47], SPINAL-64 (small phase incremental alternation) [48], WALTZ-16 (wideband alternating phase low-power technique for zero residue splitting) [49] and FLOPSY-8 (flip-flop spectroscopy) [50] schemes, and compared them to a conventional

continuous wave (CW) decoupling. The ^{13}C chemical shift spectra acquired using different decoupling schemes are presented in Fig. 3. The ^1H RF field strength was 20 kHz and the decoupler frequency was set to obtain the best resolution in the most crowded ^{13}C spectral region, 30–36 ppm, by applying a CW irradiation. For the acyl chain ^{13}C signals, the performances of TPPM, SPINAL-64, and FLOPSY-8 decoupling sequences are comparable and

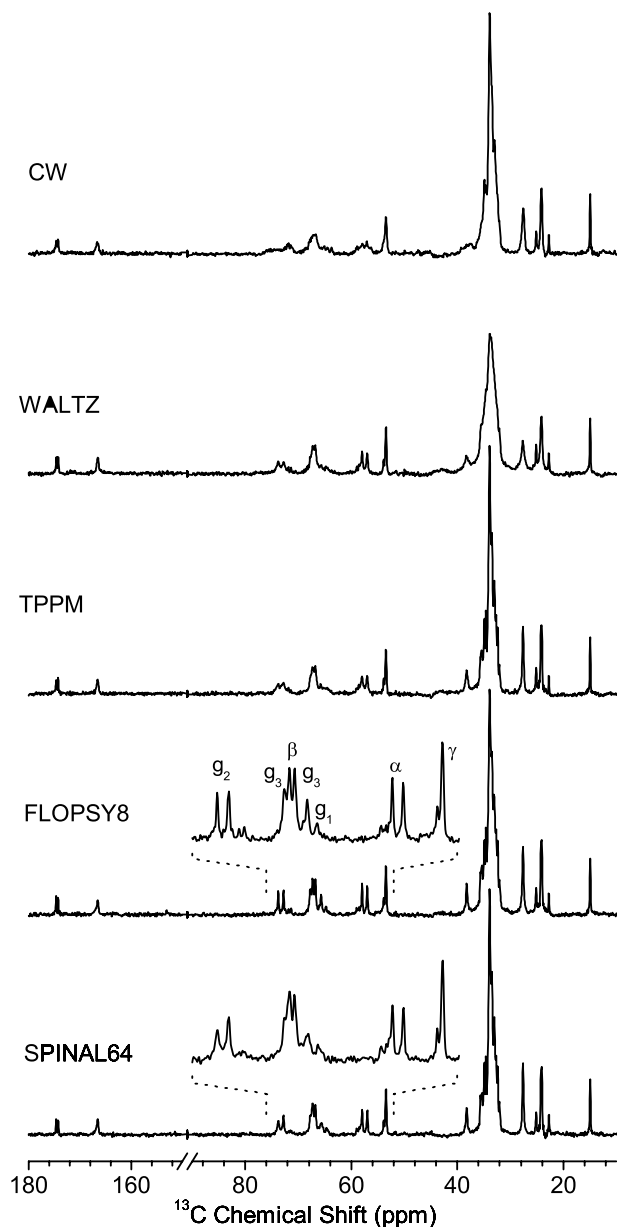


Fig. 3. ^{13}C spectra of DMPC/DHPC bicelles at 37 °C measured with different heteronuclear decoupling sequences. The proton decoupler RF field strength was set at 20 kHz. The RF decoupler frequency was set to a value that resulted in an optimal resolution for the crowded chain-carbon region when CW irradiation was used. The pulse durations were optimized for WALTZ-16 and FLOPSY-8 sequences, while both pulse durations and RF phase shifts were optimized for TPPM and SPINAL-64 schemes. The spectral region containing signals from the lipid head group and glycerol sites is expanded for an easy comparison of spectra obtained using the FLOPSY and SPINAL decoupling sequences.

significantly better than CW and WALTZ sequences. Excellent resolution was achieved using the FLOPSY-8 pulse sequence scheme, which was originally designed for the broadband homonuclear cross-polarization in liquids [50], but was also used for heteronuclear decoupling in lyotropic liquid crystalline systems [51]. Compared to the SPINAL-64 decoupling, use of the FLOPSY-8 sequence improved the spectral resolution for the head group and glycerol carbons; in particular for g_2 , g_3 , and β sites (Fig. 3). In addition the FLOPSY sequence is relatively easy to set up since it requires only adjusting the single parameter, the 180° pulse length, and is very tolerant to the miscalibration of the pulse length.

