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Editing ^{13}C-NMR spectra of membranes

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We report the carbon-13 'magic-angle' sample-spinning nuclear magnetic resonance (NMR) spectra of several lipid-water systems, under a variety of radiofrequency excitation conditions. Our results show that complex lipid or membrane spectra can be greatly simplified by using 'spectral editing' techniques. For example, in a 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)-water mesophase, the glycerol (C-1, C-2 and C-3) carbons are readily distinguished from the headgroup C^{α}, C^{β} and C^{γ} carbons, on the basis of their mix-time behavior in a cross-polarization (CP) experiment, while in the more complex DMPC/cholesterol-water system, many of the more rigid cholesterol carbon resonances can be edited from the phospholipid peaks. In very complex systems, such as human myelin membranes, editing permits the unambiguous observation of the mobile lipid headgroup carbon resonances, as well as the much more rigid sterol ring carbons. We also report the observation of a large differential CP due to C-H vector 'magic-angle' orientational effects in the DMPC/desipramine system. Thus, both motional or orientational reduction of the C-H dipolar interaction can lead to considerable simplifications of complex membrane spectra, and are of interest from both spectral assignment and membrane dynamics aspects.

Introduction

In this paper, we discuss some recent applications of solid-state 'magic-angle' sample spinning (MAS [1,2]) nuclear magnetic resonance (NMR) spectroscopy to the study of lipid and biological membrane structure. Our results show that considerable simplifications of ^{13}C-NMR spectra of complex systems, such as phospholipid-sterol model membranes and human myelin, can be achieved by using cross-polarization (CP) techniques [3,4], and these simplifications are of use in making spectral assignments, as well as giving fundamental information on molecular mobility, and in one case, on C-H vector orientation.

To a greater or lesser extent, we find that choline headgroups can be differentiated from glycerol headgroup carbons; cholesterol ring resonances from lipid resonances; mobile myelin headgroup carbons from rigid residues; and in addition we demonstrate a more subtle effect, due to C-H vector orientation, which can lead to large differences in CP behavior for different methine carbons located in the same aromatic ring, due this time to a 'magic-angle' orientation effect [5].

Materials and Methods

NMR spectroscopy

^{13}C-MAS-NMR spectra were obtained on a 'home-built' NMR spectrometer, which operates at 500 MHz for ^{1}H, using an Oxford Instruments (Osney Mead, UK) 2.0-inch bore, 11.7 T superconducting solenoid, together with a Nicolet (Madison, WI, USA) Model 1280 computer system, a Henry Radio (Los Angeles, CA, USA) Model 1002 radiofrequency amplifier, an Amplifier Research (Souderton, PA, USA) Model 200L radiofrequency amplifier, and a 5-mm Doty Scientific (Columbia, SC, USA) MAS-NMR probe. Dipolar decoupled (40 W ^{1}H) Bloch decays were recorded using 8.5–9.0-μs 90° pulse widths, spinning rates of approx. 3.0 kHz and CYCLOPS phase cycling. CP/MAS experiments used about the same ^{1}H power levels.

Temperatures were regulated by using a Doric (San Diego, CA, USA) Model DC7000 controller, and the values reported are gas-flow temperatures monitored

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with a thermocouple located near the sample. All spectra were referenced with respect to an external chemical shift standard of adamantane ($\delta = 38.5$, 29.5 ppm from TMS), using the convention that high frequency, low field, paramagnetic or deshielded values are denoted as positive (IUPAC δ-scale).

Sample preparation

1,2-Ditetradecanoyl-*sn*-glycero-3-phosphocholine (dimyristoylphosphatidylcholine, DMPC) was obtained from Sigma (St. Louis, MO, USA). Cholesterol was from Aldrich (Milwaukee, WI, USA), and was recrystallized three times, from ethanol, before use. Desipramine was also from Sigma, and was used as received.

