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ARTICLE

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Order parameters of unsaturated phospholipids in membranes and the effect of cholesterol: a ^{1}H–^{13}C solid-state NMR study at natural abundance

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Abstract Most biological phospholipids contain at least one unsaturated alkyl chain. However, few order parameters of unsaturated lipids have been determined because of the difficulty associated with isotopic labeling of a double bond. Dipolar recoupling on axis with scaling and shape preservation (DROSS) is a solid-state nuclear magnetic resonance technique optimized for measuring ^{1}H–^{13}C dipolar couplings and order parameters in lipid membranes in the fluid phase. It has been used to determine the order profile of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine hydrated membranes. Here, we show an application for the measurement of local order parameters in multilamellar vesicles containing unsaturated lipids. Taking advantage of the very good ^{13}C chemical shift dispersion, one can easily follow the segmental order along the acyl chains and, particularly, around the double bonds where we have been able to determine the previously misassigned order parameters of each acyl chain of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). We have followed the variation of such order profiles with temperature, unsaturation content and cholesterol addition. We have found that the phase formed by DOPC with 30% cholesterol is analogous to the liquid-ordered (l_o) phase. Because these experiments do not require isotopic enrichment, this technique can, in principle, be applied to natural lipids and biomembranes.

Keywords Unsaturated lipids · Phospholipid cholesterol interactions · Liquid-ordered phase · Order parameters · Dipolar recoupling on axis with scaling and shape preservation

Abbreviations DOPC: 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine · DPPC: 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine · DROSS: Dipolar recoupling on axis with scaling and shape preservation · INEPT: Insensitive nuclei enhanced by polarization transfer · l_o: Liquid-ordered · MAS: Magic-angle spinning · POPC: 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine · TPPM: Two-pulse phase modulation

Introduction

It has been known for over 30 years that, in biomembranes, the *sn*-2 chain of phospholipids is predominantly unsaturated (Wood and Harlow 1969). Although the effects of such lipids on membrane properties are crucial (Binder and Gawrisch 2001), many studies have been performed on membranes made of saturated lipids. For example, order parameters of CH bonds in lipid membranes have often been determined through ^{2}H NMR experiments (Oldfield et al. 1971; Seelig 1977; Davis 1983). A considerable amount of information regarding lipid phases, the influence of temperature, lipid mixtures, cholesterol and lipid/protein interactions has been gathered. The application of ^{2}H NMR to unsaturated lipids has been retarded owing to synthesis difficulties of specifically ^{2}H-labeled unsaturated acyl chains. Consequently there have been very few such studies,with or without cholesterol (Seelig and Waespe-Sarcevic 1978; Yeagle 1985; Baenziger et al. 1991; Habiger et al. 1992; Thewalt and Bloom 1992; van Duyl et al. 2003). The prohibitively low natural abundance of ^{2}H (0.01%) makes it mandatory to use isotopically enriched lipids and proscribes the direct study of natural biological membranes. Notable exceptions of fully ^{2}H-labeled natural lipids have been studied with *Escherichia coli* (Gally et al. 1979) or *Acholeplasma laidlawii* lipids (Rance et al.

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1980). In addition, for each new lipid, deuteration must be performed at each position separately and many syntheses are necessary to get a full order profile from ^{2}H spectra. An alternative technique that can probe naturally abundant lipids was introduced in 1997. Dipolar recoupling on axis with scaling and shape preservation (DROSS) is one of many NMR techniques devised for measuring dipolar couplings in solids (Gross et al. 1997). Under magic-angle spinning (MAS), this technique allows proton magnetization to evolve solely under the recoupled ^{1}H–^{13}C dipolar couplings, then transfers the magnetization from protons to carbons and detects it. It produces a 2D NMR spectrum that correlates, in the first dimension, a high-resolution ^{13}C NMR spectrum of the molecule with, in the second dimension, wide doublets that reflect the ^{1}H–^{13}C residual dipolar couplings sensed by the molecule at each corresponding carbon. Such couplings are straightforward to analyze and provide segmental *order* parameters, rather than *structural* parameters, similar to those obtained with ^{2}H NMR. This result is very encouraging since, although the natural abundance of ^{13}C is low (1.1% of all carbon isotopes), a 2D spectrum can be obtained overnight using a conventional MAS NMR probe, without the need to isotopically enrich the sample. Furthermore, it provides all couplings in a single 2D spectrum. DROSS has not been used very often (Gawrisch et al. 2002) and yet it is an ideal technique to study natural lipids, including unsaturated lipids. Rather than analyzing order parameters in terms of molecular structure, we chose to use them as local probes and to observe their variations with external conditions. In that respect, order parameter variation *at* and *right next to* the double bond provides a good picture of the heart of the biomembrane. We made DROSS measurements on 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) but they can be extended to lipids having more unsaturations, including natural lipids from plant or animal membrane cells.

Materials and methods

POPC and DOPC were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol was purchased from Sigma Chemical (Saint Quentin Fallavier, France). All lipids were used without further purification. Lipid mixtures were dissolved in chloroform/methanol (3/1) and dried at room temperature with a rotary evaporator, three consecutive times. Residual organic solvent was removed by pumping overnight with a mechanical vacuum pump. Samples were then hydrated by adding 50% in weight of buffer in H_2O [100 mM tris(hydroxymethyl)aminomethane, 100 mM NaCl, 2 mM EDTA; pH 7]. Samples with a single lipid were hydrated with 50% in weight of plain D_2O although no difference was found whether buffer or D_2O was added to the lipid powder. The lipid dispersions were vortexed and freeze-thawed a couple of times before approximately 100 mg of sample was transferred to the 4-mm ZrO_2 NMR rotor.

All NMR experiments were performed and processed with a Bruker AVANCE DMX400-WB NMR spectrometer (^{1}H resonance at 400 MHz, ^{13}C resonance at 100 MHz) using a Bruker 4-mm MAS probe with the spinning rate set at 5 kHz, controlled to within 5 Hz. The DROSS pulse sequence (Gross et al. 1997) with a scaling factor $\chi = 0.393$ and $\epsilon = 0$ was used with carefully set pulse lengths and refocused insensitive nuclei enhanced by polarization transfer (INEPT) (Gross et al. 1995) with delays set to $1/8J$ and $1/4J$ and a J value of 140 Hz, a good compromise both for sp^2 and sp^3 carbons. Typical pulse lengths were ^{13}C (90°) = 4 μs, ^{1}H (90°) = 5 μs and ^{1}H two-pulse phase-modulation (TPPM) decoupling at 30 kHz with a phase modulation angle of 50°. For each of the 64 free induction decays acquired, 512 scans were summed with a recycle delay of 3 s, a spectral width of 16 kHz and 4,000 complex points. The total acquisition time was 14 h. Prior to 2D Fourier transformation, the data were zero-filled to 8,000×256 points, exponentially multiplied with 10-Hz line broadening in each dimension and treated with automatic baseline correction.