Fig. 4 compares the ^{13}C chemical shift spectra of a bicellar sample obtained at different RF power levels using the FLOPSY-8 decoupling. Notably, an RF field strength as low as 16 kHz is sufficient to achieve adequate resolution comparable to that obtained using other sequences at much higher fields. It is interesting to note that very strong

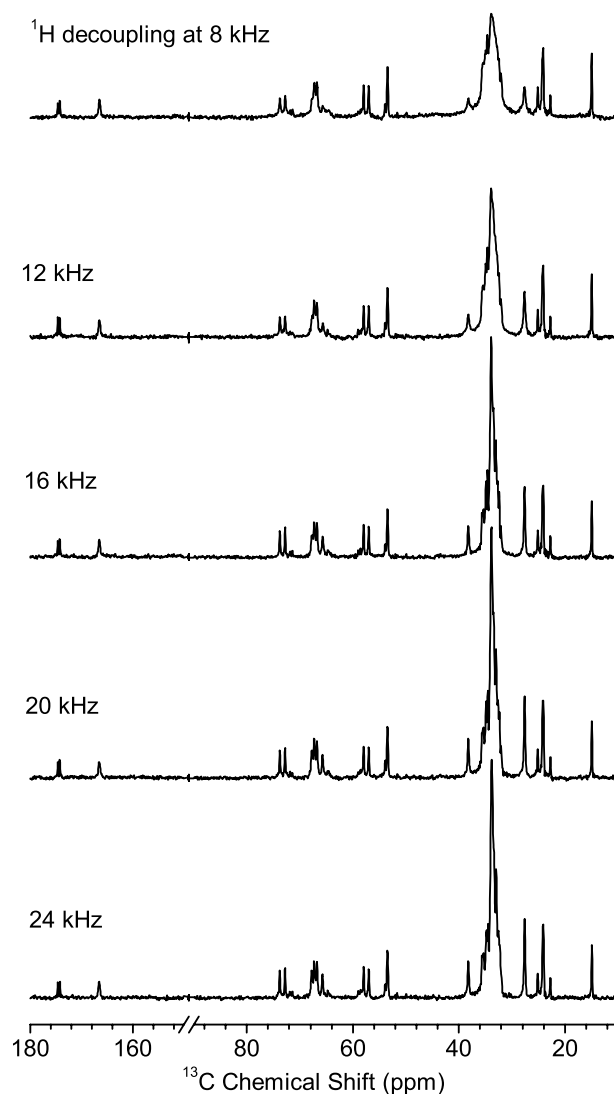


Fig. 4. ^{13}C spectra of DMPC/DHPC bicelles at 37 °C obtained using the FLOPSY-8 proton-decoupling sequence at different decoupling power levels.

decoupling fields, up to 80 kHz, were commonly employed in similar systems [45]. It should be pointed out that a previous study on magnetically aligned bicelles containing a peptide suggested that a SPINAL-16 [48] decoupling sequence provided a better decoupling performance than other decoupling sequences analyzed [52]; however, the performance of the FLOPSY sequence was not examined in that study. Our results suggest that broadband sequences like FLOPSY can provide significant advantage in bicellar systems.

2.4. Nitrogen-14 NMR spectra of bicelles

The electric field gradient around the ^{14}N nucleus, and hence the quadrupole coupling, is considerably reduced due to the near-tetrahedral symmetry of the choline groups of DMPC and DHPC molecules [53–57]. The dynamics of the lipid head group further reduces the quadrupole coupling to about 20 kHz. Therefore, the detection of ^{14}N in bicelles requires neither extremely short pulses nor a wide spectral window.