Pure phospholipid samples were prepared for NMR spectroscopy by the addition of 50 weight% ^2H_2O (Sigma). Phospholipid/cholesterol samples were prepared by co-dissolving the appropriate amounts of lipid and sterol in $CHCl_3$, then removing the solvent under an N_2 stream, followed by evacuation overnight. Sample purity was monitored periodically via thin-layer chromatography, on Eastman Chromagram silica-gel sheets no. 6061 (Eastman Kodak, Rochester, NY, USA) using a $CHCl_3$:MeOH:7 M NH_4OH (230:90:15 (v/v/v)) solvent system [6], with molybdenum blue as the visualizing agent [7].

Human brain samples were obtained from Prof. M.A. Moscarello at the Hospital for Sick Children in Toronto (Toronto, Ontario, Canada) and from Dr. W.W. Tourtellotte of the National Neurological Research Bank, Veterans Administration Medical Center Wadsworth Division (Los Angeles, CA, USA). All samples were received frozen and formalin free. Human myelin was isolated using a sucrose gradient, as described elsewhere [8,9].

Results and Discussion

By way of introduction, we show in Fig. 1A–C the ^{13}C-MAS-NMR spectra of DMPC (50 weight% in ^2H_2O) at 37°C, in the glycerol backbone and choline headgroup spectral region, obtained by using either a simple Bloch decay with dipolar decoupling sequence (Fig. 1A), or by using ^1H-^{13}C cross-polarization, with a mixing time of either 200 μs (Fig. 1B) or 200 ms (Fig. 1C). As anticipated from the previous work of Halladay et al. [10] at a 200-MHz ^1H resonance frequency, and our previous work [11–13], all six choline headgroup and glycerol backbone carbons are clearly visible in the proton decoupled Bloch decay spectrum (Fig. 1A). In sharp contrast, when using a very short mix-time (200 μs, Fig. 1B), only the three glycerol backbone carbons, C-2 (at approx. 71.6 ppm [12]), C-3 (at 64.5 ppm) and C-1 (at 64.0 ppm), are observed, due to their strong C-H dipolar interaction. At very long mix-times (200 ms, Fig. 1C), the three choline headgroup carbons: C^β (at approx. 67 ppm), C^α (at 60 ppm) and C^γ (at 55 ppm) are by far the largest contributors to the observed spectrum, due in this case to very inefficient cross-relaxation, and long ^1H $T_{1\rho}$ values, as anticipated for a highly mobile headgroup. Thus, the spectrum shown in Fig. 1A can be simplified, or edited, by using the CP process to yield two 'subspectra'. At very short mix-times, only the rigid glycerol backbone carbons are readily observed, Fig. 1B, while at very long mix-times, the mobile choline headgroup C^α, C^β and C^γ carbons dominate the spectrum (Fig. 1C). Related effects are seen in other regions of the spectra, e.g., the terminal methyl groups are not observable at short contact times (data not shown).