The absolute value of the *segmental* order parameter, $|S_{CH}|$, can be extracted several ways. The most accurate one was described in the original paper (Gross et al. 1997) and consists in performing numerical lineshape simulations of the aforementioned experiment. By design, the recoupled lineshape is similar to the static one, scaled down by the χ factor, but undistorted. In that case, the order parameter can also be extracted, as in ^{2}H NMR, by measuring the dipolar splitting directly on the spectra, dividing it by χ and then by 20.2 kHz for a methylene carbon or 20.8 kHz for a vinylic carbon (Seelig 1977). The error on the measurement is $\pm 2\times10^{-3}$, roughly corresponding to the size of the symbols in the figures. The two methods gave identical results in the case of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine. If the splitting corresponds to a coupling between the carbon and its geminal proton then the distance is known and the coupling depends only on the bond orientation and dynamics. For example, for methylene carbons away from the double bond, a *molecular* order parameter, S_{mol}, can easily be deduced, as $S_{mol} = -2S_{CH}$ (Seelig and Niederberger 1974). For other carbons, the calculation is more complex (Seelig and Waespe-Sarcevic 1978) as we will see later.

Results and discussion

Lipid order profiles for unsaturated lipids

2D ^{1} H–^{13}C DROSS spectra were obtained for all lipid samples at several temperatures and 1D slices were extracted from the 2D spectra at each ^{13}C resonance. Assignment of the ^{13}C resonances followed that of previously published data from solution NMR in organic solvents and from solid-state NMR in hydrated

lipids (Batchelor et al. 1974; Husted et al. 1993; Hong et al. 1995a). The internal reference was chosen to be the very intense lipid headgroup γ-$^{13}CH_3$ resonance assigned to 54.5 ppm. For unsaturated lipids, several specific ^{13}C resonances provide more information in the heart of the bilayer. In POPC and DOPC, the two vinylic carbons involved in each double bond resonate at 130.0 and 129.8 ppm, assigned to C9 and C10, respectively. This assignment will be confirmed by our own measurements. The resonance at 27.6 ppm corresponds to methylene carbons right next to the vinylic carbons, i.e., C8 and C11 from each unsaturated chain overlapped. Rather than showing the full 2D ^{1}H–^{13}C DROSS spectra, Fig. 1a focuses on the double-bond region of POPC, DOPC and DOPC/cholesterol 2/1 at 37°C. Figure 1b shows several dipolar slices extracted from such spectra. In the absence of cholesterol, the dipolar slices show either no splitting (129.8 ppm), one splitting (27.6 ppm) or two splittings (130.0 ppm, for DOPC). This point will be further discussed later. All remaining dipolar slices are shown in the supplementary material (Fig. S1).

^{1}H–^{13}C dipolar couplings were deduced from the measured splittings and the absolute value, $|S_{CH}|$, of the segmental order parameters was calculated from these values. Signs could be obtained from additional S-DROSS experiments (Gross et al. 1997). Figure 2 shows the lipid order profiles obtained at 37°C, compared with the most complete set of data obtained from ^{2}H NMR, i.e., 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) at approximately the same temperature (Gally et al. 1975, 1981; Douliez et al. 1995). Resonances

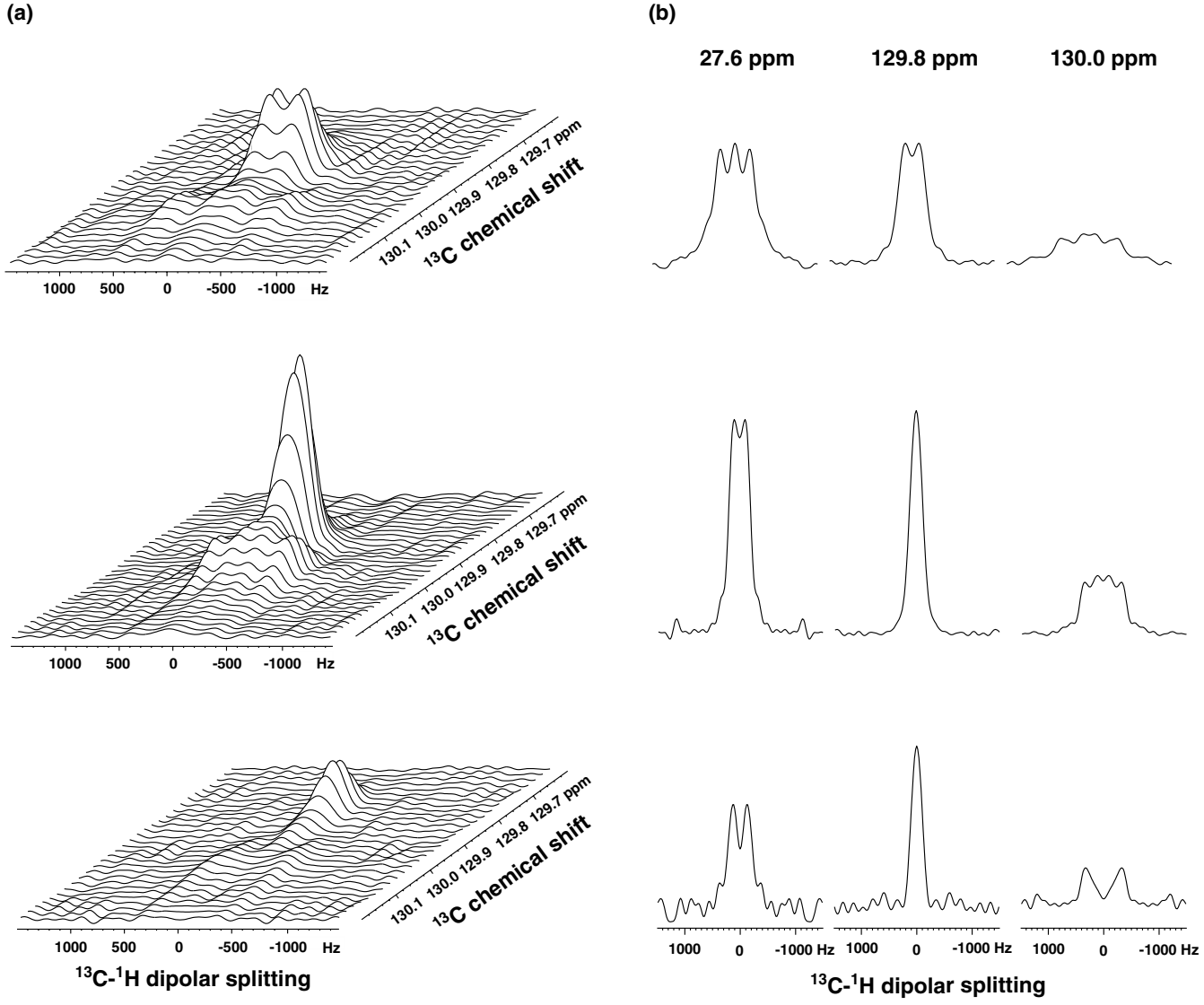

Fig. 1 **a** 2D ^{1}H–^{13}C dipolar recoupling on axis with scaling and shape preservation *(DROSS)* spectra of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (*POPC*) (*bottom row*), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (*DOPC*) (*middle row*) and DOPC/cholesterol 2/1 (*top row*) at 37°C. ^{13}C chemical shift region between 129.6 and 130.2 ppm. **b** ^{1}H–^{13}C dipolar slices extracted at the indicated ^{13}C chemical shift (methylenes C8 and C11 overlapped at 27.6 ppm, vinylic C9 and C10 at 130.0 and 129.8 ppm, respectively)

