Phase Diagram of Ternary Cholesterol/Palmitoylsphingomyelin/Palmitoyloleoyl-Phosphatidylcholine Mixtures: Spin-Label EPR Study of Lipid-Raft Formation

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SUPPORTING MATERIAL

S.1 Intersubtractions, tie-line endpoints and spin-label partition

The normalized two-component EPR spectrum, $\hat{S}$, in a region of L_α–L_o phase coexistence can be expressed as:

$$\hat{S} = \alpha\hat{S}_{L_o} + (1-\alpha)\hat{S}_{L_\alpha} \tag{S.1}$$

where α is the fraction of spin-labelled lipid probe in the L_o-phase. The carets indicate that the individual spectral components $\hat{S}_{L_o}$ and $\hat{S}_{L_\alpha}$ are also normalized to their second integral. The values of α can be obtained by intersubtractions between normalized spectral line shapes, $\hat{S}_A$ and $\hat{S}_B$, for two compositions $\mathbf{X}_A$ and $\mathbf{X}_B$ that lie along the same tie line. The (unnormalised) spectral line shapes S_{L_α} and S_{L_o} of the pure L_α and L_o phases are given by:

$$S_{L_\alpha} = \hat{S}_A - k\hat{S}_B \tag{S.2}$$

$$S_{L_o} = \hat{S}_B - k'\hat{S}_A \tag{S.3}$$

where k and k' are the subtraction factors corresponding to the L_α- and L_o-endpoints, respectively. From Eqs. S.1–S.3, the experimental intersubtraction endpoints, k and k', are given by (cf. ref. 33):

$$k = \frac{\alpha_A}{\alpha_B} \tag{S.4}$$

$$k' = \frac{1-\alpha_B}{1-\alpha_A} \tag{S.5}$$

from which the two values of α, viz., α_A and α_B, can be determined.

The partition coefficient, K_p, of the spin label between L_α and L_o phases is defined by:

$$K_p = \left(\frac{\alpha}{f}\right) \Big/ \left(\frac{1-\alpha}{1-f}\right) \tag{S.6}$$

where f is the fraction of total lipid in the L_o phase. The latter is given by the lever rule (44):

$$f = \frac{\mathbf{X} - \mathbf{X}_{L_\alpha}}{\mathbf{X}_{L_o} - \mathbf{X}_{L_\alpha}} \qquad \mathbf{X} \equiv (x_1, x_2, x_3) \tag{S.7}$$

where x_1, x_2 or x_3 are the mole fractions of POPC, PSM and cholesterol, respectively, in the ternary mixture ($x_1 + x_2 + x_3 = 1$). The vectors $\mathbf{X}_{L_o}$ and $\mathbf{X}_{L_\alpha}$ specify the coordinates of the ends of the tie line at the boundaries with the L_o- and L_α-phases, respectively. From Eqs. S.6 and S.7, the fraction of spin-labelled lipid in the L_o-phase is therefore:

$$\alpha = \frac{K_p(\mathbf{X} - \mathbf{X}_{L_\alpha})}{\mathbf{X}_{L_o} - \mathbf{X}_{L_\alpha} + (K_p - 1)(\mathbf{X} - \mathbf{X}_{L_\alpha})} \tag{S.8}$$

Note that Eq. S.8 gives three independent expressions for α, in terms of the x_1, x_2 or x_3 compositional coordinates, from which the vectors $\mathbf{X}_{L_o}$ and $\mathbf{X}_{L_\alpha}$, and the single value of K_p can be determined.