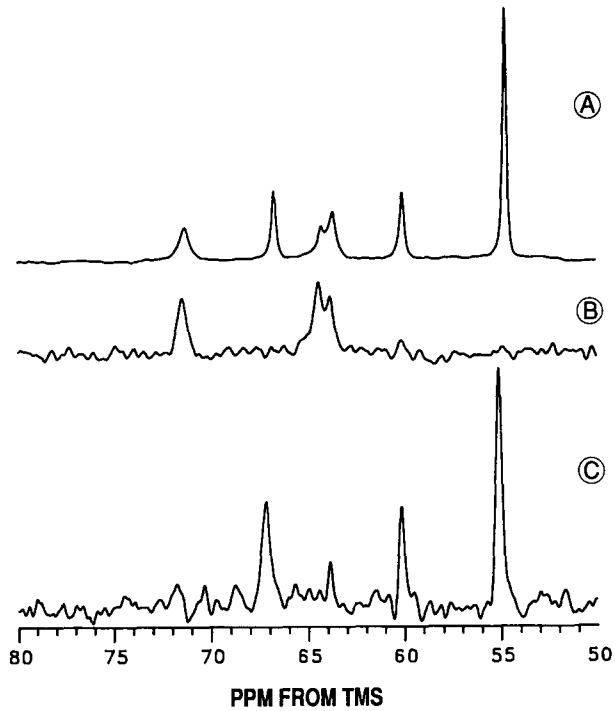

Fig. 1. Proton-decoupled ^{13}C-MAS-NMR spectra of 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (dimyristoylphosphatidylcholine, DMPC)/^2H_2O (1:1 (w/w)) at 37°C, recorded at a ^{13}C Larmor frequency of 125.7 MHz (11.7 T) and using different excitation conditions. A, Bloch decay; B, CP/MAS spectrum, 200 μs contact; C, CP/MAS spectrum, 200 ms contact. The MAS rate was approx. 3.6 kHz in each case and 128 scans at a 5-s recycle delay were used. Spectra were apodised by using a 20-Hz exponential (Lorentzian) linebroadening function.

Upon addition of cholesterol (CHOL) at a 1:1 molar ratio with DMPC, a number of new peaks appear in the ^{13}C spectrum, due to CHOL itself [12]. Fig. 2 shows a representative portion of the spectrum of DMPC/CHOL (1:1) in excess water, at 37°C, in the range 10–80 ppm from TMS. In the proton-decoupled Bloch decay spectrum (Fig. 2A) there is a new peak at approx. 72 ppm, arising from CHOL-C-3 (superimposed on the glycerol C-2 carbon), plus two additional well

resolved peaks, one at 57.7 ppm (CHOL-C-14,17) and another at 51.2 ppm (CHOL-C-9). In addition, there are a number of peaks in the range 37–43 ppm, due to CHOL-C-13,4,12,24,1,10,20 and 22 [12,15], as well as additional features to even higher field.

We show in Fig. 2B and 2C the ^{13}C CP/MAS NMR spectra of the same sample as used in Fig. 2A, but recorded using either a 500 μs mix-time (Fig. 2B) or a 50 ms mix-time (Fig. 2C). As can be seen in Fig. 2B, there are a number of major changes in peak intensity compared with the Bloch decay result (Fig. 2A). All of the intense, narrow line peaks seen in Fig. 2A are decreased in intensity, consistent with the results obtained for DMPC alone (Fig. 1B), and the major new features can be assigned to the rigid sterol ring carbons: C-3 (71.4 ppm), C-14,17 (57.7 ppm) and C-9 (51.0 ppm). In the 37–44 ppm chemical shift range, the peaks at 43.2 and 37.3 ppm are decreased in amplitude, consistent with their assignment to CHOL-C-13 and CHOL-C-10, the two quaternary (aliphatic) carbon resonances, which are expected to have long T_{CH} values, due to the absence of directly bonded hydrogens.

At long mix-times (Fig. 2C) most of the broad features seen at short mix-times (Fig. 2B) are edited away, and the major features seen are, as with pure DMPC-H_2O, those from the choline C^{α}, C^{β} and C^{γ} carbons,