Nitrogen-14 spectra of aligned bicelles are given in Fig. 5. Two sets of narrow doublet peaks are observed in the spectra. The doublet with a larger quadrupole splitting arises from DMPC as it is similar to the spectra obtained from mechanically aligned DMPC or POPC bilayers [54], while the doublet with a smaller splitting corresponds to the DHPC molecule. Flipped bicelles [58] are characterized by the orientation of the bilayer normal along the external magnetic field. As demonstrated in Fig. 5b, in a flipped bicelle sample the quadrupolar splittings are nearly doubled compared to that of unflipped bicelles in correspondence with the direction of the alignment.

As seen from spectra of bicelles containing ligands, ^{14}N is very sensitive to the ligand–lipid interactions that alter the electric field gradient surrounding the ^{14}N nucleus. For example, the presence of an amphipathic, α -helical antimicrobial peptide, MSI-78 [59], significantly reduces the quadrupole splitting of DMPC (Fig. 5d and e). Since MSI-78 is cationic with a net charge of +9 and interacts with the lipid headgroups, it could interfere with the cross-linking of lipids. This would enhance the symmetry of the positively charged choline group and therefore reduce the quadrupole splitting. Similar effects have been utilized in probing the interaction of antidepressants with membranes in mechanically aligned bilayers. A sample spectrum of bicelles containing an antidepressant, desipramine, is given in Fig. 5c, which shows the expected reduction in the ^{14}N quadrupole splitting [54]. Interestingly, the presence of an anionic lipid, DMPG, slightly increases the quadrupole coupling of DMPC as well as DHPC while the line width depends on the concentration of salt in the sample (Fig. 5f and g). While more experimental analyses are needed to understand the observed changes in the ^{14}N quadrupole coupling, these results suggest that ^{14}N NMR spectroscopy of magnetically aligned bicelles can be used as a ‘voltmeter’ to measure the interaction of

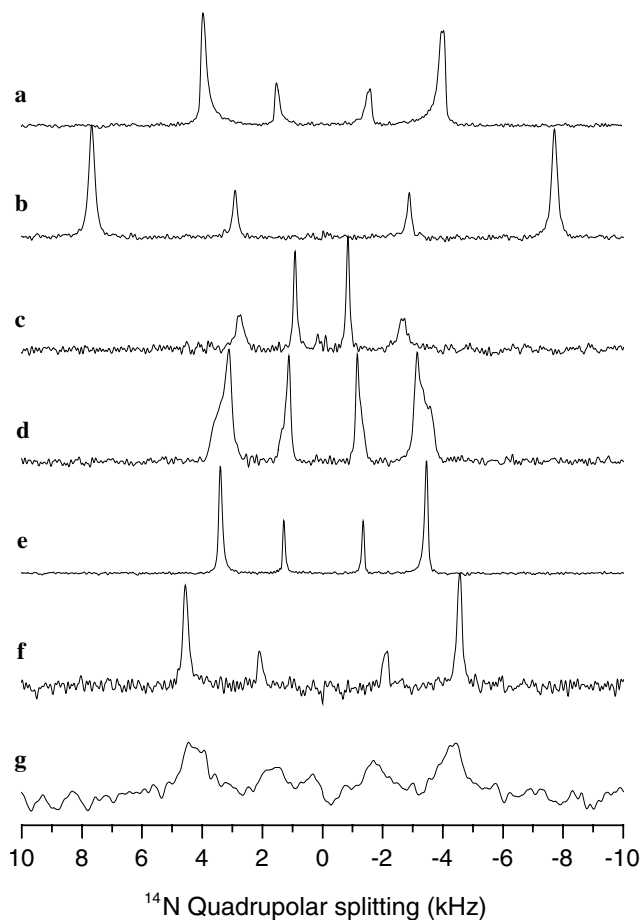


Fig. 5. ^{14}N quadrupole coupling spectra of magnetically aligned DMPC:DHPC bicelles, $q = 3.5$, showing the influence of admixing different substances: (a) Pure DMPC:DHPC bicelles; (b) in the presence of Yb^{3+} ions, resulting in ‘flipped’ bicelles; (c) 2.0 mol% desipramine; (d) 2.0 mol% MSI-78; (e) 0.5 mol% MSI-78; (f) 20.0 mol% negatively charged lipid DMPG in the presence and (g) absence of 150 mM NaCl.

ligands with membranes. It should be mentioned here that the measurement of the ^{14}N quadrupole coupling of the choline group was recently utilized to understand the membrane-interaction of charged molecules such as antimicrobial peptides in mechanically aligned bilayers and also in multilamellar vesicles under static and MAS conditions [60–62].

