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Supplemental Information

The Affinity of Sterols for Different Phospholipid Classes and Its Impact on Lateral Segregation

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Supporting material

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Methods

Equilibrium distribution of CTL between mβCD and large unilamellar vesicles

CTL partitioning between large unilamellar vesicles (LUVs) and mβCD was measured as described previously (1, 2). In brief, multilamellar vesicles of different PLs with 2 mol% CTL were prepared in buffer. LUVs were prepared by extrusion using membranes with 100-nm pores (Whatman International, Maidstone, UK). The LUVs (final concentration 0.4 mM) were mixed with cyclodextrin (0–1 mM), and the samples were incubated until the equilibrium distribution of CTL between LUVs and cyclodextrin was reached. The steady-state anisotropy of CTL was measured in samples with different mβCD concentrations, and the molar concentration of CTL, C_{CTL}^{LUV}, in the LUVs in each sample was calculated from the measured anisotropies according to

$$C_{CTL}^{LUV} = C_{CTL}\frac{(r_i - r_{CD})}{(r_{LUV} - r_{CD})} \quad [1]$$

where C_{CTL} is the total concentration of CTL in the samples, r_{LUV} is the anisotropy of CTL in the specific PL bilayer, r_i is the CTL anisotropy in the sample, and r_{CD} is the anisotropy of CTL in the CTL–mβCD complex. K_X was calculated by plotting the calculated molar concentrations of CTL in the PL bilayers against the mβCD concentration and fitting the obtained curves with the following equation:

$$C_{CTL}^{LUV} = \frac{C_L - C_{CTL} + (C_{CD})^n/K_X}{2} \times \left(\sqrt{1 + 4\frac{C_L C_{CTL}}{[C_L - C_{CTL} + (C_{CD})^n/K_X]^2}} - 1\right) \quad [2]$$

Here, C_L is the PL concentration, C_{CD} is the cyclodextrin concentration, and C_{CTL}^{LUV} is the cholesterol concentration in lipid bilayers. The PL concentration was determined after anisotropy measurements in all samples so that the correct concentration was used in the calculations. For this, the samples were freeze dried and re-dissolved in methanol, and the PL concentration was determined according to Rouser et al (3). The relative partitioning coefficient K_R was calculated by dividing the K_X obtained with different PL samples with the K_X obtained from samples with only POPC.

Equilibrium distribution of cholesterol between donor and acceptor vesicles

The equilibrium partitioning of cholesterol between large unilamellar donor vesicles (donor LUVs) and large unilamellar acceptor vesicles (acceptor LUVs) was determined using an anisotropy-based assay. Donor LUVs were composed of POPC, 25 mol% cholesterol and

DPH-PC (1 mol%). In studies of unsaturated PL-cholesterol interactions, the acceptor LUVs were composed of pure POPC, OSM, POPE, POPS or POPG. When the interactions between cholesterol and saturated PLs was studied the acceptor LUVs were composed of 80 mol% POPC and 20 mol% DPPC, PSM, DPPE, DPPS, or DPPG. LUVs were prepared as follows. First, the lipids were mixed in organic solvent, and then the solvent was evaporated under a constant stream of nitrogen. The dry lipid film was hydrated in buffer at 60 ºC, after which it was vigorously vortexed and bath sonicated at 60 ºC for 5 minutes. To prepare the LUVs, the vesicles were passed through membranes with a pore size of 100 nm 11 times using an Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL). The PL concentrations were determined after extrusion according to a published procedure (3).

For the experiment, 50 μM donor and 50 μM acceptor LUVs (PL concentration) were mixed together in a glass tube and incubated overnight at 37 ºC, after which the steady-state anisotropy of DPH-PC in the donor LUVs was measured. The amount of cholesterol in the donor LUVs was determined using a standard curve, which was determined for POPC with 0 to 30 mol% cholesterol and 1 mol% DPH-PC at the appropriate temperature. When the total amount of cholesterol was known (determined from the standard curve in samples consisting of only donor LUVs), the cholesterol content in the acceptor LUVs could be calculated, and K_R could also be calculated by dividing the amount of cholesterol in the acceptor LUVs with that in the donor LUVs.