S.2 Bottom of the Gibbs triangle: low-chol region

Chol 3 mol%

The dependence of the 14-PCSL EPR line shapes on PSM content in POPC/PSM mixtures that contain 3 mol% chol is similar to that for binary POPC/PSM mixtures without cholesterol. Two overlapping components corresponding to the coexistence of L_α and L_β phases are observed at the same contents of PSM as in the absence of cholesterol. Within experimental error, the *s*-component from the L_β-phase has the same motional anisotropy, represented by $2\langle A_{\max}\rangle$, as without cholesterol, which corresponds practically to that for PSM alone. However, at equal PSM contents, the fraction of *s*-component from mixtures containing 3 mol% chol is decreased relative to that for 0 mol% chol. The outer hyperfine splitting, $2\langle A_{\max}\rangle$, of the *w*-component increases slightly with increasing cholesterol content: e.g., for 67 mol% PSM, $2\langle A_{\max}\rangle$ = 3.20 mT at 0 mol% chol and 3.26 mT at 3 mol% chol. This indicates that cholesterol is incorporated in the L_α phase. The set of spectra from samples containing 3 mol% chol displays isosbestic points, i.e., corresponds to a tie line, over the range 40–77 mol% PSM. The average partition coefficient of 14-PCSL between L_α and L_β phases is $K_p(L_\alpha{:}L_\beta)$ = 1.14±0.15 for this tie line.

Chol 10 mol%.

Conventional V_1-EPR spectra of 14-PCSL in POPC/PSM mixtures that contain 10 mol% chol are shown in the upper panel of Fig. S.1. For PSM contents up to 40 mol%, the EPR spectra consist solely of the *w*-component with an outer hyperfine splitting in the range $2\langle A_{\max}\rangle$ = 3.24–3.53 mT, which is characteristic of the L_α-phase. Two-component EPR spectra consisting of both *s*- and *w*-components appear first at ~50 mol% PSM. Initially,

the presence of the *s*-component appears as a broadening in the wings of the low-field line, as compared to the lower PSM contents. The lower panel in Fig. S.1 gives the dependence of the central-component amplitude (I_o) of the normalized spectra on PSM content. This exhibits a break at 40–50 mol% PSM that corresponds to the boundary between the L_α-phase and L_α+L_β coexistence regions. The EPR line shapes for mixtures containing 80 and 83 mol% PSM clearly show the presence of two components, with a predominant contribution from the *s*-component, but the *w*-component has a large anisotropy and corresponds to the L_o-phase, not to the L_α-phase. These latter two spectra do not fit into the isosbestic set formed by 40, 50, 60 and 70 mol% PSM, which define a tie line for L_α+L_β phase coexistence (see Fig. S.1 top). The outer hyperfine splitting ($2\langle A_{\max}\rangle$) of the *s*-component is approximately the same for 10 mol% chol as that for 0 mol% chol, indicating that the composition of the L_β-phase of PSM/chol mixtures does not change appreciably up to 10 mol% chol.

S.3 Right side of the Gibbs triangle: low-POPC region

Conventional V_1-EPR spectra of 14-PCSL in PSM/chol mixtures that contain 4.5, 7 or 16.1 mol% POPC and different contents of cholesterol are shown in the top, middle and bottom panels, respectively, of Fig. S.2. Tie lines for L_o+L_β coexistence are found at each of these contents of POPC, as indicated by the isosbestic points in the three sets of spectra. Boundaries of both two-phase coexistence regions, L_o+L_β and L_α+L_β, were verified from inspection and analysis of the EPR spectra from samples with adjacent compositions.

S.4 L_α–L_o tie-line end points

Judging by their line shapes, spectra for the compositions POPC:PSM:chol = 60:30:10 and 30:45:25 mol/mol/mol in the upper panel of Fig. 5 of the main text are reasonably close to the L_α- and L_o-endpoints of the tie line, respectively. Therefore, these compositions were used for determining the single-component endpoints according to Eqs. S.2 and S.3. Note that the EPR spectra for the intermediate compositions, POPC:PSM:chol = 50:35:15 and 40:40:20 mol/mol/mol, can be obtained by superposition of the above spectra – see Fig. 5 top for the latter composition.

