Supporting Information

Molecular rheometry: direct determination of viscosity in $L_o$ and $L_d$ lipid phases via fluorescence lifetime imaging.

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(1) The molecular rotors

Two fluorescent molecular rotors based on the BODIPY structure were interchangeably used in this work: BODIPY-O-C_{10}H_{21} and BODIPY-O-C_{12}H_{25}. Both dyes were synthesized by adaptation of the published procedure.\textsuperscript{1} Spectroscopically both dyes are identical. The viscosity-dependent lifetimes of both dyes are plotted in Figure S1. Based on this data we conclude that a single calibration graph is sufficient to assign unique microviscosity values to their fluorescence lifetimes.

![Fluorescence lifetime of BODIPY-O-C_{10}H_{21} (•) and BODIPY-O-C_{12}H_{25} (■) recorded in methanol/glycerol mixtures of various compositions, plotted against viscosity.](chart.png)

**Fig. S1:** Fluorescence lifetime of BODIPY-O-C_{10}H_{21} (•) and BODIPY-O-C_{12}H_{25} (■) recorded in methanol/glycerol mixtures of various compositions, plotted against viscosity.

(2) The self-quenching/aggregation of BODIPY in lipid vesicles.

In order to determine the optimal [lipid]:[dye] ratio for viscosity measurements we have measured fluorescence and excitation spectra of BODIPY in DOPC large unilamellar vesicles (LUVs) at increasing ratios from 20 to 200, Figure S2. The formation of the aggregated BODIPY species is recorded at ratios below 100 by the presence of the fluorescence band centered at 700 nm, Figure S2 inset.

The time resolved fluorescence measurements (Figure S3) confirmed that the observed BODIPY decays are strongly wavelength dependent. We established that in the presence of the aggregates the BODIPY decays are biexponential in the wavelength range 500-700 nm and the aggregate emission is characterised by a longer lifetime than that of the monomer, Figure S3a.
Fig. S2: The excitation and emission spectra of BODIPY recorded in LUVs made of DOPC at varied [lipid]:[dye] ratios from 20 to 200. [BODIPY] is 2.5 µM. The formation of the aggregates is manifested by the appearance of a new emission band at 650 nm (inset). The black line corresponds to the spectrum of pure water (blank).

Fig. S3: (a) Wavelength-dependent time resolved fluorescence decays of BODIPY recorded in LUVs made of DOPC at [DOPC]:[BODIPY] ratio of 20; [BODIPY] is 2.5 µM; (b) The amplitudes resulting from the biexponential fitting of the data in (a) with $\tau_1 = 0.80\pm0.05$ ns and $\tau_2 = 4.5\pm0.2$ ns. It is clear that while the monomer spectrum maximum is at ca 500 nm, the aggregate emission maximum is red shifted to ca 650 nm.
(3) The photophysical study of BODIPY in gel phase DPPC vesicles.

We have detected biexponential decays of BODIPY in gel-phase LUVs, even at a high [lipid]:[dye] ratio of 500 (Figures 2, 5, 6 main text). We have performed additional experiments to ensure that the biexponential nature of the decays does not result from the aggregation of the dye. Firstly we have recorded time resolved fluorescence decays in LUVs made of DPPC, where [DPPC]:[BODIPY] ratio was 500:1; [BODIPY] is 2.5 µM, Figure S4. Unlike the traces shown in Figure S3a, no evidence of the aggregate formation is observed in this case, i.e. the traces have no wavelength dependence.

We have further confirmed this observation by recording the time-resolved emission spectra of BODIPY on DPPC LUVs, Figure S5. Since no changes in the emission spectra are observed between 0.2 and 20 ns, we conclude that no aggregates are formed at these conditions. This implies that the biexponential decay of the BODIPY is due to the inhomogeneous nature of the environment in the gel phase of DPPC at room temperature.

![Emission decays: λ<sub>ex</sub> = 467 nm](image)

**Fig. S4:** Wavelength-dependent time resolved fluorescence decays of BODIPY recorded in DPPC LUVs at room temperature. [DPPC]:[BODIPY] ratio of 500:1; [BODIPY] is 2.5 µM. Excitation wavelength was 467 nm.
Fig. S5: Time-dependent fluorescence spectra of BODIPY in DPPC LUVs at room temperature, recorded between 300 ps and 20 ns. [DPPC]:[BODIPY] ratio of 500:1; [BODIPY] is 2.5 μM. Inset: point-by-point time resolved fluorescence decay obtained from these data.

(4) Viscosity measurements in various PC vesicles.

Fig. S6: Apparent Arrhenius activation energies for L_d phase viscosity dependence on temperature of five PC lipids, as measured by BODIPY lifetime. All values are derived from linear (lnτ) vs (1/T) plots above a gel-to-liquid transition temperature for each lipid.

(5) Ternary lipid mixtures, cholesterol sensing.

Initially, we have examined the effect of cholesterol on the rotor lifetime in two cases: (i) in the phases containing fixed amount 5 mol % of sphingomyelin and (ii) in the phases containing fixed amount 5 mol % of DOPC, Figure S7.

