

Communication

# $^1\text{H}$ – $^{13}\text{C}$ polarization transfer in membranes: A tool for probing lipid dynamics and the effect of cholesterol

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## Abstract

Phospholipid bilayers with over 20% cholesterol can form a liquid-ordered ( $l_o$ ) phase, which can be found in lateral domains, called *rafts*, in biomembranes. We show here that high-resolution  $^{13}\text{C}$  and  $^1\text{H}$  solid-state NMR are well suited to explore this phase, intermediate between gel and fluid. This approach can be applied to artificial or natural membranes, with no isotopic enrichment and with the help of magic-angle spinning (MAS), taking advantage of the high resolution and sensitivity of these nuclei. The sensitivity of magnetization transfer schemes to different lipid states has allowed us here to discriminate between various phases. We show that the phase composed of *unsaturated* phospholipids and cholesterol differs, in terms of lipid dynamics, both from the previously described  $l_o$  phase and from the liquid-disordered phase.

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## 1. Introduction

The regulation of cholesterol content in cells is crucial for the control of membrane fluidity and other vital functions. Excess cholesterol, either from dietary ingestion or altered metabolism, is a major cause for atherosclerosis and cardiovascular disease [1]. On the other hand, specific domains of cell membranes, called *rafts*, have been found to be rich in cholesterol and necessary for some proteins to function properly [2]. We are interested in understanding how lipid structure and dynamics are modified in the presence of cholesterol. The phase formed by *saturated* phospholipids and cholesterol has been abundantly studied, in particular by deuterium NMR [3–6]. It is intermediate in many ways between the gel and the fluid phase of lipids and has been called the  $l_o$  phase, or “liquid-ordered” phase. Although biomembranes usually contain cholesterol and *unsaturated*

phospholipids, these phospholipids were less studied mostly because of isotopic labeling difficulties. At first, they were thought to resemble the  $l_o$  phase formed by their saturated counterpart [7,8]. Recently, measuring lipid diffusion by pulsed-gradient  $^1\text{H}$  NMR methods, Lindblom and co-workers [9] have observed a difference between saturated and unsaturated lipids in presence of cholesterol. They have inferred that unsaturated lipids and cholesterol formed a phase closer to the classical  $l_d$  (liquid disordered) phase. On the other hand, we have recently measured, using  $^1\text{H}$ – $^{13}\text{C}$  DROSS NMR experiments, a large increase in order parameters of unsaturated lipids when adding cholesterol to the  $l_d$  phase [10].

To explain these discrepancies, we decided to explore lipid *local* dynamics, rather than translational diffusion, using high-resolution  $^{13}\text{C}$  NMR under magic-angle spinning (MAS). In a previous article [11], we have compared the efficiency of various polarization transfer schemes in phospholipid membranes. Because lipid dynamics affect the homogeneous nature of  $^1\text{H}$ – $^1\text{H}$  dipolar interactions, the efficiency varies depending on the

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lipid phase. This pitfall can be used to probe lipid dynamics in those phases. Thereby, we have better characterized the mysterious phase formed by unsaturated phospholipids and cholesterol, which is probably close to the phase found in biomembranes. The advantage of using  $^{13}\text{C}$  NMR over  $^2\text{H}$  NMR is mostly the ability to study natural lipids with no isotopic labeling and with a very good spectral resolution. This resolution is also better than that of  $^1\text{H}$  MAS NMR and allowed us to observe local effects of cholesterol on phospholipid acyl chains, as shown below.

## 2. Results

### 2.1. Gel phase vs. fluid phase

High-resolution  $^{13}\text{C}$  MAS NMR spectra of lipid vesicles can be obtained at spinning rates as low as 3 kHz. This holds true with  $^1\text{H}$  MAS NMR *only* if lipids are in a *fluid* phase. It stems from the nature of  $^1\text{H}$ – $^1\text{H}$  dipolar interactions, generally homogeneous unless the spin pairs are all aligned [12]. This is the case for lipid protons in the fluid phase, mostly because of their rapid axial diffusion, as explained previously [13,14]. Similarly, different polarization transfer schemes from protons to carbons can be chosen depending on the lipid state. Although NOE transfers can be used independent of the phase, cross-polarization should be used in the gel phase whereas liquid-state NMR schemes, like INEPT, should be preferred for lipids in the fluid phase [11,15,16]. INEPT transfer has the advantage over cross-polarization to be independent of the CH bond orientation with respect to the magnetic field.