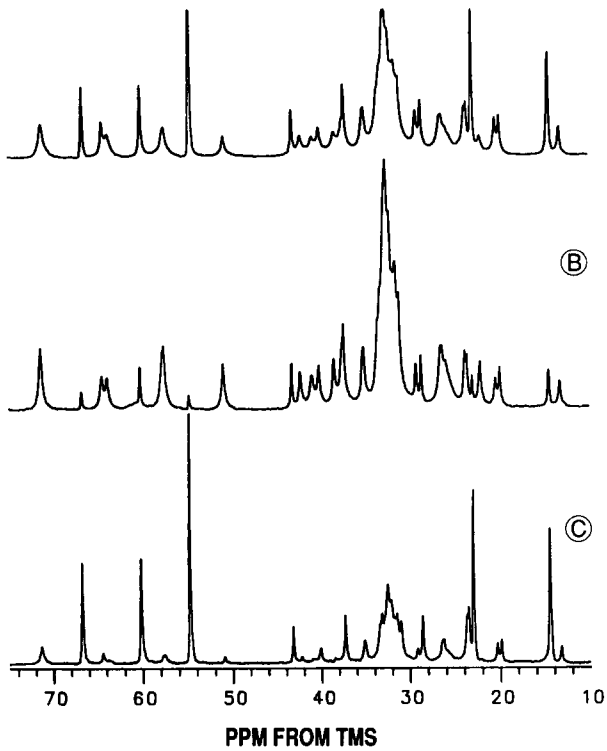

Fig. 2. Proton-decoupled ^{13}C-MAS-NMR spectra of DMPC-CHOL (1:1)/50 weight% $^{2}H_2O$, at 37°C. A, Bloch decay; B, CP/MAS spectrum, 500 μs contact; C, CP/MAS spectrum, 50 ms contact. Other conditions basically as in Fig. 1, except that 2500 scans were recorded and a 5-Hz linebroadening applied.

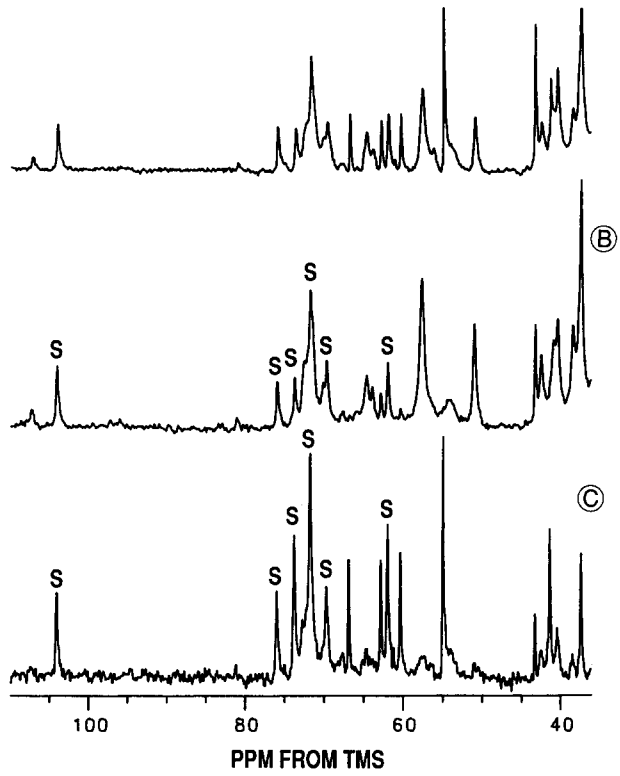

Fig. 3. Proton-decoupled ^{13}C-MAS-NMR spectra of adult human myelin in excess H_2O, at 37°C. A, Bloch decay; B, CP/MAS spectrum, 500 μs contact; C, CP/MAS spectrum, 30 ms contact. Other condition basically as in Fig. 1, except that 1500 scans were recorded and a 7-Hz linebroadening applied. Peaks labelled 'S' arise from sugar headgroup carbons.

plus in addition, the two quaternary CHOL carbons, C-10 and C-13, and the C-14 and C-13 lipid acyl chain resonances.

We now apply these editing pulse sequences to the much more complex system, adult human myelin, and show in Fig. 3 the proton-decoupled Bloch decay spectrum (Fig. 3A), a proton-decoupled CP/MAS spectrum recorded by using a 500 μs cross-polarization mix-time (Fig. 3B), and a CP/MAS spectrum obtained by using a 30-ms mix-time (Fig. 3C). We have used progressively shorter mix-times for the long mix-time experiment on transition from DMPC (200 ms) to DMPC-CHOL (50 ms) to myelin (30 ms), since, consistent with the early work of Cornell et al. [18], there is a progressive decrease in T_{CH} and (^{1}H) $T_{1\rho}$ on addition of CHOL and protein (in myelin), such that if very long mix-times are used with myelin, there is an unacceptable loss of signal amplitude for very long mix-time values (data not shown).