Equilibrium distribution of TF-cholesterol between donor and acceptor vesicles

The distribution of TF-cholesterol between large unilamellar donor vesicles (donor LUVs) and large unilamellar acceptor vesicles (acceptor LUVs) was studied using a Förster resonance energy transfer (FRET) approach, in which the FRET donor was DPH-PC and the FRET acceptor was TF-cholesterol. Donor LUVs were prepared from 100 nmol PL POPC and 0.5 mol% DPH-PC and 0.5 mol% TF-cholesterol. Acceptor vesicles were prepared from 100 nmol PLs. Lipids and fluorophores were mixed in organic solvent, and then the solvent was evaporated under a constant stream of N_2 at 40 ºC. The dry films were re-dissolved in chloroform, and subsequently the solvent was again evaporated. The dry lipid film was hydrated at 60 ºC and vortexed vigorously. The resulting multilamellar vesicles were then filtered 11 times through a polycarbonate filter with a pore size of 100 nm (Whatman International, Maidstone, UK) above 60 ºC. The quality of the formed LUVs was verified with a Malvern Zetasizer Nano-S (Malvern Instruments, Worcestershire, UK).

Donor and acceptor LUVs were mixed in water (total lipid concentration 80 μM) and 0.5 mM mβCD. The donor/acceptor LUV ratio was 1 if not otherwise mentioned. All samples were then incubated at 37 ºC overnight to ensure equilibrium partitioning. Next, the fluorescence lifetime of DPH-PC was measured at the equilibration temperature using a PicoQuant Fluotime 200 spectrometer (PicoQuant, Berlin, Germany) mounted with a 378 nm PDL 800-D pulsed diode laser and a PicoHarp event timer. In addition, the lifetime of DPH-PC in the donor LUVs without cyclodextrin and acceptor LUVs (τ_{INI}) and the lifetime of DPH-PC in donor LUVs without TopFlour-cholesterol (τ_0) were measured at the indicated temperature. The average lifetimes of all samples were calculated using Fluofit software (PicoQuant).

From the time-resolved data, the FRET efficiency (E) was calculated for all samples according to

$$E = 1 - \frac{\tau_i}{\tau_0} \quad [3]$$

As the FRET efficiency has a linear dependence on the concentration of TopFlour-cholesterol in the donor LUVs, the fraction of TopFlour-cholesterol in the donor LUVs (X_D) could be calculated from the FRET efficiency using the following equation (assuming that E is 0 upon total removal of TopFlour-cholesterol from the donor LUVs):

$$X_D = \frac{E_{INI} - E_i}{E_{INI}} \quad [4]$$

where E_{INI} is the FRET efficiency without transfer (with donor LUVs only) and E_i is the FRET efficiency in the particular mixture of donor and acceptor LUVs. When the molar fractions of TopFlour-cholesterol in donor and acceptor LUVs are known, the partitioning coefficient (K) can be calculated according to

$$K = \frac{X_A C_A}{X_D C_D} \quad [5]$$

where X_A and X_D are the molar fraction sterols in the acceptor and donor LUVs and C_A and C_D are the concentrations of acceptor and donor LUVs. The K values are normalized to the K obtained for POPC–POPC partitioning (which was always close to equality), which gives the K_R.