An illustration of the aforementioned phenomena is given in Fig. 1 with simple 1D MAS NMR spectra. At 37 °C, DPPC is in the gel phase and resonances from the frozen acyl chains and glycerol backbone have totally disappeared from the  $^1\text{H}$  spectrum (Fig. 1A) or the  $^{13}\text{C}$  refocused INEPT spectrum (Fig. 1C). By contrast, almost all resonances are visible when DPPC is in the fluid phase (Figs. 1B and D). With NOE enhanced  $^{13}\text{C}$  spectra, the difference between the gel and the fluid phase is less pronounced and is mostly due to differences in  $^{13}\text{C}$   $T_2$  relaxation. The differences in relative intensities and linewidths between refocused INEPT and NOE enhanced  $^{13}\text{C}$  spectra of lipids in the fluid phase (Figs. 1D and F) have been explained previously [11].

### 2.2. “ $l_o$ ” phases

Fig. 2 shows 1D MAS NMR spectra of several phospholipid:cholesterol (2:1) mixtures at 30 °C. Clearly, on each spectrum, more resonances are visible than on any corresponding spectrum of the bottom row of Fig. 1. This is a first indication that lipids with cholesterol are more mobile than in the gel phase. While NOE enhanced  $^{13}\text{C}$  spectra are almost unaffected by the presence of cholesterol (right column) some resonances have been dramatically reduced in the refocused INEPT  $^{13}\text{C}$  and  $^1\text{H}$  spectra (middle and left columns), showing a restricted mobility compared to a liquid-disordered phase. Quantitative comparison between various spectra in Fig. 2 is difficult to make because all measurements are not made at the same *reduced temperature*,  $T_r = (T - T_c)/T_c$ , [17], where  $T_c$  is the main transition temperature. On the other hand, according to the diagram of Vist and Davis [4],  $T_c$  becomes irrelevant in

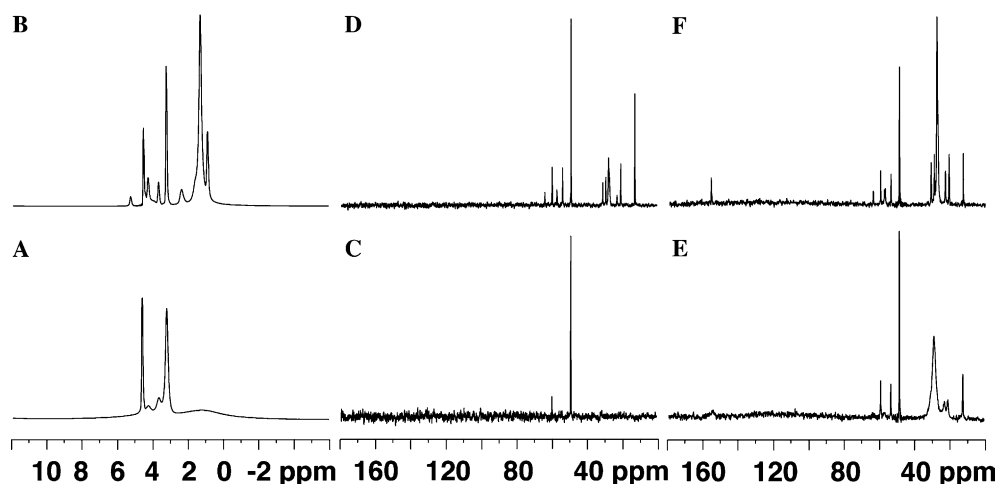


Fig. 1. One-dimensional MAS NMR spectra of DPPC (transition temperature is 41 °C). (A)  $^1\text{H}$  spectra at 37 °C; (B) at 50 °C; (C) refocused INEPT  $^{13}\text{C}$  spectra [15] at 37 °C; (D) at 50 °C; (E) NOE enhanced  $^{13}\text{C}$  spectra [11] at 37 °C; (F) at 50 °C. One thousand twenty-four scans were summed with a recycle delay of 5 s, a spectral width of 30 kHz and 4k complex points. Total acquisition time was 90 min. Prior to Fourier transformation, the data were zero filled to 8k and exponentially multiplied with 10 Hz line broadening. The internal reference was chosen to be the very intense lipid headgroup  $\gamma\text{-CH}_3$  resonance assigned to 54.5 ppm for  $^{13}\text{C}$  and 3.25 ppm for  $^1\text{H}$ , and the assignment of other resonances has been published elsewhere [10].