Because the exact line shapes of the isolated L_α- and L_o-components are not known exactly, criteria are required to establish the subtraction endpoints, particularly for the L_o-component. A criterion for obtaining the L_α-endpoint is the appearance of "anomalies" in the spectral wings. Criteria for the L_o-component are a symmetric high-field line shape (Y1≈Y2) and a maximum value for the ratio (Y1+Y2)/2d (see inset to Fig. 5). The first criterion (Y1/Y2) is found to be more sensitive. From Eqs. S.2 –S.5, the fraction of spin label in the L_o phase is α = 0.91–0.87 and 0.144–0.137 for compositions POPC:PSM:chol 60:30:10 and 30:45:25 mol/mol/mol, respectively. Because both L_α- and L_o-phases are fluid it is reasonable to assume that the 16-PCSL partition coefficient is $K_p(L_o{:}L_\alpha) \approx 1$. Hence from Eq. S.8, the tie-line endpoints are: $\mathbf{X}_{L_\alpha}$ (POPC:PSM:chol) = (0.63–0.65, 0.28–0.27, 0.08–0.07) and $\mathbf{X}_{L_o}$ = (0.244, 0.478, 0.278)

in mole fractions, for the L_α and L_o phases respectively. End points for the tie line corresponding to the lower spectral series in Fig. 5 were determined in a similar manner.

S.5 First-harmonic nonlinear EPR

Figure S.3 compares the linear and nonlinear first-harmonic EPR spectra for 14-PCSL in membranes of two compositions (POPC/PSM/chol = 24:50:26 and 30:50:20 mol/mol/mol) in the L_α+L_o region, and of one composition (POPC/PSM/Chol = 10:80:10 mol/mol/mol) in the L_o+L_β coexistence region. All these membrane samples were prepared in an argon atmosphere and Ni perchlorate was added to the aqueous phase to a final concentration of 5 mM. The conventional in-phase V_1-EPR spectra were recorded under standard conditions of no saturation, whereas the out-of-phase V_1'-EPR spectra were recorded under saturating conditions at a microwave field of $\langle H_1^2 \rangle^{1/2}$ = 18 μT, as described in the Materials and Methods section.

The appreciable difference between the V_1'-EPR and V_1-EPR line shapes in Fig. S.3 is indicative of the composite nature of the EPR spectra for these regions of the phase diagram. The difference arises because two (or more) spectral components differ in their electron spin-lattice relaxation time, $T_{1,e}$ (see refs. 29, 35, 30). Paramagnetic Ni perchlorate enhances the differences in $T_{1,e}$ of the lipid spin label because it partitions differently into the coexisting lipid phases (see ref. 30).

S.6 Spectral simulations and lipid dynamics

Spectral simulations were made on the basis of the stochastic Liouville equation, which is valid in both fast and slow regimes of molecular rotation (38, 39, 40). The dynamic model is parameterised by an order parameter, S_{zz}, characterising the time-average orientation relative to the director (i.e., membrane normal), and the diffusion coefficient, $D_{R\perp}$, for rotation of the long axis of the lipid molecule.

Simulations of conventional 14-PCSL EPR spectra were made first for different spectral series that correspond to compositions with constant low PSM contents (0, 15 and 25 mol%), from the left side of the Gibbs triangle. For 0 mol% PSM, the lipid-chain order parameter increases monotonically, without any breaks, from S_{zz} = 0.13 for 9 mol% chol to S_{zz} = 0.365 for 60 mol% chol. The diffusion coefficient for off-axis rotation increases only weakly, from $D_{R\perp} = 1 \cdot 10^8$ s^{-1} to $D_{R\perp} = 1.3 \cdot 10^8$ s^{-1}, over the same range of cholesterol contents. Qualitatively similar monotonic changes with increasing cholesterol content, without breaks, are found for 15 mol% and 25 mol% PSM contents, although the absolute values of S_{zz} and $D_{R\perp}$ increase with increasing PSM content. For instance, $S_{zz} \approx$ 0.43 and $D_{R\perp} \approx 10^9$ s^{-1}, for POPC:PSM:chol = 35:15:50 mol/mol/mol. Such simultaneous increases in S_{zz} and $D_{R\perp}$ are indicative of a gradual transition from the L_α- to the L_o-phase.