in the glycerol backbone and the upper part of the acyl chains (C2 and C3 methylenes) often present several large splittings at each position but only the most intense one is reported here, when it is measurable. For example, at the C2 position, both ^{2}H NMR and DROSS detect three splittings (one for the *sn*-1 chain and two for the two inequivalent protons of the *sn*-2 chain). It has been called the "spectral fingerprint" of phospholipids, and it is almost independent of the physical state or chemical composition (Gally et al. 1979). Similarly, order parameters in the lipid headgroup are, at a constant ionic strength, relatively independent of the sample phase. The main differences in order profiles of one lipid to another are located in the acyl chains. For convenience, whatever the acyl chains, the last position (ω) is assigned to C18, as in the oleoyl chains. Several carbon methylene resonances overlap at around 30.1 ppm, namely C4–C13 in palmitoyl chains and C4–C7 and C12–C15 in oleoyl chains. The measured dipolar coupling is a superposition of many couplings and therefore is dominated by the narrower (more intense) one, corresponding to the methylenes closest to the terminal methyls (ω-3). On the other hand, C4–C7 carbons correspond to the well-known plateau (Seelig 1977) where order parameters also overlap in ^{2}H NMR. Similarly C8 and C11 ^{13}C resonances of oleoyl chains overlap but only one splitting is observed at this chemical shift (see Fig. 1 at 27.6 ppm). For reasons that will be explained later, we believe the order parameter deduced should be assigned to the C11 position only.

DROSS NMR vs ^{2}H NMR

^{1}H–^{13}C DROSS is inherently more sensitive than ^{2}H-NMR in terms of signal-to-noise ratio because the gyromagnetic ratio of ^{13}C is almost twice that of ^{2}H. The sensitivity of ^{1}H–^{13}C DROSS is sufficient to investigate natural abundance membranes, which is unconceivable with ^{2}H NMR. The possibility of uniformly labeling lipids with ^{13}C would, of course, allow the acquisition of similar data with less material, including minor components in a complicated mixture (Gross et al. 1995). On the other hand, the *precision* in the absolute value of the order parameter obtained by ^{2}H NMR is higher than that obtained by DROSS because ^{1}H–^{13}C dipolar couplings are about an order of magnitude smaller than ^{2}H quadrupolar couplings. This is particularly crucial when the coupling to be measured is small, as in the headgroup. ^{1}H–^{13}C DROSS is currently not adapted to resolve couplings below about 150 Hz, corresponding to segmental order parameters around 0.02. If high precision is necessary and if labeling is possible, ^{2}H NMR may still be a better approach. Nevertheless, because of problems associated with chemical deuteration, ^{2}H NMR has provided complete order profiles for only a few lipids. For example, to determine the order profiles in Fig. 2, ^{2}H NMR data were obtained from many experiments on many different samples incorporating ^{2}H labels, whereas DROSS NMR data were obtained from a single experiment for each lipid and with no isotopic labeling. In addition, assignments of C9–^{2}H and C10–^{2}H in ^{2}H NMR are very difficult since it is almost impossible to label solely one of those two vinylic protons. On the other hand, the splittings of C8–^{2}H and C11–^{2}H can be assigned unambiguously for different samples. The situation is reversed in ^{13}C NMR, where ^{13}C9 and ^{13}C10 are resolved, whereas ^{13}C8 and ^{13}C11 are overlapped.

Finally, ^{2}H NMR-derived order parameters are systematically a little larger than those determined by DROSS (Gross et al. 1997). Several factors can explain this discrepancy: First and foremost, Baenziger et al.

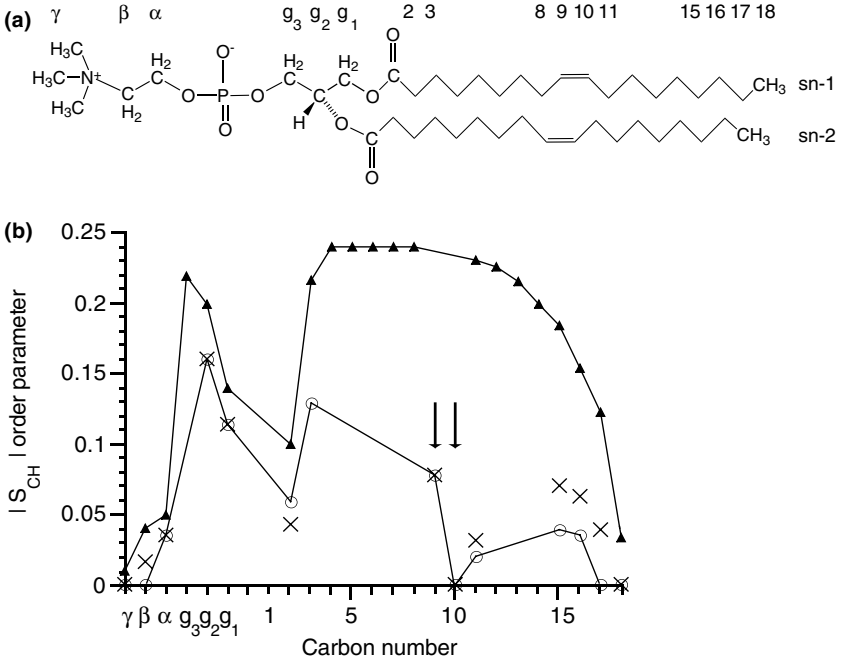

Fig. 2 **a** Schematic representation of DOPC. **b** Lipid order profile for DOPC (*squares*) and POPC (*crosses*) at 37°C, as extracted from DROSS NMR, and for 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (*triangles*), as extracted from published ^{2}H NMR data (Gally et al. 1975, 1981; Douliez et al. 1995). When several splittings are observed at one carbon position and for sake of clarity, only the most intense coupling is reported but the other splittings are discussed in the text. The double bond is at positions 9 and 10, indicated by the *arrows* in **b**

(1991) observed a large ^{1}H–^{2}H coupling affecting the ^{2}H lineshape when ^{1}H was not decoupled. Most reported ^{2}H NMR experiments were done without proton decoupling (Seelig and Waespe-Sarcevic 1978; Rance et al. 1980; van Duyl et al. 2003), which causes an overestimation of the quadrupolar couplings. In our case, protons are decoupled. Second, Rance et al. (1980) observed a large ^{2}H T_2 anisotropy that renders ^{2}H spectra dependent on the quadrupolar echo settings. Third, ^{1}H–^{13}C NMR and ^{2}H NMR have slightly different timescales: some motion could be considered slow for ^{2}H NMR and fast for ^{1}H–^{13}C NMR, which could also explain the observed differences.

