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Polarization Transfer in Lipid Membranes

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Polarization transfer is a key experiment for the detection of insensitive nuclei by NMR. Transfer in liquids is often achieved through *J*-coupling using the INEPT experiment, while in solids the dipolar coupling is used with cross polarization. Liquid crystals, including lipid membranes, are intermediate cases between solids and liquids. In the present article, we compare several transfer methods for lipid membranes spinning at the magic angle. It is shown that the most commonly used cross polarization technique is, in most cases, advantageously replaced by refocused INEPT or even by the NOE enhancement experiment, a method that is not normally used in that context. In principle, these enhancement techniques could be applied to other systems, including biological tissues and, more generally, soft matter systems that are neither solid nor liquid by NMR standards. © 2000 Academic Press

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Polarization transfer from protons to nuclei of low abundance or low sensitivity is a crucial part of numerous NMR experiments. In the case of carbon atoms, it can be used for sensitivity enhancement, for inverse detection of these nuclei by protons, for two-dimensional heteronuclear correlation, for filtering of some specific carbon nuclei, or for speeding up the relaxation process using the proton relaxation pathway.

In liquids, the original insensitive nuclei enhanced by polarization transfer (INEPT) (*1–5*) scheme, and its variations, are efficient transfer methods between bonded nuclei that provide all the aforementioned capabilities. The coherence transfer can be performed either way and it depends on T_2 relaxation since it takes place in the transverse plane, in approximately 1 to 4 ms, using the proton–carbon one bond *J* coupling of ca. 135 Hz. In most solids, even under state-of-the-art high-resolution solid-state NMR, lines are too broad to permit observation of *J* couplings. In other words, coherences should vanish in the transverse plane before the transfer could take place, although efforts are being made in this direction to overcome this limitation (*6–8*). Less versatile but sometimes useful is the heteronuclear nuclear Overhauser effect (NOE) coupling between closely spaced nuclei (*9–11*). It takes place in the longitudinal plane and depends on T_1 relaxations, gyromagnetic ratios, and NOE build-up rates of both nuclei. For these reasons, two-dimensional heteronuclear NOE spectroscopy

(HOESY) is favorable in the case of ^{31}P–^1H nuclei but unfavorable in the case of ^{13}C–^1H nuclei (*12, 13*).

In solids, the most popular method is cross polarization (CP), often combined with magic angle spinning (MAS) (*14–16*), using the strong coherent dipolar coupling and the Hartmann–Hahn transfer scheme. As we will see below, implementing and maintaining the Hartmann–Hahn condition under MAS is sometimes hard, especially with weak dipolar couplings and/or high spinning speed, but improved versions of CP try to circumvent this problem (*17–21*). CP in liquids is also used for some specific applications and has gained recent renewed interest for selective coherence transfer (*22–24*).

