Supporting Information

Using Phase-Resolved Vibrational Sum-Frequency Imaging to Probe the Impact of Head-group Functionality on Hierarchical Domain Structure in Lipid Membranes

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Comparison of Spectral Line-Shapes

As mentioned in the main text, the overall SFG signals are given by the product between molecular density, ρ, an orientational order coefficient, O, an Euler transformation based on the average molecular orientation, $\tau(\bar{\theta}, \bar{\phi}, \bar{\psi})$, and the molecular hyperpolarisability, β. This is shown in Eq. S1 as well as Eq. 2 in the main text.

$$S^{SFG} \propto \rho \cdot O \cdot \tau(\bar{\theta}, \bar{\phi}, \bar{\psi}) \cdot \beta \qquad (S1)$$

When comparing the signals from the lipid monolayers investigated in this work, as they predominantly arise from equivalent tail-group moieties, they are probing the same molecular hyperpolarisabilities.[1,2] This parameter can hence be absorbed into the proportionality constant; leaving the SFG signals to only depend on density, orientational order, and the average orientation.

If we consider the impact of the average molecular orientation on the SFG signals, taking only the out-of-plane contribution results in the in-plane Euler angle, $\bar{\phi}$, becoming redundant. This hence leaves only the average tilt and twist angles ($\bar{\theta}$ and $\bar{\psi}$, respectively). Both of these parameters can strongly influence the amplitudes of the observed resonant features in the out-of-plane contribution.[2,3] However, given that the lipids within a self-assembled monolayer are generally fairly well-packed and consistently have mostly 'upright' tail geometries, the variability in these two orientational parameters is likely small. This hence suggests that the τ parameter in Eq. S1 can also be absorbed into the proportionality coefficient, leading to the out-of-plane SFG signals being given by Eq. S2, which is equivalent to Eq. 3 shown in the main text.

$$S_{OP}^{SFG} \propto \rho \cdot O_{OP} \qquad (S2)$$

To assess the validity of this assumption, we turn to a comparison of the spectra from the different lipid monolayers, particularly comparing their symmetric-to-antisymmetric CH_3/CD_3 ratios. The spectra are shown in Figure S1, having normalised each spectrum based on the amplitude separation of these two resonances (which are highlighted by dashed boxes). Comparing these normalised spectra shows that they have incredibly similar line-shapes, without significant differences in their symmetric-to-antisymmetric ratios. This hence suggests that neglecting any variation in the τ factors will have only minor impacts on the quantitative analysis of the SFG signals.

The overall similarity of the spectra in Figure S1 also shows that they share other equivalences. Particularly, the similarity of the DPPC reference and two LC spectra in the C-H region demonstrates that they not only likely have similar average molecular orientations, but also that they generally have similar orientational distributions i.e., similar orientational order parameters. This gives rise to the assumption described by Eq. 7 in the main text. Equally, while the two LC spectra in the C-D region are relatively noisy due to their lower absolute amplitudes, they nevertheless display similar line-shapes as the pure dPOPC reference. This hence gives rise to the other assumption that is described by Eq. 8 in the main text; that they too have equal out-of-plane orientational order coefficients. Finally, given that all five of the presented C-D spectra are highly overlapping, this indicates that they all likely have relatively similar levels of oxidation of the unsaturated tail-group. As mentioned in the main text, this is expected given their similar sample

preparation procedures. Furthermore, as this involves a ~2-hour exposure to ambient conditions prior to casting, previous studies in the literature[4] would suggest that the dPOPC is likely to be almost entirely oxidised during this time. This hence means that the relation between the molecular density of dPOPC and the effective CD_3 density should be equal across all samples.