In the DOPC/Chol mixtures containing fixed 5 % of Sph, no phase separation is indicated in the phase diagrams available from literature. However, a biexponential fitting was always
required for the fluorescence decays recorded in these mixtures. Fitting revealed approximately 12% contribution from the short-lived component (~1 ns). Since the lifetime of these short-lived species is even lower than the lifetime of the rotor in the pure DOPC membrane, we believe that this fraction corresponds to the oddly localised population of the dye, which does not appropriately reflect the viscosity of the bilayer. When the cholesterol content is increased from 0 to 40%, a change in the long lifetime component is observed from $2.1 \pm 0.1$ to $2.2 \pm 0.1$ ns (303 to 350 cP), reflecting overall increase in the decay time of the trace, Figure S7. While this change is small and is associated with some uncertainty, we believe that it is real, since the visual inspection of the trace indicates the increase in the decay time.

In the main manuscript text we have used the phase diagram of Smith and Freed\textsuperscript{2} which reports the tie-lines essential for the design of our experiment and the interpretation of our data. We chose it since we believe that the tie-lines obtained from the EPR measurements are more appropriate than those based on the microscopy images\textsuperscript{1} as the EPR is not subjected to the limited optical resolution. On the other hand, the phase diagram\textsuperscript{3} mainly concentrates on the region of liquid-liquid coexistence and does not describe the situation in the Sph rich regions: gel, gel-Lo phases. To make our experimental design clear we have produced a “hybrid phase diagram” (Figure S7c) which combines the phase diagrams given in the main manuscript text (Figure 6b) and the diagram given in the manuscript of Veatch and Keller.\textsuperscript{3} Incidentally, this hybrid diagram is very close to that reported recently.\textsuperscript{4}

In the Sph/Chol mixture containing fixed 5 mol% of DOPC, the phase behaviour is expected to be rather complex. At increasing levels of cholesterol between 0-40%, firstly, pure gel phase is present, followed up by the L\textsubscript{o} and gel coexistence region and finally the L\textsubscript{o} phase is formed.\textsuperscript{3,4} However, in our experiments, we did not detect optical phase coexistence up to 30% cholesterol.
Fig. S7 a) Time-resolved fluorescence decays of BODIPY in the DOPC/Sph (5 %)/Chol and DOPC (5 %)/Sph/Chol mixtures containing 0 % (black), 10 % (blue), 20 % (olive), 30 % (red), and 40 % (orange) of cholesterol. b) Dependence of the long-lifetime decay component on the cholesterol content in DOPC/Sph (5 %)/Chol (●) and DOPC (5 %)/Sph/Chol (■). c) Schematic phase diagram combined from refs 2, 3.

By fitting the BODIPY time resolved traces obtained for 0 to 30 % cholesterol to a biexponential decay we detected that the longer lifetime component decreased from 3.9 ± 0.1 to 3.4 ± 0.1 ns, corresponding to viscosity change from 980 to 755 cP (see Figure S7b).

(6) Localisation of the probe in the bilayer: acrylamide quenching.

We have used quenching of BODIPY fluorescence with acrylamide to obtain additional information on the localisation of the dye in the bilayers. Based on the lifetime reduction upon the addition of acrylamide we concluded that the deactivation of the probe is dominated by the collision-based quenching. The higher the quenching efficiency, the steeper is the slope of the Stern-Volmer plot, Figure S8. Thus the gradient characterises accessibility of BODIPY to the quencher and by this, the more outward localisation of the dye within the membrane.
**Fig. S8** Stern-Volmer plots for the BODIPY quenching with acrylamide when incorporated in LUV bilayers containing DOPC at 25 °C (■), DOPC/Chol (75/25) at 25 °C (●), DPPC at 25 °C (▲), and DPPC at 55 °C (▲).

Figure S8 clearly demonstrates that the rotor accessibility to quenching differs significantly upon the phase change of the bilayer. In the pure DOPC bilayer as well as in the 75/25 DOPC/Chol bilayer, the addition of acrylamide results in only a small amount of quenching, which suggests localisation of the fluorophore deep in the membrane. Conversely, in the DPPC bilayer at room temperature (gel phase), the quenching efficiency is significant, which suggests that the fluorophore (or at least one fraction of it) is more exposed to quenching and is probably localised towards the outside of the membrane. This is consistent with the presence of the shorter lifetime component (ca 50 % 1.5 ns) in DPPC bilayers in gel phase. Importantly, when DPPC-containing LUVs are heated above the phase transition temperature (41°C), the quenching is reduced to the level observed in DOPC. Thus the phase change forces the rotor to re-localise inwards. The localisation data reported above give us confidence to conclude that the viscosity changes measured by the BODIPY rotor in the liquid phase bilayers are indeed real.

(7) **Lipid mixtures where the biexponential decay was observed**

Table S1. Representative instances of BODIPY biexponential decay in lipid mixtures at room temperature

<table>
<thead>
<tr>
<th>Lipid/mixture</th>
<th>τ₁ (A₁)</th>
<th>τ₂ (A₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td>1.5 ns (50%)</td>
<td>4.5 ns (50%)</td>
</tr>
<tr>
<td>Sph/Chol 70:30</td>
<td>2.0 ns (50%)</td>
<td>4.8 ns (50%)</td>
</tr>
<tr>
<td>DOPC/Chol/Sph 55:40:5</td>
<td>1.0 ns (12%)</td>
<td>2.2 ns (88%)</td>
</tr>
<tr>
<td>DOPC/Chol/Sph 5:30:65</td>
<td>1.0 ns (12%)</td>
<td>3.4 ns (88%)</td>
</tr>
</tbody>
</table>
(8) References.


