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Supplemental Information

The Phase Behavior and Organization of Sphingomyelin/Cholesterol Membranes: A Deuterium NMR Study

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SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

^{2}H NMR spectroscopy

Spectral subtraction

The boundaries of the **so** + **lo** coexistence region are determined by the spectral subtraction method (18). Within this two-phase region, a ^{2}H NMR spectrum consists of different amounts of endpoint spectra characteristic of the two phases in equilibrium and the phase boundaries, x_s and x_f, can be calculated:

$$x_s = \frac{(1 - x_B)x_A - K(1 - x_A)x_B}{(1 - x_B) - K(1 - x_A)} \tag{S1}$$

$$x_f = \frac{(1 - x_A)x_B - K'(1 - x_B)x_A}{(1 - x_A) - K'(1 - x_B)}, \tag{S2}$$

where K is the ratio of **lo**-phase lipid fractions in the two samples, K' is the ratio of **so**-phase lipid fractions in the two samples, and x_A and x_B are the cholesterol concentrations of two lipid/cholesterol MLDs. The spectral subtraction method is valid as long as certain assumptions hold. The spectra of the two phases, S_s and S_f, have to be sufficiently different that one can easily distinguish and carry out the subtraction procedure. The exchange of labeled lipid between two kinds of domains must be slow on the NMR timescale so that it can be neglected. In addition, the domain must be sufficiently large, so that the signal from the lipid on the boundary of the domains is negligible. Also, this method assumes that both phases have the same relaxation time T_{2e}, which is not true in our case. Depending on the temperature, the **lo** phase has a T_{2e} two to six times larger than the **so** phase T_{2e} and therefore the **so** component decays faster than the **lo** component (the signal $\propto e^{-t/T_{2e}}$). Thus, at any given quadrupolar echo time 2τ, the ^{2}H NMR spectrum will contain a smaller **so** component S_s than is representative of the sample. Due to this T_{2e} effect f_A and f_B in Eqs. S1 and S2, which should be denoted as $f_A(t = 2\tau)$ and $f_B(t = 2\tau)$, do not reflect the actual fractions of fluid lipid in the samples. Thus, the K and K' (which should be denoted as $K(t = 2\tau)$ and $K'(t = 2\tau)$, respectively) determined from the spectral subtraction will not be correct, leading to a deviation of x_s and x_f from the actual values. To incorporate this T_{2e} effect, corrected f_A and f_B values (i.e., $f_A(t = 0)$ and $f_B(t = 0)$) are calculated by extrapolating the height of the respective echo signal back to $t = 0$ using the measured T_{2e} for a given temperature, and then the corrected K and K' values (i.e., $K(t = 0)$ and $K'(t = 0)$) can be derived and expressed in terms of the experimentally determined K and K' (i.e., $K(t = 2\tau)$ and $K'(t = 2\tau)$), and T_{2e}s. Assuming the gel component of a mixed spectrum will have decayed by an extra factor R relative to the liquid ordered component, then (R1)

$$K_{t=0} = C\,K_{t=2\tau} \tag{S3}$$

$$K'_{t=0} = \left(\frac{1}{C}\right)K'_{t=2\tau} \tag{S4}$$

where the coefficient C is given by

$$C = \frac{1 + f_A\left(\frac{1 - R}{R}\right)}{1 + f_B\left(\frac{1 - R}{R}\right)} \tag{S5}$$

Using Eqs. S1 and S2 with these corrected K and K' values, the T_{2e}-corrected values of x_s and x_f are obtained.

RESULTS AND DISCUSSION

Phase Boundaries at Higher Cholesterol Content and Low Temperature: so/(so+lo)/lo