Segmental vs molecular order parameter

Order parameters measured by NMR depend on molecular structure and dynamics. Assuming that all CH bonds attached to saturated chains have the same average orientation with respect to the bilayer normal, one can deduce a relation between the saturated *segmental* chain order profile measured by NMR and some sort of lipid flexibility profile called the *molecular* order profile (Seelig and Niederberger 1974). Since vinylic CH bonds have a *different* average orientation; the order profiles of unsaturated chains determined by NMR reflect a combination of flexibility and structure effects and their analysis is not as straightforward. To determine the molecular order parameter of the double bond, an order matrix with three independent elements is required (Dufourc et al. 1983; Baenziger et al. 1991). In the case of POPC or DOPC, only two segmental order parameters are available and an accurate determination of the molecular order is, in principle, impossible. Nevertheless, it was assumed that the distinct dip observed in the segmental order profile of POPC was compatible with a structural rather than with a dynamic effect. To prove it, in 1978, Seelig established the molecular order profile of POPC by using a third local order parameter obtained from IR dichroism experiments on egg lecithin at room temperature (Seelig and Waespe-Sarcevic 1978). Indeed, a slight tilt angle of the double bond was enough to explain the dip, without assuming a very different molecular order parameter. This has helped prove that the molecular order profile is smooth and is almost the same in a saturated chain and an unsaturated chain at the same *reduced* temperature, $T_r = (T - T_c)/T_c$, where T_c is the main transition temperature (Seelig and Browning 1978).

Although qualitatively the approach of Seelig and Waespe-Sarcevic is satisfactory, it should be used with caution if one wants to determine a precise molecular order parameter value (J. Seelig, personal communication). Values obtained by IR spectroscopy depend greatly on sample, temperature, hydration and cholesterol content (Binder 2003) and they are rarely obtained in the same conditions as in NMR. For example, no IR data are available for DOPC or, for that matter, for any lipid with a double bond on the *sn*-1 chain. In addition, because the values measured by IR spectroscopy are much higher than those measured by NMR, the matrix diagonalization is slightly biased: the resulting molecular order parameter will necessarily be close to the (uncertain) IR one and the resulting tilt angle will necessarily be small. For example, if we take the range of values reported by IR spectroscopy (between 0.20 and 0.40), we typically find, for the double bond, molecular order parameters of 0.30 ± 0.10 and tilt angles of $7 \pm 5°$. In the case of POPC, an additional uncertainty affects the *sign* of the segmental order at position C10, which Seelig assumes to be positive, while Hong measures it to be negative (Hong et al. 1995a). Finally, the uncertainty on the molecular order parameters determined by this method also depends on the angles that the CH (or CD) bonds make with the bilayer normal. When this angle is close to the magic angle, the molecular order parameter is deduced with very high uncertainty. In POPC and DOPC, this is the case for the molecular order parameter at position C11.

Consequently, if we follow the same approach using DROSS NMR results, we find approximately the same values as Seelig for POPC, with a slightly smaller tilt angle of the double bond ($5 \pm 3°$ instead of 7°). Transposing this approach to DOPC, assuming that the vinyl order parameter (0.36) measured by IR spectroscopy of egg lecithin is appropriate, we obtain similar molecular order parameters and tilt angles of $3 \pm 2°$ and $5 \pm 3°$, for the *sn*-1 and *sn*-2 chain double bonds, respectively. For a more likely smaller molecular order parameter (around 0.20 instead of 0.36), the tilt angles would increase to $5 \pm 3°$ and $9 \pm 4°$, respectively. Nevertheless, as shown before, these values are inherently highly uncertain. We feel that, in such cases, converting the accurate dipolar splittings or segmental order parameters into uncertain molecular order parameters is not justified. Furthermore, in the case of DPPC, POPC and DOPC, the large differences in the transition temperatures prevented us from performing experiments at the same reduced temperature. On the other hand, since segmental order profiles determined by NMR are instantaneous pictures of the membrane, we can exploit the *variation* of S_{CH}, indicating a qualitative change in flexibility and/or conformations along the chains.

Variation of S_{CH} order parameter with temperature

Although NMR (^{2}H or DROSS) can provide order parameters of lipids at nearly each CH bond, not all order parameters are as interesting in terms of sensitivity to changes in external parameters like sample composition, temperature and phase. In this article we want to develop a standard protocol to monitor the lipid state using DROSS and, for that matter, we focus on the lipid acyl chains. To probe the efficiency of the DROSS approach, we measured the well-known temperature dependence of lipid order parameters. Figure 3 shows

the lipid order profiles for POPC and DOPC chains at 23 and 37°C, as extracted from DROSS spectra. Similar results were obtained for saturated lipids, like DPPC, except only the last carbons provide data (not shown). The *second* splitting observed for DOPC at C9 is also reported, providing two order profiles, one with a solid line and one with a dotted line, that will be further discussed later.

After correcting for geometric factors introduced, for example, by a double bond, different lipids are thought to have the same order profile at the same *reduced* temperature (Seelig and Browning 1978). Indeed, our results demonstrate that, at the same temperature, order profiles shift down with more unsaturations (Fig. 2). For the same reason, DROSS-derived order profiles of unsaturated lipids collectively decrease when increasing the temperature (Fig. 3), consistent with data from the literature obtained with saturated lipids (Davis 1983) or with saturated chains of unsaturated lipids (Holte et al. 1995). A less expected result, although also observed by Seelig (Seelig and Waespe-Sarcevic 1978), is that the saturated chain of POPC, away from the glycerol backbone, has approximately the same molecular order as its unsaturated counterpart. We infer this from observing single splittings on DROSS spectra of POPC. Since DROSS spectra display a *sum* rather than an average of splittings, the intermediate values of the order parameters obtained for POPC therefore indicate that the order parameters of palmitoyl and oleoyl chains, from C11 to C18, are equal, within the precision of DROSS.