Lipids form an essential component of biological membranes. Model membranes composed of lipids and water can be designed with the desired size and lamellarity (*25, 26*). Multilamellar vesicles (MLV) are very useful models for NMR studies: they can be very concentrated in lipids and the local constraints are similar to that found in a biological membrane. The slow tumbling and the small curvature of MLV fail to average out the dipolar interactions and the chemical shift anisotropy, thus MAS is required to obtain high-resolution spectra. Such systems could then be considered as solids, but lipids in the fluid phase are not real rigid solids: (1) Dipolar couplings are attenuated by *gauche–trans* isomerization, lateral and axial diffusion. (2) Spin diffusion is attenuated as well and therefore ^1H T_1 are not as short as in a rigid solid. (3) In ^1H NMR, MAS even at moderate spinning speeds provides high-resolution spectra due to the particular nature of the ^1H–^1H dipolar interaction, rendered inhomogeneous by fast axial diffusion (*27*). Fast-limit, large-amplitude motions reduce the size of anisotropic interactions but motional averaging is incomplete and the residual interactions may be employed for solid-state NMR types of experiments. Because MLV require MAS for high-resolution NMR spectra, CP-MAS has been thought for years to be the ideal method for polarization transfer. Only recently we have shown that INEPT and TOCSY transfers are possible, and sometimes preferable, in lipid/water systems in the fluid phase under MAS (*28, 29*). In the present article we compare several different ^1H–^{13}C transfer methods in lipid bilayers and point out their respective advantages.
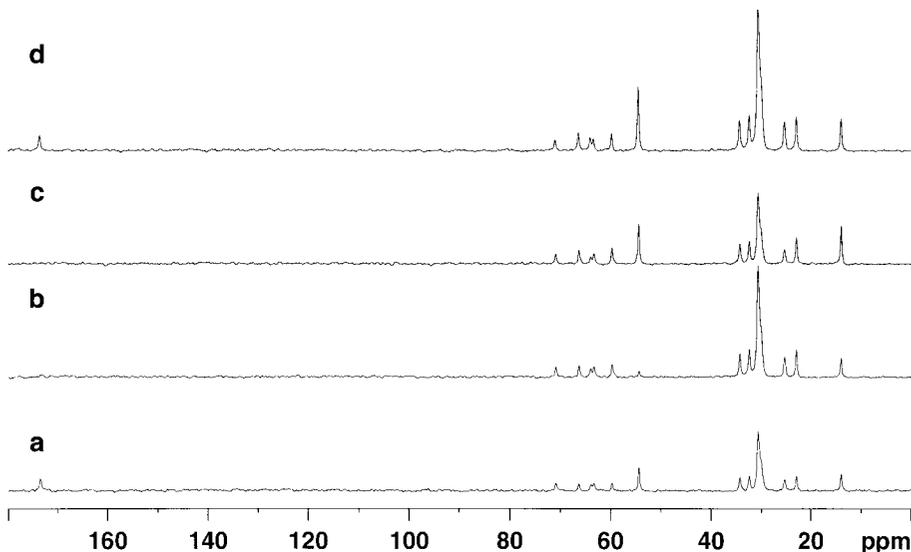
FIG. 1. 100.61 MHz ^{13}C MAS spectra with inverse gated ^{1}H TPPM decoupling of dimyristoylphosphatidylcholine (DMPC) in 50% D_2O, at 30°C, in a 4-mm rotor spinning at 5 kHz. (a) Standard Bloch decay spectrum. (b) RAMP-CP spectrum with a 5 ms spin-lock. (c) Refocused INEPT spectrum with 1.79 and 1.20 ms delays. (d) NOE enhanced spectrum. All experiments were performed on a Bruker DMX 400 MHz wide bore spectrometer, equipped with a nonlinear 1 kW ^{1}H amplifier and a linear 300 W ^{13}C amplifier and with the same following parameters: same receiver gain, 1024 scans, 5 s relaxation delay, ^{13}C (90°) pulse ca. 3 μs, ^{1}H (90°) pulse ca. 2.5 μs, 30 kHz spectral width, 2048 points, zero filling to 4096, 30 Hz line broadening, automatic baseline correction.

All experiments will be compared, with same plotting parameters, to a standard ^{13}C MAS Bloch decay experiment with inverse gated ^{1}H two-pulse phase modulation decoupling (TPPM (30)) shown on Fig. 1a. As stated previously, for lipids in the fluid phase, spinning the sample at the magic angle at several kilohertz easily averages out ^{13}C chemical shift anisotropy and ^{13}C–^{1}H dipolar couplings. Hence, most experiments presented here were performed at a spinning frequency of $\omega_r =$ 5 kHz. TPPM decoupling efficiency was compared to the more traditional GARP, WALTZ16, or CW decoupling sequences. Due to the small residual ^{1}H–^{13}C couplings in lipids, all sequences were efficient at high ^{1}H r.f. power ($\omega_H > 80$ kHz), even at high spinning speeds. TPPM was slightly more efficient at lower powers and was used in all cases presented here.

Standard cross polarization from ^{1}H to ^{13}C was performed with a spin-lock following a ^{1}H (90°) pulse and ^{13}C acquisition. Several spin-lock durations and powers were tested and the best compromise for uniform ^{13}C excitation was found to be 5 ms spin-lock, longer than values generally used for rigid solids. Montez *et al.* (31) showed that shorter or longer spin-lock times could be used for selective excitation (and assignments) of headgroup vs. glycerol peaks, or for better carbonyl excitation, but relative intensities become unreliable. Spin-lock powers up to 75 kHz r.f. field were tested and showed significant improvement with increasing power, up to approximately 30 kHz, and then negligible improvement above that value. The Hartmann–Hahn profile (a.k.a. "the finger pattern") and the modified Hartman–Hahn condition for MAS ($|\omega_C - \omega_H| = \omega_r$) was measured in each case presented here for $\omega_C = 30$ kHz. A

resulting ω_H of approximately 20 to 25 kHz, corresponding to the -1 "finger," was chosen for the 1D spectrum.