As with the DMPC/CHOL system, the short mix-time spectra result in the attenuation of the mobile choline/ethanolamine headgroup carbon resonances, concomitant with a relative enhancement of the CHOL ring carbons, which is particularly pronounced for, e.g., CHOL-C-14,17 (approx. 57.5 ppm) and C-9 (50.9 ppm).

A number of resonances attributable to sugar headgroup carbons are also quite prominent when using a 500-μs mix-time (denoted by 'S' in Fig. 3B; see also Fig. 3C). At much longer mix-times, essentially all of the rigid CHOL ring carbons are effectively edited from the spectrum (Fig. 3C) and the well-defined peaks can almost all be assigned to the polar headgroup carbon atoms: at 104.1 ppm (sugar C-1); 76.0 ppm (sugar C-5); 73.8 ppm (sugar C-3); 71.9 ppm (sugar C-2); 69.8 ppm (sugar C-4); 66.8 ppm (PC, SM C^{β}); 62.8 ppm (PE C^{α}); 62.0 ppm (sugar C-6); 60.3 ppm (PC, SM C^{α}); 54.9 ppm (PC, SM C^{γ}); 41.3 ppm (PE C^{β}) and 40.8 ppm (PEpl C^{β}). The sharp peak at 43.2 ppm arises, we believe, from CHOL-C-13, a quaternary carbon. Thus, essentially all of the intense, narrow peaks in the carbon-13 NMR spectrum of adult human myelin arise from the polar headgroups of the phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, plasmalogen and cerebroside (sugar) lipids in myelin [8,15]. Editing permits the relatively unambiguous observation (and assignment) of some features, e.g., C^{β} of PE and PE plasmalogen, that are otherwise buried under the much more intense features attributable to cholesterol. Also, our results suggest that there may be a relatively wide range of sugar headgroup mobilities, since sugar groups are seen at both short and long mix-times, an observation that would be consistent with the observation of very complex phase behavior with the cerebrosides [16–18].

Finally, we show a quite different type of spectral editing using the CP/MAS technique, one based upon the observation [5,19] that there may be significant linewidth (or T_{CH}) differences for C-H vectors attached to a rigid moiety (e.g., an olefinic group or an aromatic ring), due to fast internal motion. This type of effect was first observed in ^{2}H-NMR by Seelig and Waespe-Šarčevič [20] who noted that the ^{2}H quadrupole splittings of the two olefinic deuterons in a lipid containing an oleoyl chain had very different values (approx. 2, 15 kHz [20]). This surprising observation was explained by invoking a simple model in which the two C-H vectors subtended different angles, θ, with respect to the major axis of motional averaging, the director axis. For one C-H vector, θ was approx. 54.7°, the magic angle. In the presence of fast axial motion, this causes a collapse of the ^{2}H quadrupole splitting. We have observed similar results in the case of a ^{2}H-labeled tricylic antidepressant drug, desipramine, intercalated into a DMPC bilayer [5], and we also noted that differential line-broadening (due to interference between the C-H dipolar and ^{13}C chemical-shift anisotropy interactions) for one site (C-2/8) on the desipramine ring was extremely small, again due to a 'magic-angle' orientation of the C-H vector with respect to a major axis of motional averaging. Analogous results were obtained by Tabeta et al. [19], who observed differential ^{13}C linewidths in a sonicated egg-lecithin-imipramine bilayer system.