Spectral subtraction

Figure S1 illustrates the spectral subtraction procedure at 32^{o}C using normalized spectra of PSM-d31/cholesterol MLDs with 11 and 22.5 mol % cholesterol, Figs. S1A(I) and S1A(II). We refer to the 11 mol % cholesterol membrane as "**so**-rich" and the 22.5 mol % cholesterol membrane as "**lo**-rich". By subtracting 24% of the **so**-rich spectrum from the **lo**-rich spectrum we obtain the difference spectrum shown in Fig. S1A(IV). The value K' = 0.24 gives the **lo**-phase end-point mole fraction x_f = 0.255 denoting the **so**+**lo/lo** boundary. This difference spectrum, renormalized in Fig. S1B(II), is compared with the normalized experimental spectrum obtained at 31^{o}C for 25 mol % cholesterol, Fig. S1B(I). The difference between the spectra in Figs. S1B(I) and S1B(II) is shown in Fig. S1B(III). Clearly, there is excellent agreement between the end-point spectrum obtained by spectral subtraction and the spectrum of the 25 mol % sample. If we subtract 17% of the **lo**-rich spectrum from the **so**-rich spectrum, then we obtain the **so**-phase end-point spectrum shown in Fig. S1A(III), corresponding to a cholesterol mole fraction of x_s = 0.082. This difference spectrum, after normalization (Fig. S1B(V)), is compared to the experimental spectrum obtained for the 8.5 mol % sample at 31^{o}C (Fig. S1B(IV)). The difference between the end-point spectrum and the 8.5 mol % spectrum is shown in Fig. S1B(VI). The spectral subtraction approach thus proves robust in separating experimental spectra of MLDs within the **so**+**lo** region into their end-point components. Errors in determining the end-point cholesterol mole fractions resulted from the uncertainty in K and K' values for a given pair of experimental spectra in the **so**+**lo** coexistence region.

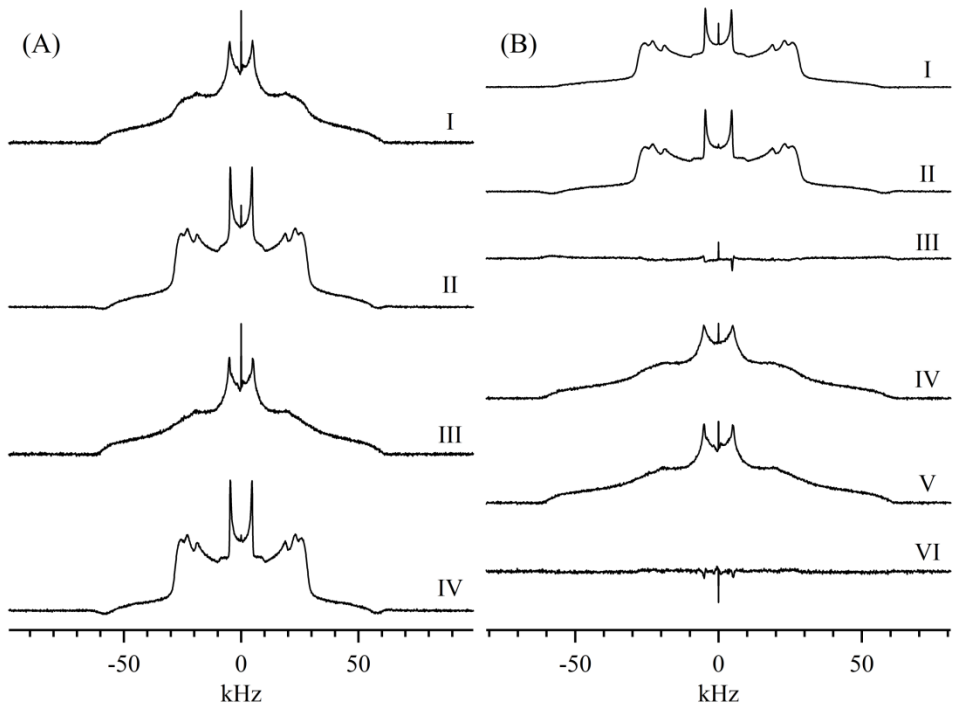

Figure S1: ^{2}H NMR spectral subtraction at 32º: (A)I and (A)II show experimental spectra of 11 mole% and 22.5 mol% cholesterol, respectively; (A)III displays the spectrum in (A)I minus 0.17 times that in (A)II; (A)IV displays the spectrum in (A)II minus 0.24 times that in (A)I. Figure S1(B) compares the end-point difference spectra with experimental spectra at 32ºC: (B)I is the spectrum of the 25 mol% cholesterol sample; (B)II is the end-point difference spectrum in (A)IV; (B)III is the difference between the spectra in (B)I and (B)II; (B)IV is the spectrum of the 8.5 mol% cholesterol sample; (B)V is the end-point difference spectrum in (A)III; (B)VI is the difference between the spectra in (B)IV and (B)V. All spectra (except those in (B)III and (B)VI) are normalized in area.