An apparent increase in chain ordering and rotation rate (S_{zz} and $D_{R\perp}$) with increasing cholesterol content is observed also for samples with fixed POPC content (see Fig. S.4, lower). For the series with 20 and 30 mol% POPC, the increases in ordering (S_{zz}) are rather sharp over the range 10–20 mol% chol, followed by only a weak increase or plateau value at higher cholesterol contents. Again, the diffusion coefficient for off-axis

rotation increases rather weakly with increasing cholesterol content: from $D_{R\perp} = 0.9 \cdot 10^8$ s-^{1} to $D_{R\perp} = 1.5 \cdot 10^8$ s^{-1} for 20 mol% POPC, and less for 30 mol% POPC. The results for the order parameter, S_{zz}, agree well with the similar dependences of the experimental anisotropy parameter, $2\langle A_{max} \rangle$, on cholesterol content (see Fig. S.4, upper). The latter also show that less abrupt and more progressive changes take place at higher POPC concentrations (viz., 60 mol%). Note that, at less than 25–30 mol% cholesterol, the values of S_{zz} in Fig. S.4 are only apparent values because they do not correspond to single-component spectra. The true values of S_{zz} in the L_o-phase region are indicated by the solid lines in the lower panel of Fig. S.4. Nevertheless, $2\langle A_{max} \rangle$ shown in the upper panel of Fig. S.4 is still a useful empirical spectral parameter to characterise the phase boundaries in this region, throughout the full range of cholesterol contents. At 60 mol% POPC, $2\langle A_{max} \rangle$ does not exhibit abrupt discontinuities with increasing cholesterol content, because this series barely crosses a region of phase coexistence (see Fig. 4). The set of lipid mixtures with 60 mol% POPC is located at the extreme border of the region of continuous transition between the L_α and L_o phases.

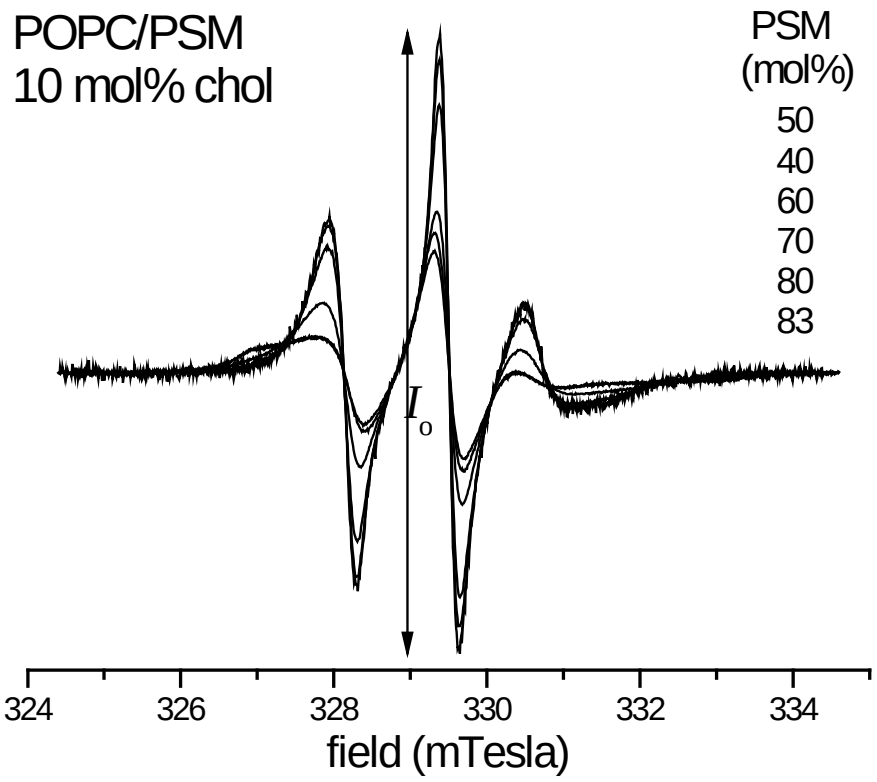
FIGURES FOR SUPPORTING MATERIAL:

Figure S.1. *Top*: First-derivative V_1-EPR spectra of the 14-PCSL spin label in POPC/PSM/chol membranes, all containing 10 mol% chol. With decreasing line height, the spectra correspond to 50, 40, 60, 70, 80 and 83 mol% PSM contents. Spectra are normalised to constant second integral. In the range 40–70 mol% PSM, the EPR spectra form an isosbestic set. *Bottom*: Dependence of the normalized line height, I_o, of the central peak in the 14-PCSL EPR spectrum (see *top* panel) on PSM content for POPC/PSM/chol membranes with 10 mol% chol. T = 23 ^{o}C.