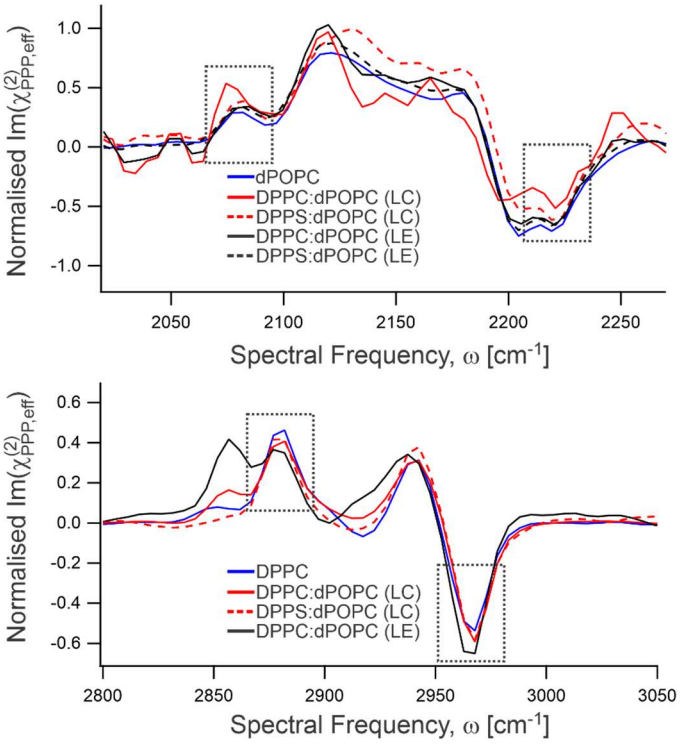

Figure S1: Out-of-plane SFG spectra in the C-D and C-H stretching regions for the two reference spectra (pure DPPC and dPOPC) as well as the LC and LE phases in each of the two lipid mixtures. It should be noted that, due to its low signal amplitudes, the normalised C-H spectrum from DPPS in the LE phase is not presented as no meaningful comparisons of the spectral line-shape can be made.

Deconvolution of SFG Signals into Structural Parameters

Having established that the SFG signals can, to a good approximation, be generally described by Eq. S2 (equivalent to Eq. 3 in the main text), they can be used to directly access the compositional densities and relative out-of-plane orientational coefficients for the LC and LE phases in both lipid samples. As discussed in the main text, the SFG signals from both frequency ranges can be averaged across each phase in the membrane, yielding four distinct spectra. If we then consider only the methyl resonances (given that the methylene signals have a much more complicated relationship with the relative order in the tail-groups), we can extract a representative value from each spectrum by taking the amplitude separation between its symmetric (~2880 cm^{-1}) and antisymmetric (~2965 cm^{-1}) stretches in the imaginary line-shape. It is also useful to note that using this separation increases the validity of the above assumption that any changes in the average molecular orientation can be neglected. This is due to orientational changes strongly modulating the ratio between the symmetric and antisymmetric stretches, but having a far less pronounced effect on their total signal amplitudes (accounting for the sign inversion). Overall, these values

hence give us four observable quantities that are related to the eight structural parameters of interest.

In addition to the SFG signals, the C-H and C-D magnitude images shown in Figure 3 (main text) can also be used to define the relative area coverages of the two phases in each sample (i.e., by using an amplitude boundary threshold). These values relate the densities of the two lipids in each phase based on Eq. S3 (equivalent to Eq. 4 in the main text), which also incorporates the information that the lipid mixing ratio is 1:1 for these samples, and thus the sums of the densities of each lipid within each phase, weighted by their area coverages, must be both equal to half the total number of molecules present in the image (i.e. the total density, ρ_{tot}, multiplied by the total area, A_{tot}).

$$\rho^{LC}_{DPP(S,C)}A^{LC} + \rho^{LE}_{DPP(S,C)}A^{LE} = \rho^{LC}_{POPC}A^{LC} + \rho^{LE}_{POPC}A^{LE}\left(= \frac{\rho_{tot}}{2}A_{tot}\right) \quad (S3)$$

These equations hence represent two further observables relating the different lipid densities to the total density, which can be trivially determined from the pressure-area isotherms shown in Figure 4a (main text).

Finally, as discussed above as well as in the main text, the generally good overlap between the spectra presented in Figure S1 for the two reference spectra (of condensed pure lipid monolayers) and the LC phases of each lipid mixture shows that they can be assumed to have the same orientational order coefficients, as described by Eqs. S4 and S5 (equivalent to Eqs. 7 and 8 in the main text). Furthermore, with the saturated DPPS and DPPC lipids generally being well-packed in the condensed phase with narrow orientational distributions and little signal cancellation, their out-of-plane order coefficients can be taken to be approximately unity, as in Eq. S4.

$$O^{ref}_{DPPC} = O^{LC}_{DPP(S,C)} = 1 \quad (S4)$$

$$O^{ref}_{POPC} = O^{LC}_{POPC} \quad (S5)$$

With these assumptions, the SFG signals for the two reference measurements of the pure lipid monolayers can be written as in Eqs. S6 and S7.