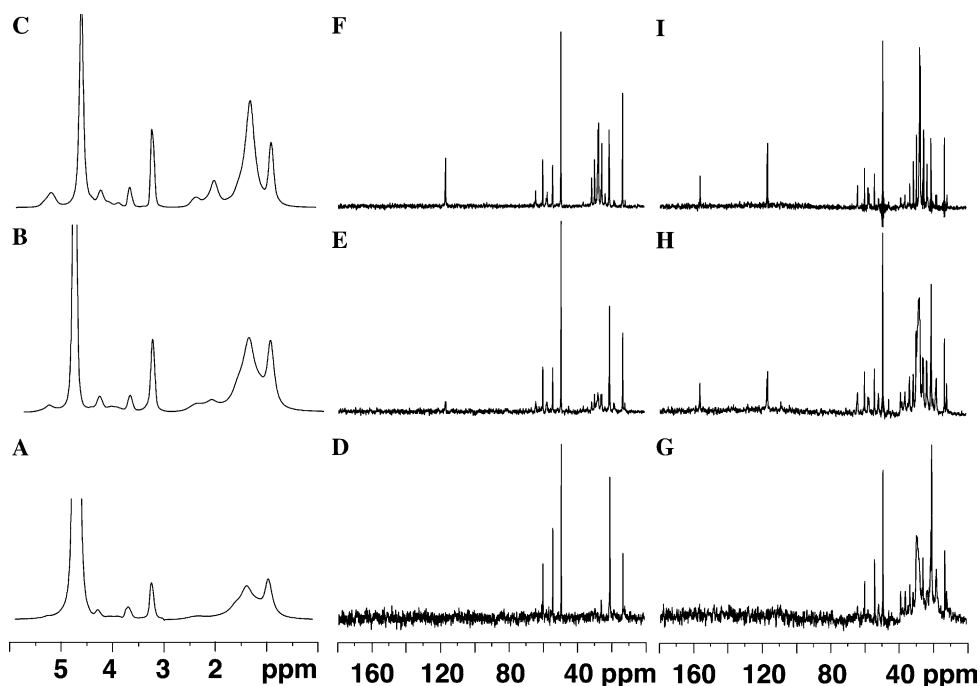


Fig. 2. One-dimensional MAS NMR spectra of phospholipid/cholesterol (67:33) mixtures at 30 °C:  $^1\text{H}$  spectra of DPPC:Chol (A), POPC:Chol (B), and DOPC:Chol (C); refocused INEPT  $^{13}\text{C}$  spectra of DPPC:Chol (D), POPC:Chol (E), and DOPC:Chol (F); NOE enhanced  $^{13}\text{C}$  spectra of DPPC:Chol (G), POPC:Chol (H), and DOPC:Chol (I).

presence of 33% cholesterol and we believe some general comments may still be drawn from Fig. 2. Furthermore, test experiments have been performed for several lipids, for at least one identical *reduced temperature* (50 °C for DPPC, 30 °C for DMPC, and 5 °C for POPC) and the spectra were qualitatively similar (data not shown).