Figure S.2. Isosbestic sets of first-derivative V_1-EPR spectra from the 14-PCSL spin label in POPC/PSM/chol membranes with constant POPC contents. *Top*: 4.5 mol% POPC – with decreasing line height, the spectra correspond to 36, 30, 26, 16 and 10 mol% cholesterol contents. *Middle*: 7 mol% POPC – with decreasing line height, the spectra correspond to 35, 30, 20 and 8 mol% cholesterol contents. *Bottom*: 16.1 mol% POPC – with decreasing line height, the spectra correspond to 20, 24 and 14 mol% cholesterol contents. Spectra are normalised to constant second integral. T = 23 ^{o}C.

Figure S.3. In-phase (V_1, dotted) and out-of-phase (V_1', solid) first-harmonic EPR spectra of 14-PCSL in two-phase regions of POPC/PSM/chol membranes at 23 ^{o}C. The POPC:PSM:chol compositions are: 24:50:26 mol/mol/mol (*top*) and 30:50:20 mol/mol/mol (*middle*), corresponding to L_α+L_o phase coexistence; and 10:80:10 mol/mol/mol (*bottom*), corresponding to L_o+L_β phase coexistence.

Figure S.4. Dependence on cholesterol content of: the outer hyperfine splitting, $2\langle A_{max} \rangle$ (*top* panel), and the order parameter, S_{zz} (*bottom* panel), of the 14-PCSL spin label in POPC/PSM/chol membranes with fixed POPC contents: 20 (circles), 30 (triangles) or 60 (squares) mol%. T = 23 ^{o}C. Broken lines in the bottom panel correspond to spectra that are not single-component; the order parameters are therefore only effective quantities that reflect the majority component.


POPC/PSM
10 mol% chol
PSM
(mol%)
50
40
60
70
80
83
I_o
324
326
328
330
332
334
field (mTesla)

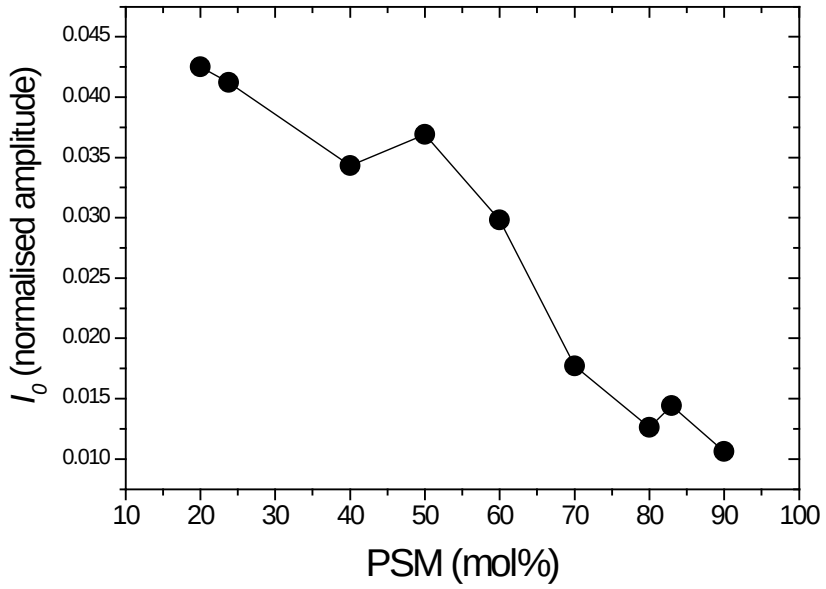

I_o (normalised amplitude)
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
10
20
30
40
50
60
70
80
90
100
PSM (mol%)