$$S^{ref}_{DPPC} \propto \rho^{ref}_{DPPC} \quad (S6)$$

$$S^{ref}_{POPC} \propto \rho^{ref}_{POPC} \cdot O^{ref}_{POPC} \quad (S7)$$

Unlike the saturated lipids, however, the out-of-plane orientational order parameter for dPOPC in the condensed phase cannot, with any confidence, be taken to be close to unity. This is because of the generally worse molecular packing of unsaturated lipids and their resulting oxidised forms, which is evident from their pressure-area isotherms.[4] In order to determine this parameter, we can nevertheless compare the SFG signals from the two reference measurements. This comparison, however, is frustrated by the fact that deuterated dPOPC is used instead of protonated POPC. This means the two reference spectra contribute to different frequency ranges and thus are governed by different molecular hyperpolarisabilities (despite the signals sourcing from equivalent moieties on the tail-groups i.e., methyl and methylene). This means the ratio of their signals is given by Eq.

S8, which includes an additional multiplication factor, γ, that describes the ratio of hyperpolarisabilities.

$$O_{POPC}^{ref} = \frac{S_{POPC}^{ref}}{S_{DPPC}^{ref}} \cdot \frac{\rho_{DPPC}^{ref}}{\rho_{POPC}^{ref}} \cdot \gamma \qquad (S8)$$

The γ constant for aliphatic C-H / C-D resonances is typically ~1.5. This is demonstrated below in Figure S2 which shows SFG spectra for equivalent monolayers of DPPC and d_{31}-DPPC (i.e., having a single chain deuterated), as well dPOPC. These spectra, like those in Figure 4b (main text) have been normalised by the average molecular density (determined from their pressure-area isotherm data), also accounting for the additional factor of 2 in the d_{31}-DPPC monolayer from only having a single contributing alkyl chain. By measuring the ratio of the symmetric-to-antisymmetric amplitude separation between the two DPPC spectra yields $\gamma = 1.43$. While this value could be directly used in Eq. S8 along with the density-normalised SFG signals for dPOPC and DPPC to calculate the order coefficient for dPOPC, this is equivalent to directly comparing the density-normalised C-D SFG signals of dPOPC and d_{31}-DPPC. Such a comparison gives a value of $O_{POPC}^{LC} = O_{POPC}^{ref} = 0.445$.

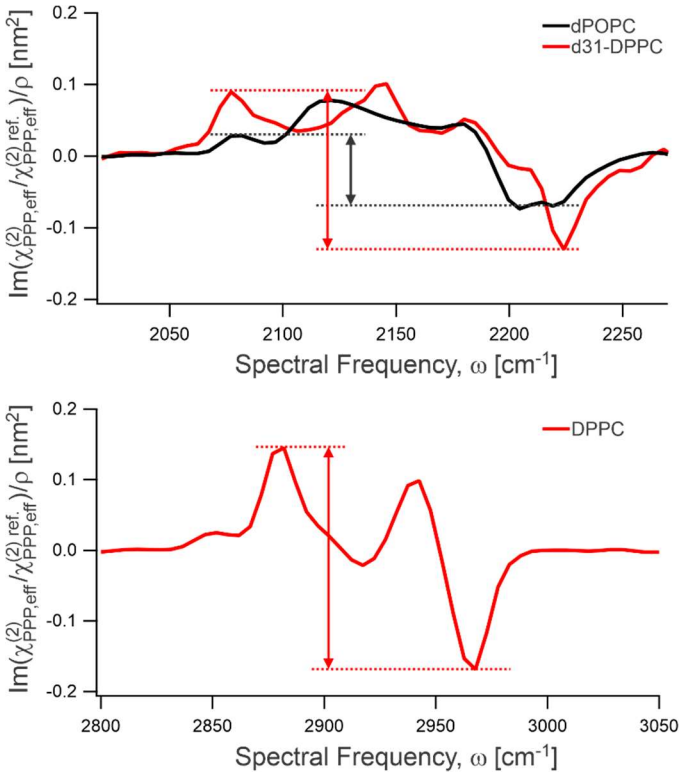

Figure S2: Out-of-plane SFG spectra of dPOPC, d_{31}-DPPC, and DPPC, having normalised based on their molecular densities (also accounting for the effective methyl density of d_{31}-DPPC being half that of DPPC by including a factor of 2 in its normalisation). The symmetric-to-antisymmetric amplitude separations are highlighted in each spectrum.