Above 5 kHz, the Hartmann–Hahn condition is more difficult to set up and maintain. This occurs because the fingers are spread apart by ω_r and their width, which is equal to the residual ^{1}H–^{13}C dipolar coupling, is smaller than in a rigid solid. Nevertheless, it can be necessary to work at such high spinning speed, in the case where CP is just part of a more complex experiment, like a recoupling experiment for example. In that case, it is useful to replace the standard CP with an improved version, like RAMP-CP (19). Figure 2 shows the comparison between Hartmann–Hahn profiles, with and without RAMP, at 5 and 10 kHz. There is no disadvantage in using RAMP-CP over standard CP in every case, even at low spinning speed, since it is a very robust sequence with respect to missettings or spectrometer instabilities. At low spinning speed, we find a gain in signal-to-noise by using RAMP-CP over standard CP, a phenomenon expected and explained by Metz *et al.* (19). Figure 1b shows such a RAMP-CP experiment, at 5 kHz spinning speed and with a 30 kHz spin-lock of 5 ms, optimized with Fig. 2b. Figures 3b and 3c show examples of CP used with two different spin-lock times (50 ms and 300 μs respectively), for better excitation of selected resonances.

Refocused INEPT from ^{1}H to ^{13}C was performed with ^{13}C detection, as described in Gross *et al.* (28). The first delay is 1.79 ms while the second one is 1.20 ms, in order to get all resonances positive, as shown in Fig. 1c. By choosing the second delay to be 2.39 ms, one can obtain spectra with CH
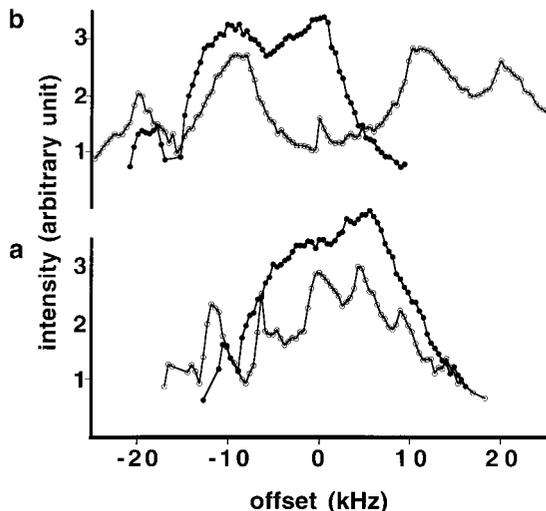
FIG. 2. $^{13}CH_2$ Hartmann–Hahn profiles of the same sample, extracted from 150 1D files, each file acquired with 64 scans and increasing ^1H spin-lock r.f. power by 300 Hz. The slice at the CH_2 chemical shift is shown for simplicity. RAMP-CP was performed using a linearly increasing ^{13}C spin-lock r.f. power, from 0.5 time the ideal power to 1.5 time the ideal power, with 252 steps. Here, $\omega_C = 30$ kHz and the signal intensity is plotted vs. $\omega_H - \omega_C$ in kHz. (a) $\omega_r = 5$ kHz. (b) $\omega_r = 10$ kHz. Open circles, standard CP. Filled circles, RAMP-CP.

and CH_3 resonances positive and CH_2 negative, for example, for help in spectral assignment, as shown in Fig. 3a.

NOE enhancement from ^1H to ^{13}C was performed with a long low power irradiation on the ^1H channel, followed by a ^{13}C (90°) pulse and ^{13}C acquisition (Fig. 1d). This experiment was found to be very insensitive to small differences around the ideal irradiation length and power which were found to be 3 s at 40 dB attenuation from the maximum ^1H r.f. power,

resulting in approximately $\omega_H = 1$ kHz. A decent transfer is already seen with a shorter ^1H irradiation of 100 ms for example. Of course, when the irradiation pulse is 3 s long, the repetition time is reduced to 2 s for a total relaxation of 5 s comparable to all other experiments.

Absolute gain in signal-to-noise ratio can be compared if one looks at the CH_2 resonance intensity. None of the tested experiments provides the theoretical maximum factor enhancement of 4. Standard CP provides a negligible enhancement factor of 1.1 (data not shown). RAMP-CP, INEPT, and NOE approximately double the signal-to-noise, with a slight advantage of NOE over the other two methods. One cannot deal with merit in signal-to-noise ratio without taking the experimental time into account. One major advantage of CP is the possibility of reducing the repetition time to ca. 5 times the ^1H T_1 rather than 5 times the ^{13}C T_1 in other experiments like the NOE enhancement. Unfortunately the economy in time is negligible here since, as stated previously, spin diffusion is attenuated in lipids in the fluid phase and ^1H T_1 are almost as long as ^{13}C T_1.