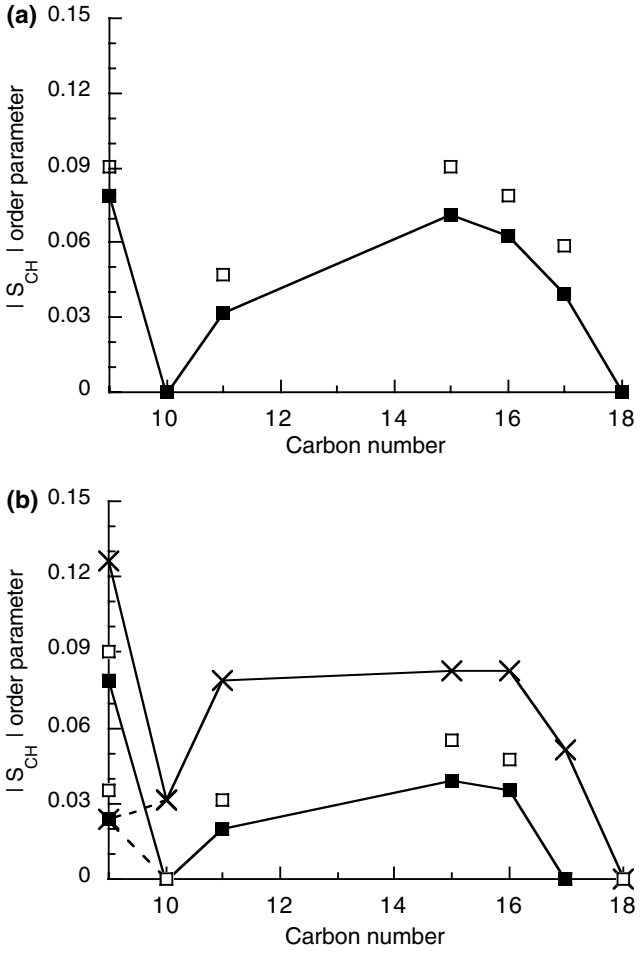

Fig. 3 Lipid chain order profile for POPC (**a**) and DOPC (**b**) at 23°C (*white squares*) and 37°C (*black squares*), and for DOPC/cholesterol 2/1 at 37°C (*crosses*), as extracted from DROSS NMR. For DOPC, with and without cholesterol, two splittings are observed at 130.0 ppm and are reported at C9. One is assigned to the *sn*-1 chain and the other one to the *sn*-2 chain. Following our assignment, the *solid line* corresponds to the *sn*-2 chain, while the *dashed line* is the order profile of the *sn*-1 chain (see text)

Variation of S_{CH} order parameter with cholesterol

After the pioneering work of Luzzati (1968), beside the usual fluid and gel phases, lipids are known to adopt various intermediate phases depending on their environment. For example, cholesterol increases the chain conformational order of saturated lipids in the fluid phase to the point where the order parameters measured by ^{2}H NMR are close to that of the gel phase. On the other hand, cholesterol does not seem to slow down the axial diffusion of the lipids, which remains similar to that of the fluid phase. Other parameters, like the bilayer thickness or compressibility, are intermediate between gel and fluid. This phase, called the liquid-ordered (l_o) phase, was described after computer simulations and ^{2}H NMR observations (Ipsen et al. 1987; Vist and Davis 1990; Almeida et al. 1992; Weisz et al. 1992). NMR spectra of lipids depend on the physical state of the observed molecules. For example, high-resolution ^{13}C NMR spectra of multilamellar vesicles can be obtained under MAS at relatively low spinning rates, but the same remark does *not* apply to ^{1}H NMR or DROSS NMR if lipids are in the gel phase. This property results from the particular molecular motions of lipids in the fluid phase, mostly their rapid axial diffusion, that render the ^{1}H spin dynamics effectively inhomogeneous, as explained previously (Oldfield et al. 1987; Forbes et al. 1988). We did not know, a priori, how DROSS NMR would behave with phospholipids in the presence of more than 20% cholesterol.

Depending on the lipid and the temperature, the effect of cholesterol was sometimes too dramatic to allow a careful interpretation via the 2D ^{1}H–^{13}C DROSS NMR experiment. In this article, only the interaction of cholesterol with DOPC is exploited quantitatively. For POPC or DPPC with cholesterol, only the order parameter at positions ω and ($\omega-1$) could be determined (not shown). The very few visible *cholesterol* resonances (C26 and C27 methyls at 22.7 ppm) corresponded to dipolar splittings equal to zero in all samples. Although more cholesterol resonances may have become visible if the sample had been spun at 15 kHz (Soubias et al. 2003), no significant differences were noticed for

phospholipid resonances when experiments were repeated at 10-kHz spinning frequency (not shown).

Figure 3b shows the lipid chain order profiles obtained for DOPC at 37°C, with and without cholesterol. Cholesterol expectedly increases the order of all carbons along the DOPC acyl chains except at ω (C18), which remains very small. With or without cholesterol, only *one* splitting is observed at each chemical shift, except at C9. This is compatible with the presence of *one* homogeneous population of DOPC interacting with cholesterol. DROSS is efficient in DOPC/cholesterol 2/1, showing that lipids are not in the gel phase, but order parameters are sometimes *tripled* when cholesterol is added, justifying the name ''ordered'' to qualify the phase formed. Whether this phase is identical or just analogous to the previously described l_o phase made of DPPC and cholesterol is a question that we will address elsewhere. We know that the measured segmental order parameters depend both on the molecular order, which is increased in the presence of cholesterol, and on the local order, which depends on specific bond conformations and which could *also* be affected by cholesterol addition. The global translation of the order profile away from the double bond, between C11 and C18 in Fig. 3b, is analogous, although much more pronounced, to the variation observed when reducing the temperature. It corresponds to a global rigidification of the chains which, as explained by Vist and Davis (1990), is an *increase* of *trans* conformation probability that occurs in the l_o phase as well as in the gel phase. The particular case of the double bond will be discussed later.

Order parameter next to the unsaturation

The ^1H–^{13}C dipolar slice measured with DROSS NMR at 27.6 ppm is comparable to that measured with a similar method, DISTINCT NMR (Hong et al. 1995a). It appears in Fig. 3a of Hong et al. (1995b) and in Fig. 4a of Hong and Schmidt-Rohr (1995), but it is not discussed any further. One may notice that dipolar splittings at this frequency can come from up to *four* CH pairs in POPC and *eight* CH pairs in DOPC. Nevertheless, in most cases only *one* splitting is observed (see, for example, Fig. 1b). This is *not* consistent with ^2H data (Seelig and Waespe-Sarcevic 1978; Rance et al. 1980) that predict a very large splitting for C8 that would be superimposed to a small splitting for C11.

How do we reconcile both results? ^2H NMR measurements at the C8 position may have suffered from lack of ^1H decoupling (Baenziger et al. 1991) and C8 and C11 may indeed have the same order parameter. Another explanation is that the splitting measured by DROSS NMR is dominated by the small dipolar coupling at C11, while the dipolar slice at C8 is too large to be visible. This hypothesis is reinforced by two other considerations: first, although DROSS NMR is not quantitative, the integral of the DROSS dipolar slice at 27.6 ppm is 2 times less than what would be expected if it included signals coming from both C8 and C11. Second, the value found is indeed much closer, although not equal, to the value determined by ^2H NMR for C11 rather than for C8. Further experiments will be needed to clarify this discrepancy but, in both cases, the deduced order parameter can safely be assigned to the C11 position in each figure. As with several other positions, the order parameter of C11 is tripled when 30% cholesterol is added (Fig. 3b). These observations are consistent with recent ^2H NMR data that, consequently, further confirm our peak assignment (van Duyl et al. 2003).

The double bond of POPC

The splitting observed at 129.8 ppm (C10) with ^1H–^{13}C DROSS is always zero, meaning that the order parameter is very small at this position. On the other hand, the splitting observed with C9 is unusually large (Fig. 1). These results are consistent with ^2H NMR results which have shown that these splittings are not due to fast or restricted motions but rather to particular orientations of the double bonds, both in POPC and DOPC (Seelig and Waespe-Sarcevic 1978; Gally et al. 1979; Hong et al. 1995a). This also confirms our assignment of C9 and C10 ^{13}C resonances.