Fig.S.1

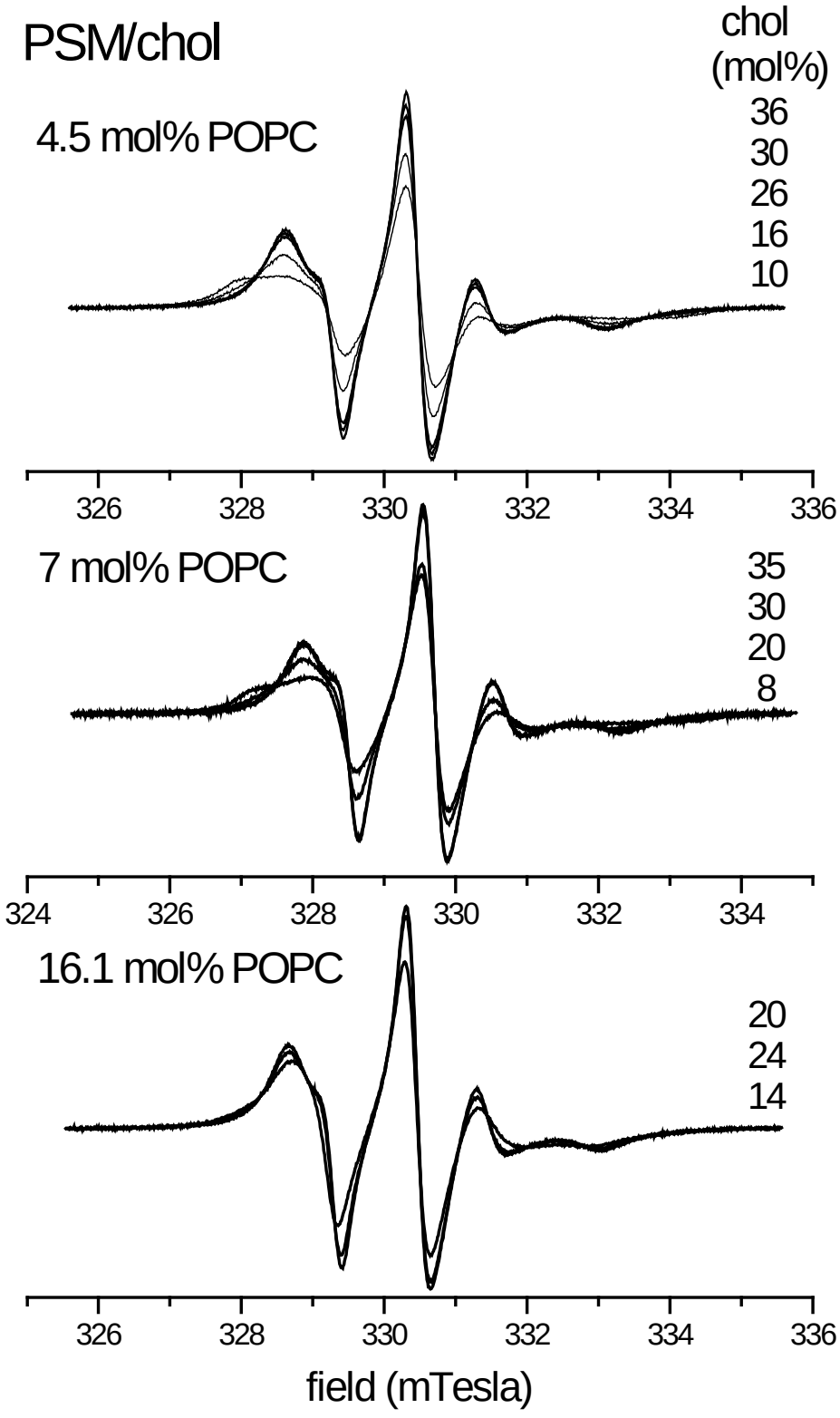

PSM/chol
chol
(mol%)
4.5 mol% POPC
36
30
26
16
10
326
328
330
332
334
336
7 mol% POPC
35
30
20
8
324
326
328
330
332
334
16.1 mol% POPC
20
24
14
326
328
330
332
334
336
field (mTesla)