Differences in Fig. 2 between DPPC (two saturated chains, transition temperature  $T_c = +41$  °C, bottom row), POPC (one saturated and one unsaturated chain,  $T_c = -3$  °C, middle row), and DOPC (two unsaturated chains,  $T_c = -18$  °C, upper row) are striking. One notices, for example, that INEPT transfer, compared to NOE as a reference, is deteriorated in presence of cholesterol, except in DOPC/cholesterol. The resolution in  $^{13}\text{C}$  NMR allows one to look more closely at specific carbon positions and Fig. 3 shows the intensity ratios of  $^{13}\text{C}$  INEPT to NOE acyl chain resonances. The bulk acyl methylenes resonance intensity, at 1.3 ppm on the  $^1\text{H}$  spectra and at 30.1 ppm on the  $^{13}\text{C}$  spectra, is reduced in presence of cholesterol and almost completely disappears in the case of the DPPC/cholesterol mixture. This effect of cholesterol is similar on carbons in the upper part of the lipid chains (below the carbonyl, C2 and C3 at 34.4 and 25.4 ppm, respectively). On the other hand, the  $^1\text{H}$  to  $^{13}\text{C}$  INEPT magnetization transfer is still efficient at both ends of the lipid in presence of cholesterol (the acyl chain terminal methyls ( $\omega$ ), the methylenes *next to* them ( $\omega - 1$ ) at 14.2 and 23.0 ppm, respectively, and the  $\alpha$ ,  $\beta$ , and  $\gamma$  headgroup resonances at 60.0, 66.5, and 54.5 ppm, respectively).

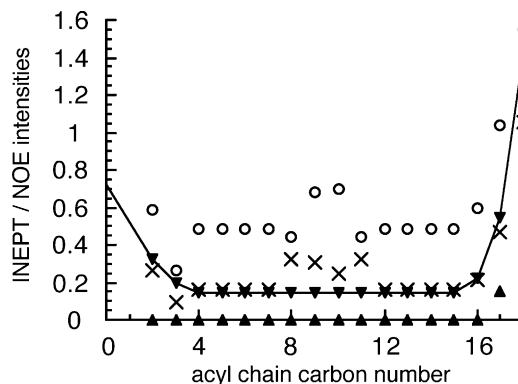


Fig. 3. Intensity ratios of  $^{13}\text{C}$  INEPT to NOE acyl chain resonances. The values are normalized so that the ratio is one for the intense lipid headgroup  $\gamma\text{-CH}_3$  resonance (54.5 ppm). DPPC ( $\blacktriangledown$ , solid line,  $l_d$  phase) at 45 °C; DPPC:Chol ( $\blacktriangle$ ,  $l_o$  phase), POPC:Chol ( $\times$ ), and DOPC:Chol ( $\circ$ ) at 30 °C. For convenience, whatever the acyl chains, the last position is assigned to carbon number 18, as in the oleoyl chains. In the oleoyl chains, the double bond is between carbons 9 and 10.

Since the effect of cholesterol on POPC is intermediate between the large effect on DPPC and the relatively small effect on DOPC, one may think that cholesterol interacts more with saturated chains, an hypothesis found in the literature [18]. Nevertheless, if one looks at the resonances around 130 ppm (the double bond), they are reduced with POPC as well, showing that both chains are affected in a comparable fashion, a result also found in the literature [19]. Similar results were obtained with DPPC and 14% cholesterol as well as with other

saturated phospholipids (DMPC or sphingomyelin) and various amounts of cholesterol (data not shown). We would like to point out that the intense peak at 22.7 ppm is a cholesterol resonance (C26 and C27 cholesterol terminal methyls) while the small peak at 23.0 ppm is a phospholipid resonance ( $\omega - 1$ ). We will now attempt to draw conclusions about phospholipid/cholesterol interactions and dynamics in the  $l_o$  phase in Section 3.

### 3. Discussion

#### 3.1. Cholesterol effect on the upper part of lipid chains

For reasons that have been explained before [13,14,20], our experimental setup does not permit us to observe many cholesterol resonances. Because we use a low spinning rate, low cholesterol content, and protonated lipids, almost only the methyl carbons of cholesterol were visible (C18, C19, C21, C26, and C27). Therefore, it is mostly the effect of cholesterol on the phospholipids that is probed here. From our results, we can predict in which cases INEPT is an appropriate transfer scheme in 2D NMR experiments like DROSS [10,21]. Unfortunately, NOE cannot be used as a coherent transfer scheme in such a 2D experiment. Consequently, in some samples containing cholesterol, only a few order parameters will be measurable with DROSS.