The carbon at 130.0 ppm (C9) sometimes shows a second, very small, splitting. This central splitting was also observed by Hong et al. (1995a) for egg phosphocholine with DISTINCT NMR. They observed two splittings at 130.2 ppm and assigned them to C9 and C13. In their case, C13 is part of a *second* double bond. In our case there is only *one* double bond and we still see *two* splittings although there is only *one* proton connected to the vinylic carbon. We think that this very small coupling can be assigned to a long-range coupling between the vinylic C9 carbon and a nongeminal proton (Caldarelli et al. 1996; Gross et al. 1997), for example, held by the second vinylic carbon C10. Hong et al. may have neglected this phenomenon and the small splitting they observed may also result from a long-range coupling.

The double bond of DOPC

Both *major* splittings observed by DROSS NMR at the double bonds of DOPC are similar to those of POPC and to those determined by ^2H NMR. Our assignment is therefore consistent with the one of Seelig and coworkers. It confirms that our ^{13}C assignment of resonances around 130 ppm is correct and compatible with literature data from solution *and* solid-state NMR (Batchelor et al. 1974; Husted et al. 1993; Hong et al. 1995a).

Compared with POPC, the additional *minor* splitting observed in DOPC by ^2H NMR was tentatively assigned by Seelig and coworkers (Gally et al. 1979; Seelig et al. 1981) to the C10$-^2H$ in the *sn*-1 oleic chain. This is

inconsistent with our observation of a single line at 129.8 ppm (C10) with ^{1}H–^{13}C DROSS (Fig. 1). With DROSS NMR, this additional splitting occurs at 130.0 ppm (C9). The size of this second splitting makes it unlikely to result from a long-range coupling and we think it should be assigned to the C9 in the *sn*-1 oleic chain. DROSS NMR results become consistent with ^{2}H NMR if they are interpreted in the following way: the small splittings result from C10, the large splitting from C9-*sn*-2 and the intermediate one from C9-*sn*-1 rather than from C10-*sn*-1. This is summarized in Table 1. Following this assignment, we can deduce that the solid line in Fig. 3b is the order profile of the *sn*-1 chain, while the dotted line is that of the *sn*-2 chain.

Upon cholesterol addition, couplings at the vinylic carbons increase and, while our assignment differs from that of Habiger et al. (1992), the amount of increase is consistent with the one they observe (Table 1). The effect of adding cholesterol is different for each acyl chain (Fig. 3b). The *sn*-2 chain order profile (solid line) is simply translated, which is compatible with an expected rigidified chain. On the other hand, the change in the order profile of the *sn*-1 chain (dashed line) cannot solely be explained by a change in flexibility. Because of the original misassignment of ^{2}H splittings, this property had never been noticed before. As explained earlier, interpretation of this order profile change in terms of quantitative structural change would be possible only if we could obtain an accurate third-order parameter at the double-bond position. However, as an example, our data fit a model with double-bond molecular order parameters of 0.80 and tilt angles of $1 \pm 1°$ and $3 \pm 1°$, for the *sn*-1 and *sn*-2 chains, respectively.

Conclusion

^{1}H–^{13}C DROSS NMR is a relatively simple technique that allows one to probe lipid segmental order S_{CH} in a variety of samples. Because of additional measurements performed in the middle of the lipid acyl chains, unsaturated lipids provide a more complete picture of the bilayer than saturated lipids. Focusing on the lipid acyl chains, we think DROSS is a useful tool to monitor the lipid state. Compared with ^{2}H NMR, both approaches have their advantages and should be used appropriately. DROSS NMR has the advantage over ^{2}H NMR that it is applicable to natural lipids without any isotopic labeling. It combines, in principle, higher sensitivity *and* spectral resolution, which renders assignment more straightforward. It has allowed us to determine the order profile of unsaturated lipids and to correct a previous misassignment at the double bond (Seelig and Waespe-Sarcevic 1978). We have carefully analyzed our measurements in terms of segmental order parameters and we have pointed out the dangers of converting them into molecular order parameters without caution. We have shown that adding cholesterol to the sample has a much more dramatic effect on the order parameters than changing the temperature, or even adding a double bond to an acyl chain. For DOPC, we have also shown that adding 30% cholesterol resulted in a lipid state analogous to the l_o phase and that the change in order profile was different for each acyl chain.

Unfortunately, because of our choice to use unlabeled lipids, overlap can be a problem, especially in the case of mixtures where the major lipid can hide a minor component. Here, for example, it was almost impossible to measure *cholesterol* ^{1}H–^{13}C couplings. The observation of cholesterol resonances could be favored by higher spinning rates, deuterated lipids, ^{1}H detection (Soubias et al. 2003) and/or ^{13}C-labeled cholesterol (Urbina et al. 1998). For monitoring unsaturated lipid order parameters, an alternative method has been proposed: it consists in studying lipids composed of one saturated and one unsaturated chain, by following the ^{2}H order parameters of the deuterated *saturated* chain (Holte et al. 1995; Eldho et al. 2003). The study of these

Table 1 Comparison of $|S_{CH}|$ values obtained in phospholipid acyl chains with ^{2}H NMR and ^{1}H–^{13}C dipolar recoupling on axis with scaling and shape preservation NMR

Sample	NMR experiment	C9		C10		C11	$\omega-3$	$\omega-2$	$\omega-1$	ω
		sn-1	*sn*-2	*sn*-1	*sn*-2					
POPC	$^{2}H^{a}$		0.10		0.02	0.05	NDA	0.08	0.06	0.02
	^{1}H–$^{13}C^{b}$		0.08		0	0.03	0.07	0.06	0.04	0
DOPC	$^{2}H^{c}$	(0.10)	0.10	(0.05)	0.02	0.05	NDA	NDA	NDA	NDA
	^{1}H–$^{13}C^{b}$	0.02	0.09	0	0	0.03	0.05	0.04	0.03	0
DOPC/cholesterol 2/1	$^{2}H^{c}$	(0.14)	0.14	(0.06)	0.03	0.08	NDA	NDA	NDA	NDA
	^{1}H–$^{13}C^{b}$	0.04	0.13	0.03	0.03	0.08	0.06	0.04	0.04	0

All ^{2}H NMR data were obtained from lipids selectively deuterated on the oleate chains. Quadrupole splittings given in kilohertz were divided by 131 kHz (for C9 and C10) and by 127 kHz (for other carbons) to obtain the reported segmental order parameters. Values in *parentheses* are the ones we believe have been misassigned. *POPC* 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, *DOPC* 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, *NDA* no data available in the literature

[a] For C9–C11, data were obtained from POPC at 27°C (Seelig and Waespe-Sarcevic 1978). For other carbons, data were obtained from *Acholeplasma laidlawii* at 25°C (Rance et al. 1980)

[b] Data obtained from unlabeled lipids at 30°C (this work)

[c] For C9 and C10, data were obtained from DOPC at 32°C (Habiger et al. 1992). For C11, data were obtained from DOPC at 30°C (van Duyl et al. 2003)

lipids with DROSS NMR would provide a complementary experiment, each technique probing a different chain of the *same* lipid.