As was discussed in the Materials and Methods section, the spectral subtraction procedure assumes that the **so** and **lo** phases have the same relaxation time, T_{2e}. Figure S2 shows that this is not true for PSM-d31/cholesterol; depending on the temperature the **lo** phase has a T_{2e} two to six times longer than that of the **so** phase. Thus the **so** component decays faster than the **lo** component and is underrepresented in the quadrupolar echo spectrum of a membrane containing both phases. Note that 5.4 and 35 mol % cholesterol compositions are used to measure relaxation times of the **so** and **lo** phases, respectively. The values of the end-point compositions, x_s and x_f, with and without T_{2e} correction are listed in Table S1. At each temperature there is a significant shift of 2.5±0.4 mol % in the **so**+**lo**/**lo** boundary to higher cholesterol concentrations. A less dramatic shift of <1.2 mol % is observed for DPPC-d31/cholesterol. Furthermore, for both PSM-d31/cholesterol and DPPC-d31/cholesterol a relatively small shift in the **so**/**so**+**lo** boundary is observed, ranging from 0.3 to 1.8 mole%.

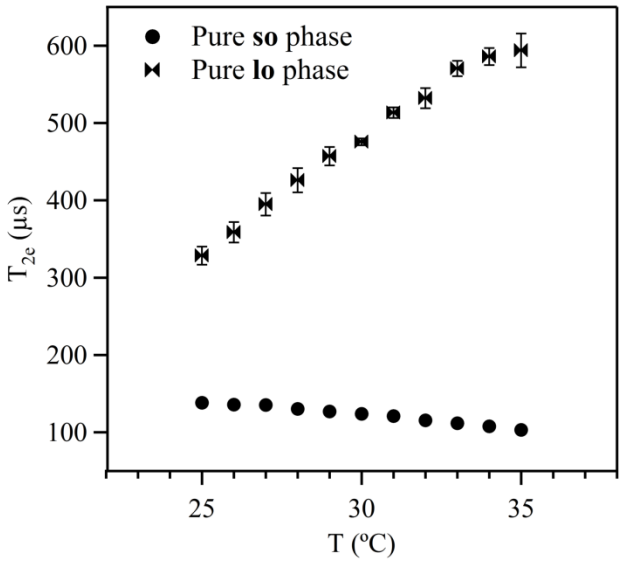

Figure S2: T_{2e} as a function of temperature for PSM-d31/cholesterol membranes with 5.4 mol% cholesterol (pure **so** phase) and 35 mol% cholesterol (pure **lo** phase).

Table S1: Comparison of the x_s and x_f values and those with T_{2e} corrections.

	No Correction		With T_{2e} Correction	
T (°C)	x_s	x_f	x_s	x_f
27	9.7 ± 0.5	27.3 ± 0.8	10 ± 0.5	30.2 ± 1.1
29	9.2 ± 0.4	26.4 ± 0.5	9.8 ± 0.5	29.2 ± 0.8
31	9 ± 0.5	25.7 ± 0.7	9.7 ± 0.5	28.3 ± 0.9
33	8 ± 0.6	25.3 ± 0.6	9.3 ± 0.5	27.8 ± 0.8
35	6.9 ± 0.7	24.9 ± 0.6	8.7 ± 0.6	27 ± 0.8

Phase diagram determination: three phase line

For DPPC-d31/cholesterol, the temperature of the transition between the **so**+**lo** and **ld**+**lo** regions of the phase diagram can be determined from the midpoint of the steep decline in the first moment (M1) vs. temperature plot for a given cholesterol concentration. For PSM-d31/cholesterol, it cannot. Thus it was necessary to devise an alternative way of measuring the transition. From an examination of depaked spectra as a function of temperature, it became apparent that there was a clear change in spectral shape at the transition (Fig. S3, right column). For DPPC-d31/cholesterol this change could be pinpointed to ± 0.5 °C and agreed extremely well with the midpoint of the steep change in M1 vs. temperature. For PSM-d31/cholesterol the transition is more subtle due to the highly ordered liquid crystalline phase(s) above the transition - the spectra do not change in width very much from **so**+**lo** to **ld**+**lo**. Even so, there are

comparable changes in spectral shape between PSM-d31/cholesterol and DPPC-d31/cholesterol at the transition. For example, a peak from one of the inequivalent C2 deuterons becomes resolved (indicated by * in Fig. S3). The other major change, observed in both PSM-d31/cholesterol and DPPC-d31/cholesterol, is a sharpening of the high-frequency peak (positioned at ~55 kHz at 33 ^{o}C) at the transition. For PSM-d31/cholesterol the transition could be determined to ± 1.0 ^{o}C.