With the above descriptions of the signals and determined LC order coefficients, we have sufficient information to determine the remaining structural parameters. The SFG signals for each lipid in the LC and LE phases can be written as in Eqs. S9-S12, having omitted the order coefficient for DPPC/DPPS in the LC phase due to it being assigned to unity.

$$S^{LC}_{DPP(S,C)} \propto \rho^{LC}_{DPP(S,C)} \tag{S9}$$

$$S^{LE}_{DPP(S,C)} \propto \rho^{LE}_{DPP(S,C)} \cdot O^{LE}_{DPP(S,C)} \tag{S10}$$

$$S^{LC}_{POPC} \propto \rho^{LC}_{POPC} \cdot O^{LC}_{POPC} \tag{S11}$$

$$S^{LE}_{POPC} \propto \rho^{LE}_{POPC} \cdot O^{LE}_{POPC} \tag{S12}$$

By combining Eqs. S9 and S11 for the two lipids in the LC phase with their corresponding reference signals described by Eqs. S6 and S7, the two densities within this phase can be directly determined, as in Eqs. S13 and S14.

$$\rho^{LC}_{DPP(S,C)} = \frac{S^{LC}_{DPP(S,C)}}{S^{ref}_{DPPC}} \cdot \rho^{ref}_{DPPC} \tag{S13}$$

$$\rho^{LC}_{POPC} = \frac{S^{LC}_{POPC}}{S^{ref}_{POPC}} \cdot \rho^{ref}_{POPC} \tag{S14}$$

Thereafter, using these values along with the total average molecular density of the film and area coverages of the two phases, we can then determine the corresponding lipid densities in the LE phase, as in Eqs. S15 and S16.

$$\rho^{LE}_{DPP(S,C)} = \frac{\frac{\rho_{tot}}{2} A_{tot} - \rho^{LC}_{DPP(S,C)} A^{LC}}{A^{LE}} \tag{S15}$$

$$\rho^{LE}_{POPC} = \frac{\frac{\rho_{tot}}{2} A_{tot} - \rho^{LC}_{POPC} A^{LC}}{A^{LE}} \tag{S16}$$

Finally, these LE phase densities can be combined with the LE signals and reference measurements to yield the two LE phase order parameters, as in Eqs. S17 and S18.

$$O^{LE}_{DPP(S,C)} = \frac{S^{LE}_{DPP(S,C)}}{S^{ref}_{DPPC}} \cdot \frac{\rho^{ref}_{DPPC}}{\rho^{LE}_{DPP(S,C)}} \tag{S17}$$

$$O^{LE}_{POPC} = \frac{S^{LE}_{DPP(S,C)}}{S^{ref}_{DPPC}} \cdot \frac{\rho^{ref}_{DPPC}}{\rho^{LE}_{DPP(S,C)}} \cdot O^{ref}_{POPC} \tag{S18}$$

A summary of the obtained values of all eight structural parameters for each lipid mixture is given in Table S1.

Table S1: Obtained structural parameters (molecular density and out-of-plane orientational order coefficients) for the two lipids within the LC and LE phases of each 1:1 lipid mixture.

	1:1 DPPS:dPOPC		1:1 DPPC:dPOPC	
	DPPS	dPOPC	DPPC	dPOPC
ρ^{LC} / nm^{-2}	1.54	0.80	1.51	0.39
ρ^{LE} / nm^{-2}	1.05	1.11	0.58	1.19
O^{LC}	1	0.45	1	0.45
O^{LE}	0.10	0.50	0.80	0.31

References

1 G. Ma and H. C. Allen, *Langmuir*, 2006, **22**, 5341–5349.

2 R. J. Feng, X. Li, Z. Zhang, Z. Lu and Y. Guo, *Journal of Chemical Physics*, 2016, **145**, 244707.

3 H. F. Wang, W. Gan, R. Lu, Y. Rao and B. H. Wu, *Int Rev Phys Chem*, 2005, **24**, 191–256.

4 L. Qiao, A. Ge, Y. Lang and S. Ye, *Journal of Physical Chemistry B*, 2015, **119**, 14188–14199.