One-dimensional spectra of lipids with 33% cholesterol (Fig. 2) exhibit narrow intense lines, which are features of fluid samples, together with some broadened resonances, as in the gel phase. This shows that the phases formed are intermediate between  $l_d$  and  $s_o$ , even for  $^1\text{H}$  and  $^{13}\text{C}$  INEPT NMR. By looking more closely (Fig. 3), one may notice that cholesterol, directly or indirectly, affects primarily the heart of the bilayer, leaving almost untouched both ends of the lipids. This is consistent with previous observations in the literature that have helped to locate cholesterol in membranes. Seelig and co-workers [8] have shown a large effect of cholesterol on the acyl chains and almost no effect on the head-group. Similarly, Kothe and co-workers [5] have observed a larger increase of order parameter in the upper than in the lower part of the acyl chains. Molecular dynamics simulations of lipid/cholesterol membranes reveal a comparable order gradient [22].

#### 3.2. Lipid chain dynamics in presence of cholesterol

Fig. 2 tells us that cholesterol has affected the  $^1\text{H}$ – $^{13}\text{C}$  INEPT polarization transfer efficiency. This implies that cholesterol has transformed the  $^1\text{H}$ – $^1\text{H}$  dipolar interactions from inhomogeneous to homogeneous, in the sense defined by Maricq and Waugh [12]. This, in turn, allows us to make assumptions on lipid dynamics affected by

cholesterol, that would change the nature of such interactions. What motions in the fluid phase are affected by the addition of cholesterol? Since lipid axial diffusion does *not* seem to be affected [4], one would expect the INEPT transfer to be as efficient in both phases, and for all resonances. What other motion is different in both phases and could explain the different behavior under MAS? Lipid collective motions are slowed down by the addition of cholesterol [5], and they could interfere with averaging by MAS [23,24]. Nevertheless, such collective motions should affect lipids as a whole, and the INEPT transfer should be as inefficient in the lower part as in the upper part of acyl chains, which is not what is observed. Another explanation, not exclusive of the previous one, relies on differences in *trans*–*gauche* isomerizations. They are very rapid in the fluid phase while they are reduced in the gel phase to the point that lipids are in an all-*trans* configuration [25,26]. The liquid-ordered phase is an intermediate case where lipids *tend* to adopt an all-*trans* conformation. We have shown previously that this is also true for mixtures of *unsaturated* lipids and cholesterol since order parameters are increased [10], an indication that *trans* conformations are favored. Kothe and co-workers [5] have reported in saturated lipids that this effect is more pronounced in the upper part of the acyl chains. It would explain why INEPT transfer is inefficient in the gel phase for all lipids and in the  $l_o$  phase for the upper part of saturated chains. Since this effect is to account for the inefficient INEPT transfer, then *trans*–*gauche* isomerizations are proven to be essential for  $^1\text{H}$ – $^1\text{H}$  interactions to be effectively inhomogeneous, and *not* axial diffusion alone. The inhomogeneous-to-homogeneous transition of the  $^1\text{H}$ – $^1\text{H}$  dipolar interactions induced by cholesterol may be the source of other unexplained phenomena such as  $T_{1\rho}$  variation in lipid bilayers [27].

In unsaturated lipids, the *cis*-double bond is fixed but the remaining bonds are thereby subject to motion with a larger amplitude, especially *below* the double bond [28]. This well-known phenomenon is at the origin of the reduction in order parameters and of the lower fluid-to-gel transition temperature. Addition of cholesterol “rigidifies” the chains, i.e., there are more *trans* configurations or/and more slow motions as explained before. Our results show that cholesterol rigidifies unsaturated lipid acyl chains to a lesser extent than saturated chains, again probably because of the *cis*-double bond. This, in turn, explains why, in presence of cholesterol, INEPT is more efficient with unsaturated than with saturated lipids.