Differences in relative intensities are more crucial. CP transfer is very sensitive to residual coherent dipolar couplings and thereby to local motion, $^{13}C–^1H$ distances, and orientations. For these reasons, the proton to carbon transfers are very poor in the following groups: the unprotonated carbonyl carbons (173.6 ppm), the highly mobile groups like the terminal methyl and the headgroup (14, 54.3, 59.7 and 66.3 ppm), and the glycerol CH bond oriented near the magic angle (64 ppm). For some of these groups, the "enhancement" is sometimes smaller than one! INEPT transfer uses the *J* coupling which is *not* orientation dependent and only slightly relaxation dependent as long as $1/J < T_2$. This last condition is verified since the multiplets are visible on an undecoupled spectrum (*28*). On the other hand, INEPT is optimized for *one* specific value of *J*, ca.
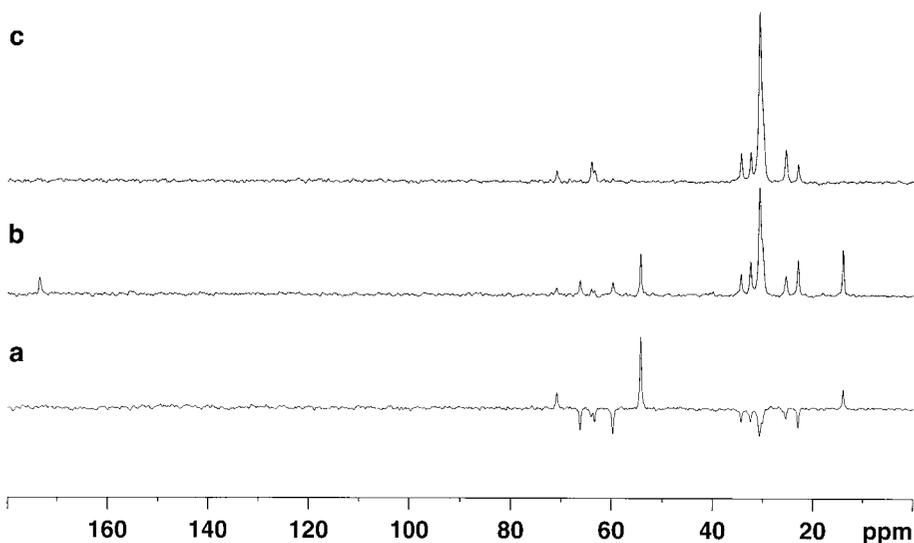

FIG. 3. 100.61 MHz ^{13}C MAS spectra of the same sample. (a) Refocused INEPT spectrum with 1.79 ms and 2.39 ms delays. (b) RAMP-CP spectrum with a 50 ms spin-lock. (c) RAMP-CP spectrum with a 300 μs spin-lock.

135 Hz for the one bond proton–carbon studied here. Slight variations around this value will affect the transfer efficiency and the resulting relative intensities, especially when changing from a CH_2 to a CH or a CH_3 group. The only crucial failure of INEPT in lipids, though, is the proton to carbonyl carbon transfer that results in the disappearance of the carbonyl line in Fig. 1c. NOE transfer is an incoherent process that is slightly sensitive to motion, $^{13}C-^{1}H$ distances, and orientations, but not as much as CP since cross relaxation makes the transfer efficient from protons to remote carbons. Here again, the most affected carbon is the carbonyl group where the enhancement is small, although larger than one. Unlike in the other two cases, there is no disappearing line.

Another parameter to be considered is the ease of implementation and, as a related problem, the stability of either experiment. Cross polarization requires special equipment, a high power ^{1}H amplifier and a CP-MAS probe rather than a HR-MAS probe, and a special set up with no ^{1}H preamplifier that would otherwise be harmed by the power involved. The Hartmann–Hahn condition is sometimes difficult to set-up, especially at high spinning speed, and it is quite unstable since the Hartmann–Hahn "fingers" are narrow and depend on both the r.f. field strengths and on the rotor spinning speed. These problems are alleviated by the use of RAMP-CP, although this pulse sequence requires the use of at least one linear amplifier. In the case where RAMP-CP has been optimized once, only the ^{1}H (90°) pulse has to be optimized in order to get a CP spectrum with a new sample, and the experiment will then be reasonably stable. ^{1}H (90°) pulse is easily measured in only a few scans, but the necessity of a preamplifier requires a hardware set up different from the one used for CP, and the measured value will then be slightly affected.