Results

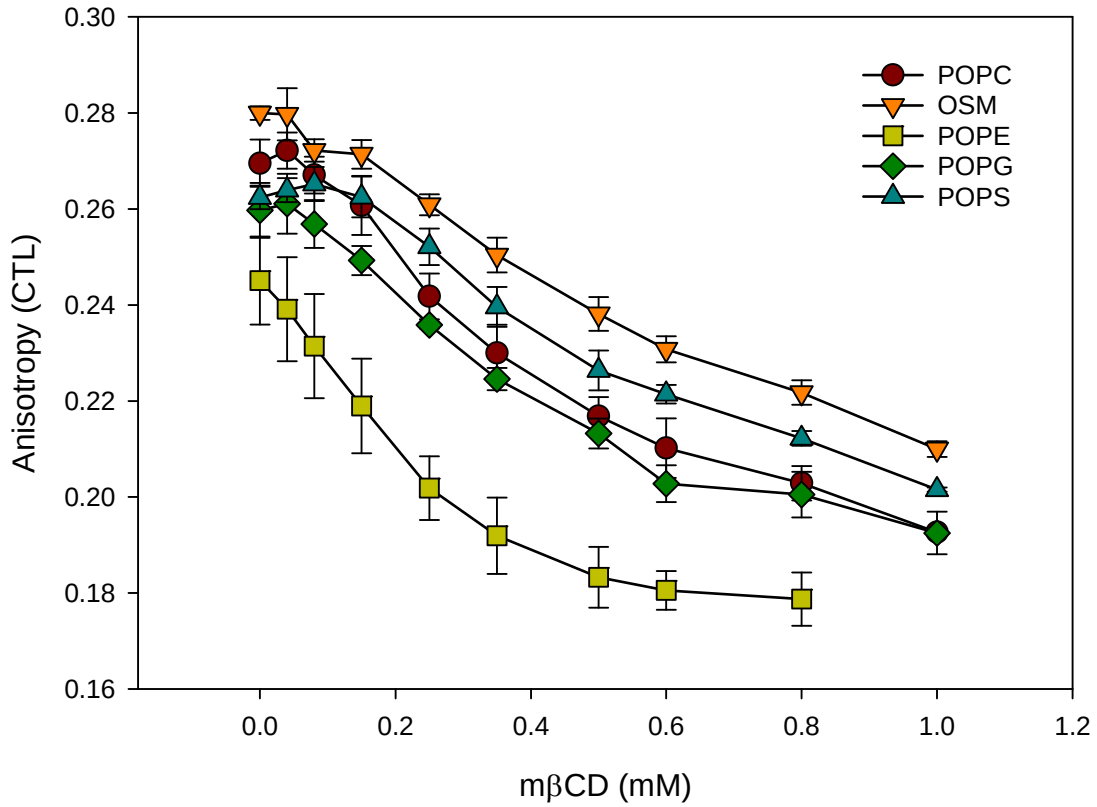

Figure S1. Equilibrium partitioning of cholestratrienol between LUVs and methyl-β-cyclodextrin. Representative data from experiments where the anisotropy of CTL was measured in samples containing different PL vesicles and 0–1.0 mM mβCD. All experiments were performed at 37 ºC.