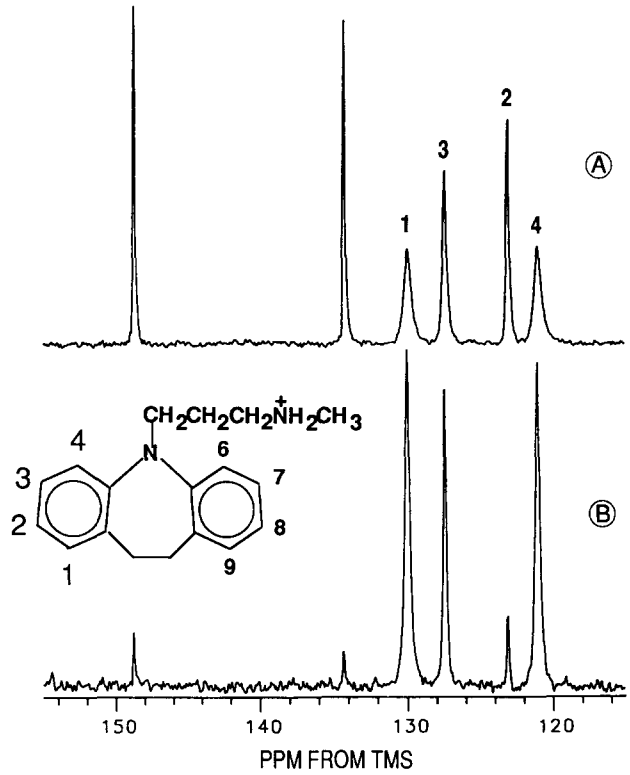

Fig. 4. Proton-decoupled ^{13}C-MAS-NMR spectra of DMPC-desipramine (64:36 molar ratio)/50 weight% ^2H_2O, at 37°C. A, Bloch decay; B, CP/MAS spectrum, 500 μs contact. Other conditions basically as in Fig. 1, except that 6000 scans were recorded. The numbers above the four lower-frequency peaks in A refer to the four different protonated aromatic carbon sites shown in B.

We thus show in Fig. 4A the aromatic part of the ^{13}C-MAS-NMR spectrum of a desipramine (36 mol%)-DMPC/H_2O bilayer at 37°C. The peak assignments have been discussed previously [21], and are shown in the figure. On cross-polarization, using a 500-μs mix-time, there are several major changes in peak intensity. The two peaks in the high-frequency region arise from the quaternary carbons and, as expected, at a 500-μs mix-time they are very weak. However, we also find that one of the protonated or methine aromatic carbons (C-2/8) is also only very weakly cross polarized (Fig. 4B), which based on our previous observation of a very small deuterium quadrupole splitting (approx. 2 kHz) for this site (vs. approx. 20 kHz for ^{2}H4/6), the observation that the ^{13}C linewidths for C-2/8 in the sonicated system are very small [19], and the lack of differential line-broadening [5], all suggest that the desipramine ring must undergo fast motions about its 'C_2' axis such that the C-2/8-H vector is close to the magic angle. Thus, the dipolar (and quadrupolar) interactions for the C-2/8 (H2/8) site are all highly motionally averaged, and cross-relaxation/cross-polarization efficiency is greatly reduced.

Conclusions

The results we have presented above are of interest for a number of reasons. First, they clearly demonstrate that simplifications of complex membrane spectra can be achieved by means of 'spectral editing'. Second, our results indicate large motional differences between polar headgroups, lipid backbones and sterol ring/sidechain carbon atoms, which permits a useful editing of the ^{13}C-NMR spectrum of human myelin. Third, our results strongly suggest that both mobile and rigid phospholipid and galactolipid headgroups exist in adult human myelin, at 37°C, in general accord with the complex phase behavior exhibited by cerebroside phospholipid systems. Fourth, our results reveal a novel differential cross-polarization effect in a model drug-lipid system, desipramine-DMPC, which can be correlated with rapid motion about the 'C_2' axis of the drug molecule, with resultant quenching of dipolar interactions for one unique carbon, due to a 'magic-angle' orientation effect. The use of these and related editing modalities in future studies of more complex membrane systems appears worthwhile.

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