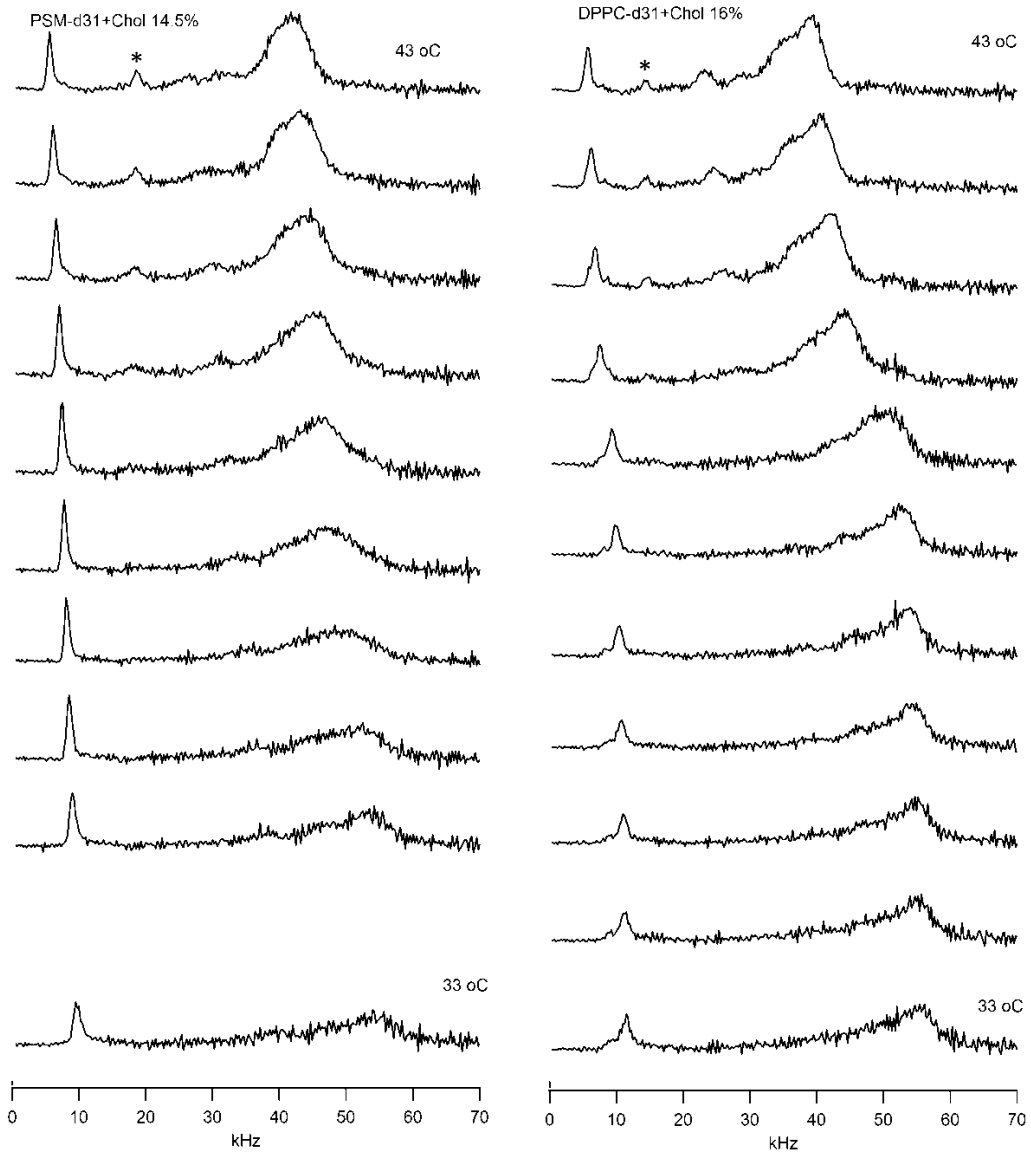

Figure S3: Determination of the temperature of the **so**+**lo** to **ld**+**lo** transition. Depaked spectra (half of each symmetric spectrum is shown) are plotted as a function of temperature at 1 degree intervals from 33 to 43 ^{o}C. Spectra of PSM-d31/cholesterol MLVs with 14.5 mol% cholesterol are shown in the left column and spectra of DPPC-d31/cholesterol MLVs with 16 mol% cholesterol are shown in the right column. The 34 ^{o}C spectrum is missing in the left column. The transition from so+lo to ld+lo occurs at 39.5 ± 0.5 ^{o}C for DPPC-d31/cholesterol, and at 38.5 ± 1.0 ^{o}C for PSM-d31/cholesterol. Asterisks indicate the resolved peak from a C2 deuteron.