The particular interest in DROSS for the study of the order near a double bond can be extended to polyunsaturated lipids, which have recently triggered renewed interest (Eldho et al. 2003; Stillwell and Wassall 2003), or to any other chemical group exhibiting a well-resolved ^{13}C chemical shift, aromatic carbons, for example. The approach described here could be applied to a variety of other samples, and DROSS would be ideally suited to tackle the fascinating problems of lipid/detergent or lipid/protein interactions, for example. More complicated mixtures of several phospholipids and cholesterol in water can also be studied, so as to better mimic the behavior of biomembranes. These conclusions make DROSS an exciting tool to study the part played by lipids in biological phenomena and, in particular, in the formation of biological lipid domains called *rafts*, where phospholipids and cholesterol coexist (de Almeida et al. 2003). This will be our future goal.

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References

Almeida PF, Vaz WL, Thompson TE (1992) Lateral diffusion and percolation in two-phase, two-component lipid bilayers. Topology of the solid-phase domains in-plane and across the lipid bilayer. Biochemistry 31:7198–7210

de Almeida RF, Fedorov A, Prieto M (2003) Sphingomyelin/phosphatidylcholine/cholesterol phase diagram: boundaries and composition of lipid rafts. Biophys J 85:2406–2416

Baenziger JE, Jarrell HC, Hill RJ, Smith IC (1991) Average structural and motional properties of a diunsaturated acyl chain in a lipid bilayer: effects of two *cis*-unsaturated double bonds. Biochemistry 30:894–903

Batchelor JG, Cushley RJ, Prestegard JH (1974) Carbon-13 Fourier transform nuclear magnetic resonance. 8. Role of steric and electric field effects in fatty acid spectra. J Org Chem 39:1698–1705

Binder H (2003) The molecular architecture of lipid membranes–new insights from hydration-tuning infrared linear dichroism spectroscopy. Appl Spectrosc Rev 38:15–69

Binder H, Gawrisch K (2001) Effect of unsaturated lipid chains on dimensions, molecular order and hydration of membranes. J Phys Chem B 105:12378–12390

Caldarelli S, Lesage A, Emsley L (1996) Long-range dipolar couplings in liquid crystals measured by three-dimensional NMR spectroscopy. J Am Chem Soc 118:12224–12225

Davis JH (1983) The description of membrane lipid conformation, order and dynamics by ^{2}H-NMR. Biochim Biophys Acta 737:117–171

Douliez J-P, Leonard A, Dufourc EJ (1995) Restatement of order parameters in biomembranes: calculation of C–C bond order parameters from C–D quadrupolar splittings. Biophys J 68:1727–1739

Dufourc EJ, Smith IC, Jarrell HC (1983) A 2H-NMR analysis of dihydrosterculoyl-containing lipids in model membranes: structural effects of a cyclopropane ring. Chem Phys Lipids 33:153–177

van Duyl BY, Ganchev D, Chupin V, de Kruijff B, Killian JA (2003) Sphingomyelin is much more effective than saturated phosphatidylcholine in excluding unsaturated phosphatidylcholine from domains formed with cholesterol. FEBS Lett 547:101–106

Eldho NV, Feller SE, Tristram-Nagle S, Polozov IV, Gawrisch K (2003) Polyunsaturated docosahexaenoic vs docosapentaenoic acid-differences in lipid matrix properties from the loss of one double bond. J Am Chem Soc 125:6409–6421

Forbes J, Husted C, Oldfield E (1988) High-field, high-resolution proton "magic-angle" sample-spinning nuclear magnetic resonance spectroscopic studies of gel and liquid crystalline lipid bilayers and the effects of cholesterol. J Am Chem Soc 110:1059–1065

Gally HU, Niederberger W, Seelig J (1975) Conformation and motion of the choline head group in bilayers of dipalmitoyl-3-sn-phosphatidylcholine. Biochemistry 14:3647–3652

Gally HU, Pluschke G, Overath P, Seelig J (1979) Structure of *Escherichia coli* membranes. Phospholipid conformation in model membranes and cells as studied by deuterium magnetic resonance. Biochemistry 18:5605–5610

Gally HU, Pluschke G, Overath P, Seelig J (1981) Structure of *Escherichia coli* membranes. Glycerol auxotrophs as a tool for the analysis of the phospholipid head-group region by deuterium magentic resonance. Biochemistry 20:1826–1831

Gawrisch K, Eldho NV, Polozov IV (2002) Novel NMR tools to study structure and dynamics of biomembranes. Chem Phys Lipids 116:135–151

Gross JD, Costa PR, Dubacq JP, Warschawski DE, Lirsac PN, Devaux PF, Griffin RG (1995) Multidimensional NMR in lipid systems. Coherence transfer through J couplings under MAS. J Magn Reson B 106:187–190

Gross JD, Warschawski DE, Griffin RG (1997) Dipolar recoupling in MAS NMR: a probe for segmental order in lipid bilayers. J Am Chem Soc 119:796–802

Habiger RG, Cassal JM, Kempen HJ, Seelig J (1992) Influence of stigmastanol and stigmastanyl-phosphorylcholine, two plasma cholesterol lowering substances, on synthetic phospholipid membranes. A 2H- and 31P-NMR study. Biochim Biophys Acta 1103:69–76

Holte LL, Peter SA, Sinnwell TM, Gawrisch K (1995) 2H nuclear magnetic resonance order parameter profiles suggest a change of molecular shape for phosphatidylcholines containing a polyunsaturated acyl chain. Biophys J 68:2396–2403

Hong M, Schmidt-Rohr K (1995) DISTINCT NMR for sign determination of C–H dipolar couplings in liquid-crystalline lipids. J Magn Reson B 109:284–290

Hong M, Schmidt-Rohr K, Nanz D (1995a) Study of phospholipid structure by ^{1}H, ^{13}C and ^{31}P dipolar couplings from two-dimensional NMR. Biophys J 69:1939–1950

Hong M, Schmidt-Rohr K, Pines A (1995b) NMR measurement of signs and magnitudes of C–H dipolar couplings in lecithin. J Am Chem Soc 117:3310–3311

Husted C, Montez B, Le C, Moscarello MA, Oldfield E (1993) Carbon-13 "magic-angle" sample-spinning nuclear magnetic resonance studies of human myelin, and model membrane systems. Magn Reson Med 29:168–178

Ipsen JH, Karlstrom G, Mouritsen OG, Wennerstrom H, Zuckermann MJ (1987) Phase equilibria in the phosphatidylcholine–cholesterol system. Biochim Biophys Acta 905:162–172

Luzzati V (1968) X-ray diffraction studies of lipid-water systems. In: Chapman D (ed) Biological membranes, vol 1. Academic, London, pp 71–123

Oldfield E, Chapman D, Derbyshire W (1971) Deuteron resonance: a novel approach to the study of hydrocarbon chain mobility in membrane systems. FEBS Lett 16:102–104

Oldfield E, Bowers JL, Forbes J (1987) High-resolution proton and carbon-13 NMR of membranes: why sonicate? Biochemistry 26:6919–6923

Rance M, Jeffrey KR, Tulloch AP, Butler KW, Smith IC (1980) Orientational order of unsaturated lipids in the membranes of *Acholeplasma laidlawii* as observed by 2H-NMR. Biochim Biophys Acta 600:245–262

Seelig J (1977) Deuterium magnetic resonance: theory and application to lipid membranes. Q Rev Biophys 10:353–418

Seelig J, Browning JL (1978) General features of phospholipid conformation in membranes. FEBS Lett 92:41–44