Fig.S.2

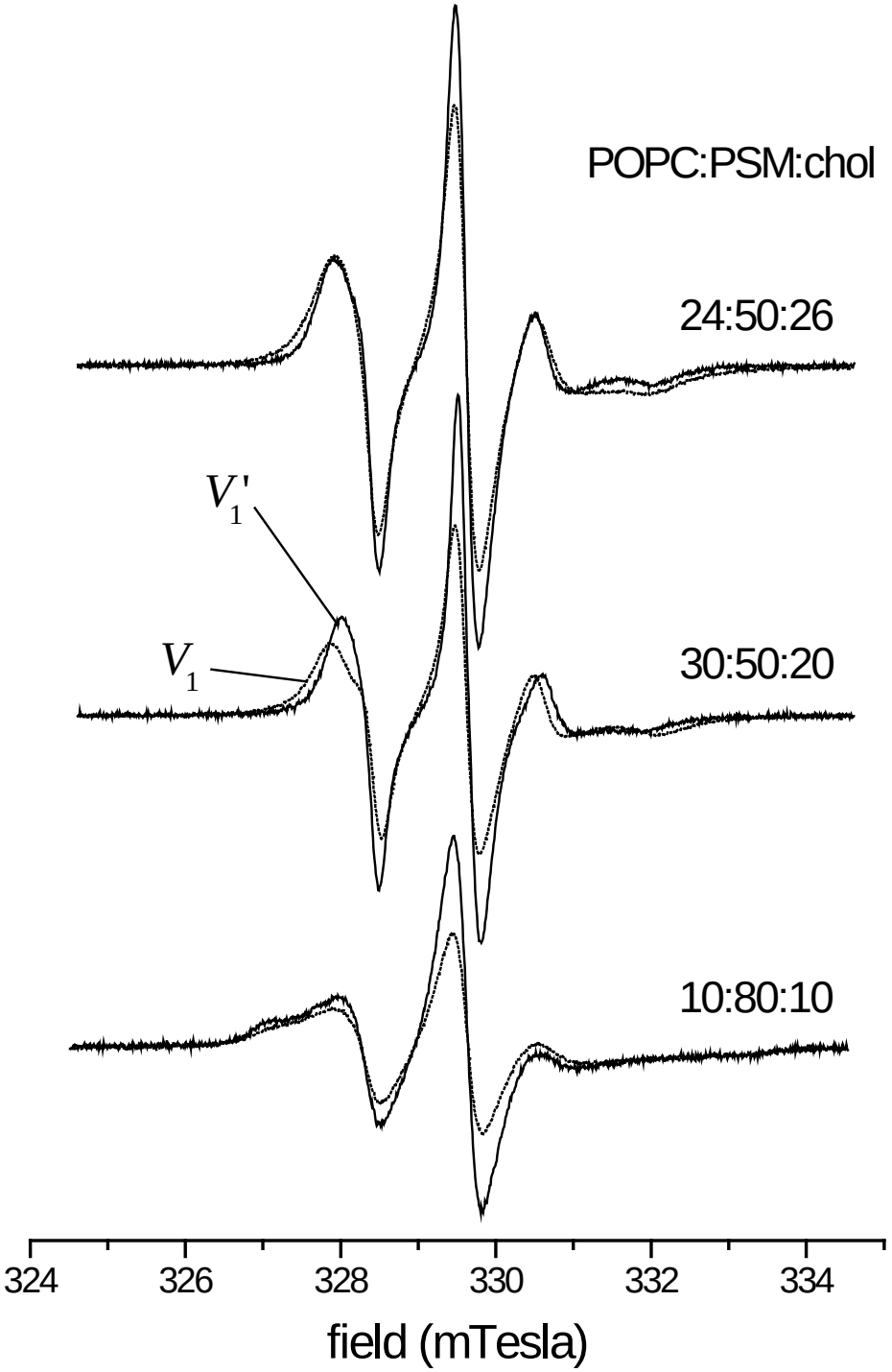

POPC:PSM:chol
24:50:26
V_1'
V_1
30:50:20
10:80:10
324
326
328
330
332
334
field (mTesla)