3. Conclusions

In this study, we investigated various practical aspects of ^{13}C and ^{14}N NMR experiments in magnetically oriented bicelles. We have demonstrated that RF heating effects in bicellar samples can easily be calibrated and taken into account by monitoring the water proton chemical shift. This approach can be used for studies on other samples like mechanically aligned bilayers and multilamellar vesicles as well. We have also demonstrated that by applying advanced broadband decoupling sequences the required decoupling RF power can be considerably reduced and

thus RF heating can be minimized. A comparison of various methods to enhance the sensitivity of experiments to detect less sensitive nuclei and to enhance the resolution of the chemical shift spectrum of less sensitive nuclei in bicelles is presented. Our results infer that ^{14}N NMR experiments on bicelles are easy to carry out with a standard commercial set-up. The detection of ^{14}N nuclei could be used as a ‘voltmeter’ to study ligand–membrane interactions. We believe that the results presented in this paper can be utilized in performing advanced multidimensional solid-state NMR experiments [63–65] on bicelles containing peptides, proteins, drugs or other types of molecules.

4. Experimental section

4.1. Sample preparation

1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphatidylcholine (DHPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). DMPC and DHPC with a molar ratio ($q = \text{DMPC}:\text{DHPC}$) of 3.5:1 was dissolved in chloroform. The solvent was slowly evaporated under a stream of nitrogen gas at room temperature and completely removed by overnight lyophilization. A 100 mM Hepes buffer at pH 7.0 was added to obtain a concentration of 37.5% (w/w) phospholipids to solution. Peptide was cosolubilized in chloroform along with the lipids at the desired molar ratios, while an appropriate amount of YbCl_3 salt was added to the Hepes buffer to yield ‘flipped’ bicelles [66,67]. The sample was vortexed until all of the lipids were solubilized in the Hepes buffer. The solubilized sample was gently sonicated in an ice-cold water bath. The final sample was obtained by several freeze and thaw cycles until a clear transparent solution was formed.

4.2. NMR measurements

All NMR experiments were carried out on a Chemagnetics/Varian Infinity-400 MHz solid-state NMR spectrometer using a 5 mm custom-modified double-resonance magic-angle spinning probe under static sample conditions. About 100 mg of sample was loaded in a 5 mm NMR glass tube of 4 cm length and was closed tightly with a Teflon tape and a cap. The sample was equilibrated prior to the measurement for about 1 h in the magnet at 37 °C. All experiments were performed at 37 °C. Phosphorus-31 chemical shift spectra were recorded to test the magnetic alignment of bicelles. A ramped-CP [44] sequence with a contact time of 5 ms was used to record the 1D ^{13}C chemical shift spectra under proton decoupling using various decoupling sequences for a comparative study. A 5 μs ^1H 90° pulse, a recycling delay of 7 s, a 25 kHz spectral width, and an acquisition time of 41 ms were used. Data were processed without any line broadening. For the NOE enhancement, protons were irradiated using a 1 kHz RF field during the recycling delay.

To record signals of ^{14}N NMR spectra the quadrupole echo sequence [68] was used with the pulse length of 4.4 μs and an echo-delay of 1 ms. With this long delay the interference effect of the probe acoustic ringing, which is severe at the ^{14}N resonance frequency of 29 MHz, was negligible. No influence of the proton decoupling on the ^{14}N spectral shape was observed. Hence, spectra presented here were acquired without the proton decoupling. Up to 4000 scans were accumulated with a repetition delay of 0.2 s.

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