Phase diagram determination, determination of the ld+lo/lo boundary:

The cholesterol concentration at the **ld+lo/lo** phase boundary can be obtained from the variation of first moment with [cholesterol] (Fig. 6). It can also be obtained from the variation of quadrupolar splitting of C12 with cholesterol concentration (Fig. S4).

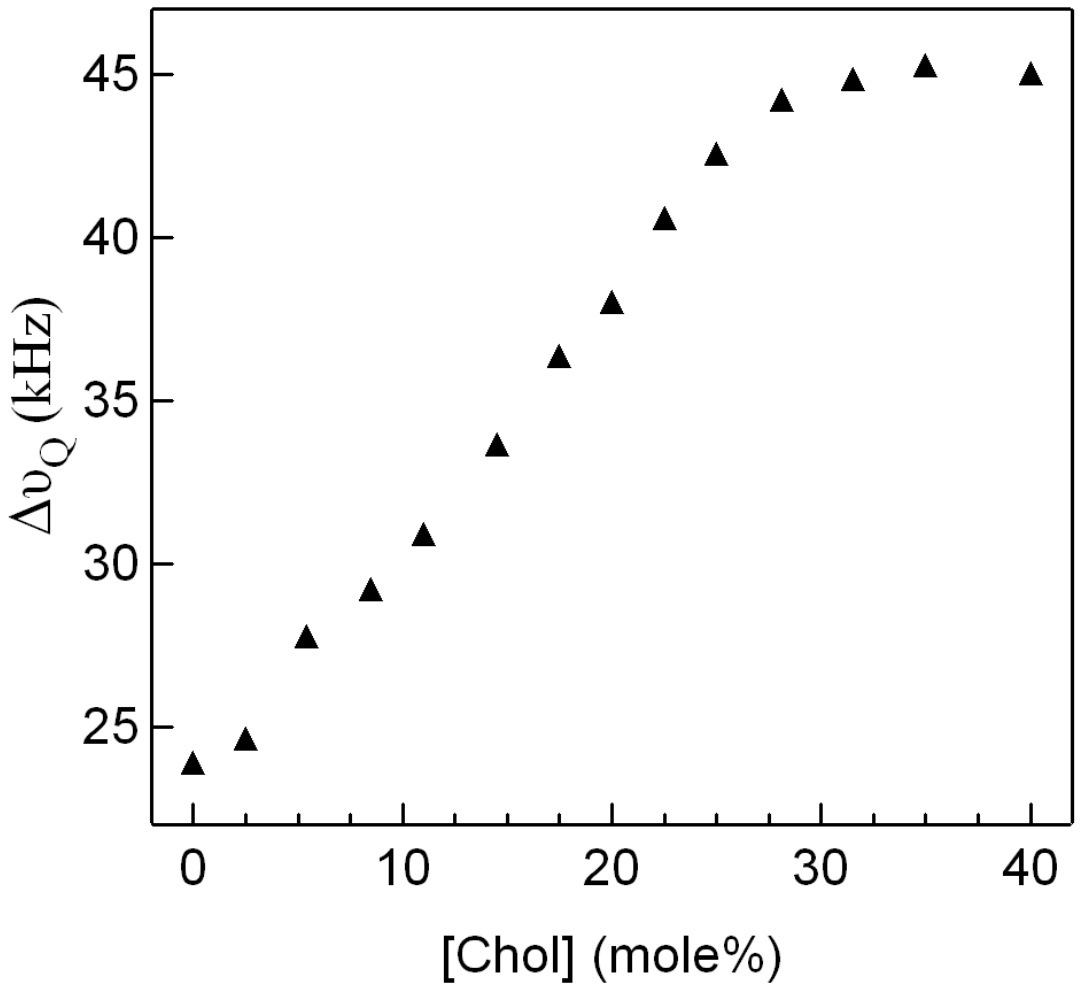

Figure S4: Quadrupolar splitting of carbon 12 on the palmitoyl chain of PSM-d31/cholesterol membranes as a function of cholesterol concentration, at T = 47ºC.

SUPPLEMENTARY REFERENCE

(R1) Morrow, M. R., R. Srinivasan, and N. Grandal. 1991. The phase diagram of dimyristoyl phosphatidylcholine and chain-perdeuterated distearoyl phosphatidylcholine: A deuterium NMR spectral difference study. *Chem. Phys. Lipids*. 58:63-72.