Fig. S.3

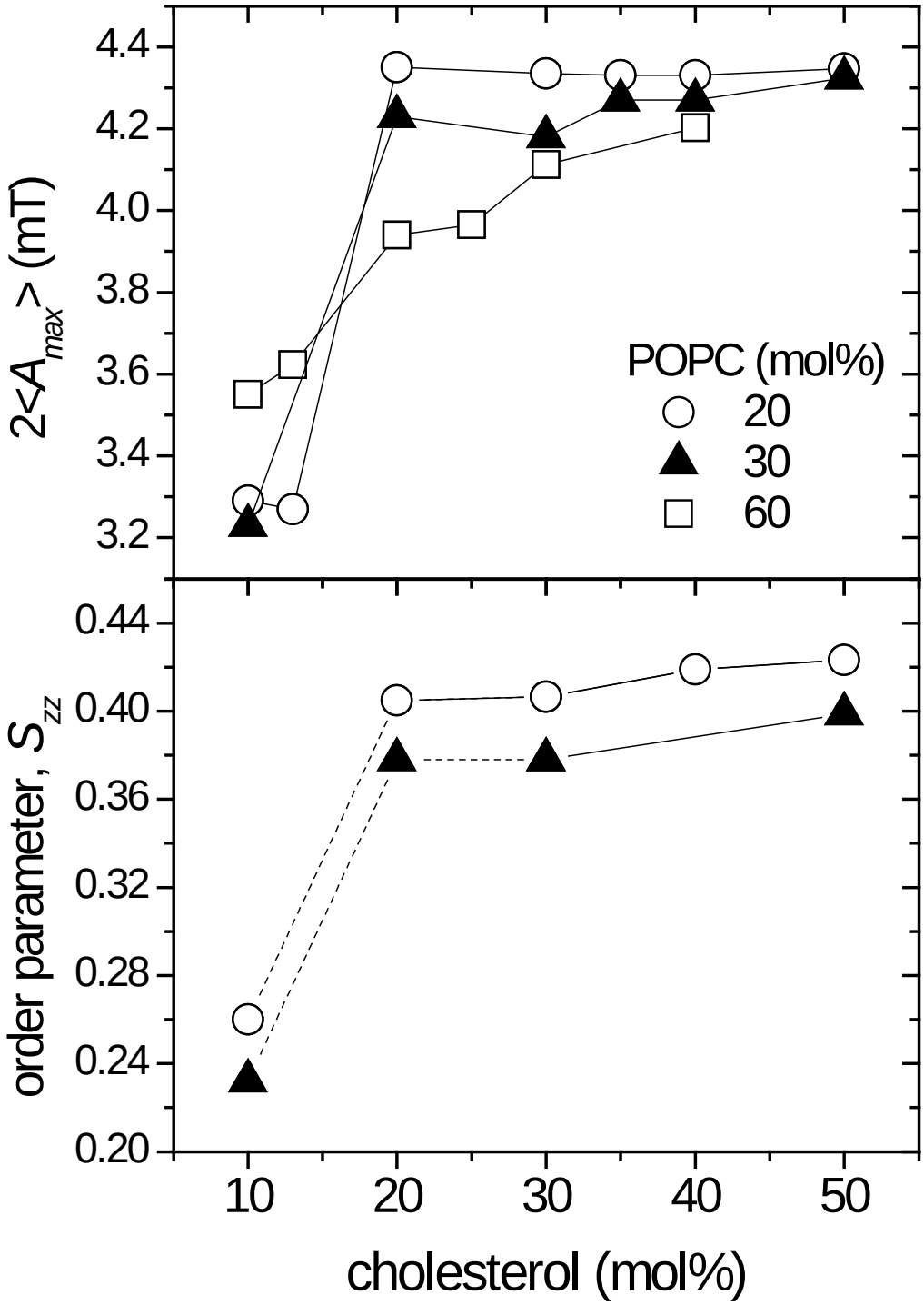

4.4
4.2
4.0
3.8
3.6
3.4
3.2
2<A_max> (mT)
POPC (mol%)
20
30
60
0.44
0.40
0.36
0.32
0.28
0.24
0.20
order parameter, S_zz
10
20
30
40
50
cholesterol (mol%)

Fig. S4