Refocused INEPT and the NOE enhanced experiment, on the other hand, require only standard hardware and are stable with respect to spinning speed variation. Refocused INEPT efficiency is sensitive to accurate (90°) pulses on *both* channels and timings have to be carefully optimized since they differ slightly from the theoretical $1/4J$ value. For the NOE enhancement experiment, only the ^{13}C (90°) pulse has to be calibrated since it is a very robust experiment with respect to small variation in the ^{1}H irradiation pulse length or strength.

Many biological samples are neither solid nor liquid by NMR standards: intact cell suspensions, biological tissues, membranes, plant extracts, natural products, etc. The scope extends even further to compounds with chemical or pharmaceutical interest like some polymers, gels, liquid crystals, micellar systems, organic molecules, and peptides bound to a solid resin support, swollen by a solvent, for solid-phase-synthesis or solution-state combinatorial chemistry. One can notice a growing interest for these heterogeneous samples as they may also benefit from a new generation of NMR probes designed for high-resolution MAS (HR-MAS) (*32–34*).

In lipid bilayers, $^{1}H-^{15}N$ or $^{1}H-^{31}P$ transfers are not as useful since there is only one nitrogen and one phosphorous nucleus per lipid, the J couplings are much smaller than J_{CH}, and the gain in sensitivity, in the case of ^{31}P, is also smaller than in the case of ^{13}C. Nevertheless, in other soft matter systems, the conclusions outlined here will apply to some extent to polarization transfers from protons to any other nuclei.

The purpose of this paragraph is to describe simple experiments that can help determine whether the conclusions drawn here are valid for a particular sample or not. One should first try to obtain a ^{1}H MAS NMR spectrum at moderate spinning speed. If all sidebands are narrow and hardly get any narrower with increasing spinning speed, then most interactions are inhomogeneous and will be averaged out by MAS at several kHz. Most likely the residual $^{13}C-^{1}H$ dipolar couplings will then be small and easily removed by heteronuclear decoupling, but it will make the Hartmann–Hahn condition difficult to set up and maintain. In addition, if ^{1}H T_1 are long, then CP transfer will not be faster than NOE enhancement. Especially if ^{1}H lines overlap, one should then try to obtain a ^{13}C (or whatever X nucleus) MAS NMR spectrum without ^{1}H decoupling. Where the $^{13}C-^{1}H$ J couplings are visible, the INEPT transfer will be efficient accordingly. Another good indication that CP is not the ideal transfer method is the narrowing of the ^{13}C lines with relatively low power ^{1}H decoupling ($\omega_H < 50$ kHz). Finally, if different lines in the spectrum have very different relaxation properties, then NOE enhancement will probably give better transfer throughout the sample than cross polarization.

Finally, recent hardware developments allow MAS at spinning speeds of 30 to 50 kHz (*35–37*). At this speed, even in some rigid solids, the ^{1}H couplings become attenuated and a whole new avenue of experiments is going to be developed that will benefit from this phenomenon. Lipids, spinning at 5 kHz, will benefit from these new experiments, but they can also serve as a prototype sample for designing such experiments using a standard hardware.

In conclusion, it had already been shown, by us and others, that CP in lipids was not the ideal proton-to-carbon transfer method (*28, 29*). In terms of absolute and relative intensities, NOE enhancement of low abundant and insensitive carbon nuclei is undoubtedly the most efficient transfer method, although it had never been used in that context before. CP transfer is the less efficient one, although it is still the most commonly used transfer method. The NOE enhanced experiment is also the easiest of the three to implement: using a standard hardware configuration with possible use of a HR-MAS probe, no need of high-power or linear amplifiers, and very robust with respect to missettings or spectrometer instabilities.

For specific applications, it can be advantageous to chose another transfer experiment: INEPT or CP can be used for two-dimensional heteronuclear correlation. INEPT offers the possibility of inverse detection of carbon nuclei by protons. INEPT or CP can also provide selective excitation of some specific carbon nuclei: CP can help distinguish between mobile

vs immobile groups and INEPT can help distinguish between CH_3 vs CH_2 vs CH groups.

We have compared here several proton-to-carbon polarization transfer methods in lipid systems and we have shown that there are advantageous alternative methods to cross polarization. Although these experiments are simple and well known, the unusual behavior of lipids make the conclusions drawn unexpected. This comparison can be extended to biological membranes or soft matter systems where signal-to-noise ratio is a dramatic problem and where NOE enhancement would probably be the solution of choice. As stated previously, we believe that the conclusions outlined here will apply to polarization transfers from protons to other nuclei in a wide range of gel-like samples, semi-solids, or samples that are neither solid nor liquid, with medical, biological, chemical, and/or pharmaceutical interest.

ACKNOWLEDGMENTS

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