Seelig J, Niederberger W (1974) Deuterium-labeled lipids as structural probes in liquid crystalline bilayers. Deuterium magnetic resonance study. J Am Chem Soc 96:2069–2072

Seelig J, Waespe-Sarcevic N (1978) Molecular order in *cis* and *trans* unsaturated phospholipid bilayers. Biochemistry 17:3310–3315

Seelig J, Tamm L, Hymel L, Fleischer S (1981) Deuterium and phosphorus nuclear magnetic resonance and fluorescence depolarization studies of functional reconstituted sarcoplasmic reticulum membrane vesicles. Biochemistry 20:3922–3932

Soubias O, Piotto M, Saurel O, Assemat O, Reat V, Milon A (2003) Detection of natural abundance 1H–13C correlations of cholesterol in its membrane environment using a gradient enhanced HSQC experiment under high resolution magic angle spinning. J Magn Reson 165:303–308

Stillwell W, Wassall SR (2003) Docosahexaenoic acid: membrane properties of a unique fatty acid. Chem Phys Lipids 126:1–27

Thewalt JL, Bloom M (1992) Phosphatidylcholine: cholesterol phase diagrams. Biophys J 63:1176–1181

Urbina JA, Moreno B, Arnold W, Taron CH, Orlean P, Oldfield E (1998) A carbon-13 nuclear magnetic resonance spectroscopic study of inter-proton pair order parameters: a new approach to study order and dynamics in phospholipid membrane systems. Biophys J 75:1372–1383

Vist MR, Davis JH (1990) Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures: 2H nuclear magnetic resonance and differential scanning calorimetry. Biochemistry 29:451–464

Weisz K, Gröbner G, Mayer C, Stohrer J, Kothe G (1992) Deuteron nuclear magnetic resonance study of the dynamic organization of phospholipid/cholesterol bilayer membranes: molecular properties and viscoelastic behavior. Biochemistry 31:1100–1112

Wood R, Harlow RD (1969) Structural analyses of rat liver phosphoglycerides. Arch Biochem Biophys 135:272–281

Yeagle PL (1985) Lanosterol and cholesterol have different effects on phospholipid acyl chain ordering. Biochim Biophys Acta 815:33–36

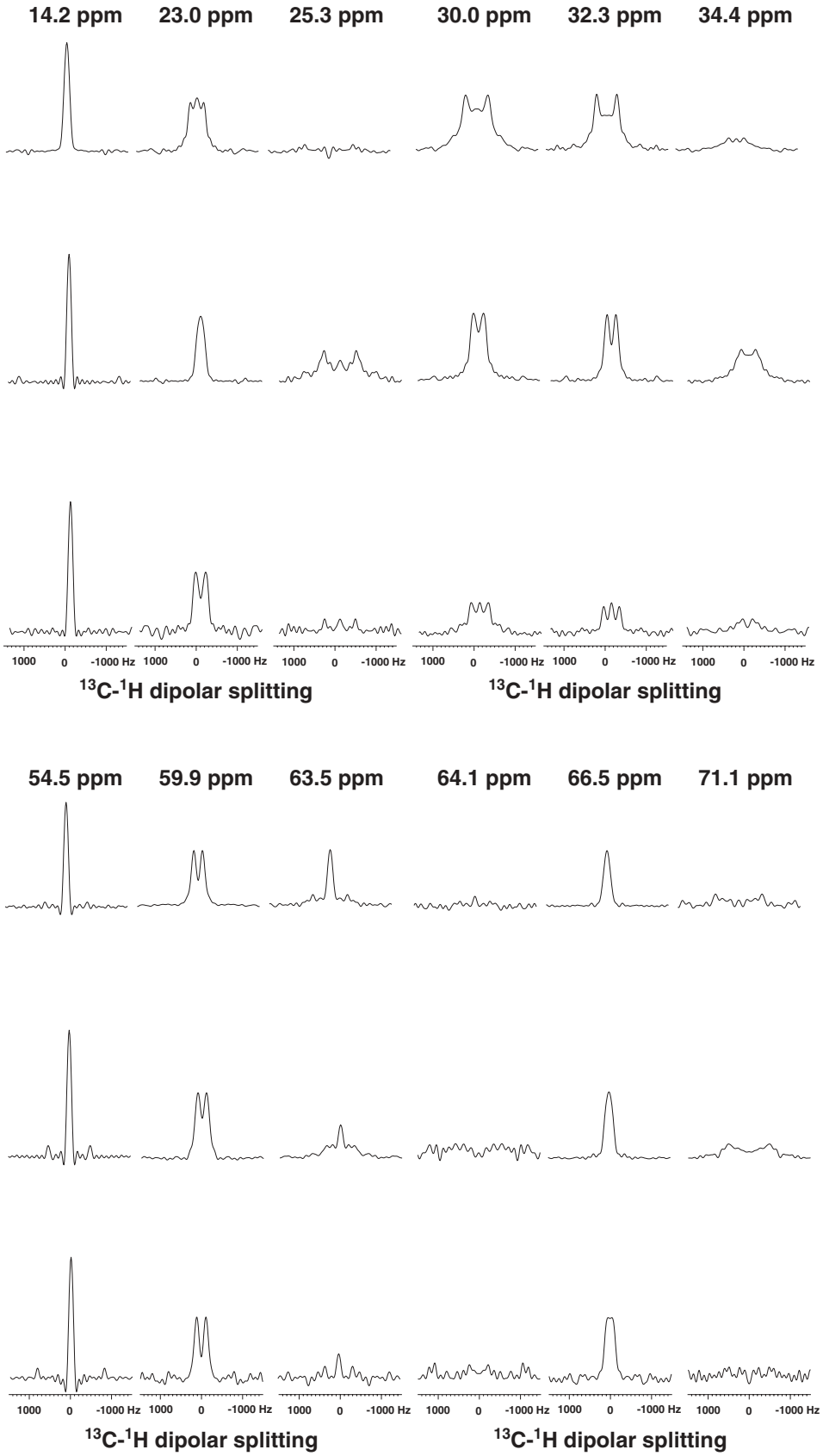

Figure S1
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Fig. S1 ^{1}H-^{13}C dipolar slices extracted at the indicated ^{13}C chemical shift of POPC (bottom row), DOPC (middle row) and DOPC/cholesterol:2/1 (top row) at 37°C. Chemical shift assignment is the following: acyl chains terminal methyls (ω) at 14.2 ppm; (ω - 1) at 23.0 ppm; C3 methylenes at 25.3 ppm; (ω - 3) at around 30 ppm; (ω - 2) at 32.3 ppm; C2 methylenes at 34.4 ppm; headgroup terminal methyls (γ) at 54.5 ppm; (α) at 59.9 ppm; glycerol g_1 at 63.5 ppm; glycerol g_3 at 64.1 ppm; (β) at 66.5 ppm; glycerol g_2 at 71.1 ppm. Dipolar splittings in the glycerol backbone and the upper part of the acyl chains are large and often multiple, resulting in a low signal-to-noise ratio. When this was the case, especially at the g_3 position, we have not reported the uncertain order parameter values.