#### 3.3. Differences between “ $l_o$ ” phases

First, let us point out that at the high *relative temperature* of our study, 55 °C above the main transition tem-

perature of DOPC, the lipid mixture is above the “bell shaped” region of the phase diagram, where two fluid phases ( $l_d$  and  $l_o$ ) would coexist [3–5,7,29]. In other words, a fair comparison with DPPC/cholesterol mixtures should be made at the same relative temperature, i.e., at around 90 °C for DPPC. Alternatively, this bell shaped region may *not* exist, as in *most* phase diagrams of lipid mixtures, because acyl chains can generally distort and mutually accommodate each other [30]. Consequently, while there can still be a phase change when adding cholesterol to DOPC at 37 °C, such a change is expected to be smoother than with DPPC at the same temperature.

Indeed, adding 33% of cholesterol to DOPC had almost no effect on the INEPT transfer efficiency while the same quantity of cholesterol added to DPPC almost totally inhibited the magnetization transfer. In this respect, our results are consistent with those of Lindblom and co-workers [9] who do not observe a major change of diffusion coefficient when adding cholesterol to DOPC, whereas they do see it with saturated lipids. Nevertheless, we do not think this is sufficient evidence to exclude that there *is* a phase transition, from  $l_d$  to another phase, when adding cholesterol to DOPC in water, as shown by the variation of *order* parameters. We have shown using DROSS NMR that although DOPC remained fluid, cholesterol more than doubled the order parameter of DOPC acyl chains [10]. The phase formed by unsaturated lipids and cholesterol is therefore *a* liquid-ordered phase but it differs from that formed by DPPC/cholesterol. Since biomembranes contain lipids with various headgroups and acyl chains that are mostly unsaturated, this phase, that we can name the  $l'_o$  phase, may be closer to physiological conditions than the *traditional*  $l_o$  phase determined with disaturated phosphocholines. To confirm the existence of a, so far putative,  $l'_o$  phase, one should build a complete phase diagram (as done in [7], for example) using NMR and calorimetry. These experiments fall beyond the scope of this communication.

#### 4. Conclusion

We have shown that magic-angle spinning is a technique ideally suited for studying lipids by  $^1\text{H}$  and  $^{13}\text{C}$  NMR. In this article, we have tackled the complex problem of lipid/cholesterol interaction and lipid dynamics in the  $l_o$  and analogous phases. Fast *trans-gauche* isomerizations appear to be necessary, in addition to axial diffusion, for  $^1\text{H}$ – $^1\text{H}$  interactions to be effectively inhomogeneous in lipids. NMR and the understanding of spin dynamics have helped us confirm that these isomerizations were reduced when adding cholesterol. Cholesterol rigidifies the membrane, but not as a switch. It appears to induce an average *increase* in the *trans* con-

formation population, and this increase occurs to different extent depending on the lipid acyl chains and also on the position along the chain.

The phase formed by DOPC and cholesterol shares with the *traditional*  $l_o$  phase the property of having much higher order parameters than in the liquid-disordered phase [10]. It differs from it by showing lateral diffusion coefficients [9] and  $^{13}\text{C}$  refocused INEPT spectra (this study) similar to those of the liquid-*disordered* phase. We have argued that this phase should be given a different name, the  $l'_o$  phase. Although we have characterized its properties, we have not attempted to accurately locate it on the DOPC/cholesterol phase diagram. Since this phase would *not* be detected by lipid lateral diffusion measurements, a combination of 1D  $^{13}\text{C}$  refocused INEPT and 2D  $^1\text{H}$ – $^{13}\text{C}$  DROSS experiments should be performed, at various temperatures, cholesterol and water concentrations. These experiments are under way.

#### 5. Experimental

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was purchased from Sigma Chemical (Saint Quentin Fallavier, France). Sample preparation was published elsewhere [10].

All NMR experiments were performed and processed on a Bruker AVANCE DMX400-WB NMR spectrometer ( $^1\text{H}$  resonance at 400 MHz,  $^{13}\text{C}$  resonance at 100 MHz) using a Bruker 4 mm MAS probe with the spinning frequency set at 5 kHz, controlled to within 5 Hz. No significant differences were noticed for phospholipid resonances when experiments were repeated at 10 kHz spinning frequency (data not shown). Typical pulse lengths were:  $^{13}\text{C}$  (90°) = 4  $\mu\text{s}$ ,  $^1\text{H}$  (90°) = 5  $\mu\text{s}$ , and  $^1\text{H}$  TPPM decoupling at 30 kHz.

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