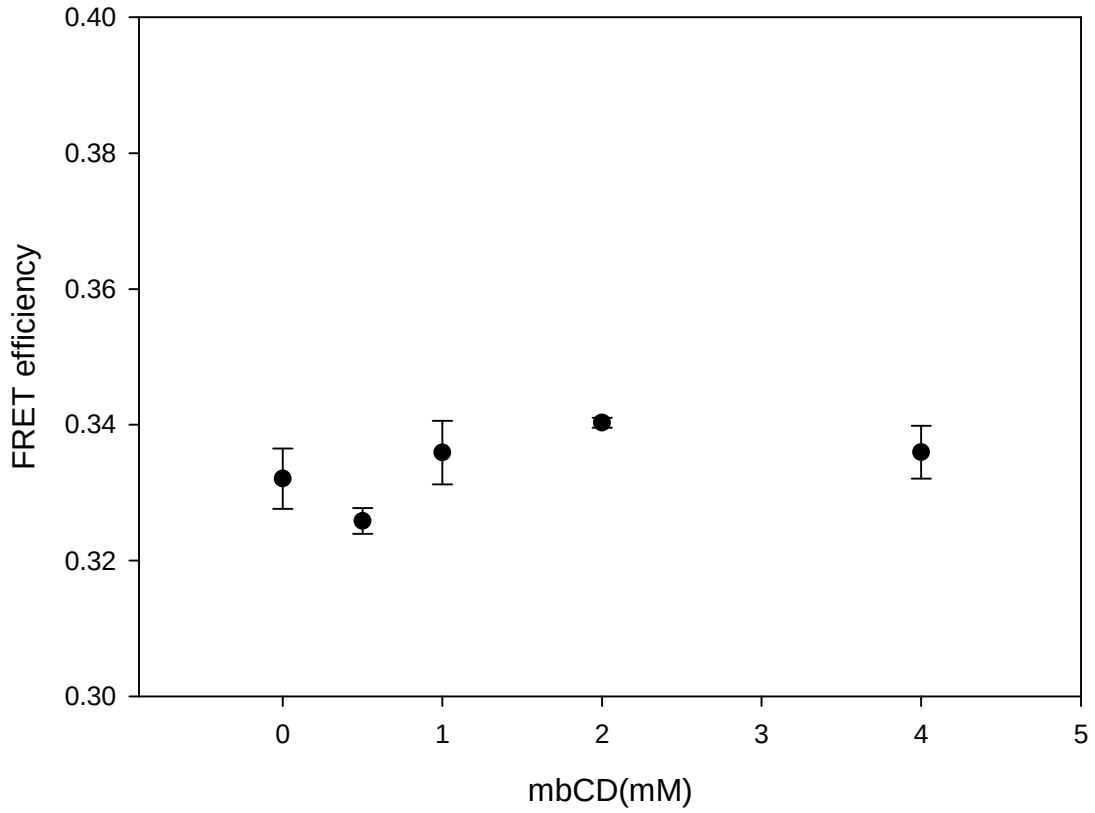

Figure S2. TopFluor-cholesterol was not removed from phospholipid bilayers by mβCD. When measuring FRET between DPH-PC and TopFluor-cholesterol in POPC bilayers in the presence of increasing amounts of mβCD no impact on the FRET efficiency was observed. This suggests that TopFluor-cholesterol was not taken up by mβCD. The experiments were made at 37 ºC, and the values are averages $\pm$ SD with n = 3.

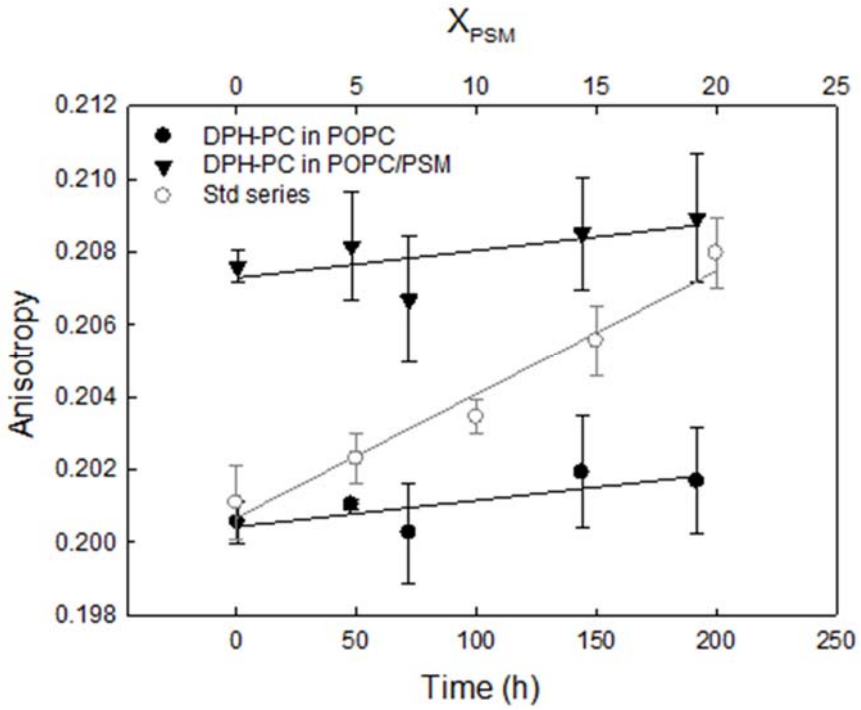

Figure S3. DPH-PC was not transferred between donor and acceptor LUVs. The anisotropy of DPH-PC was measured at 37 ºC in POPC LUVs mixed with POPC/PSM (80:20) LUVs without fluorophores and vice versa. During 200 h of measurements, the DPH-PC anisotropy remained unchanged, indicating that no exchange of probe between the vesicles occurred. The standard curve shows the DPH-PC anisotropy at the same temperature in POPC bilayers as a function of PSM content. Values are average $\pm$ SD with n = 3.

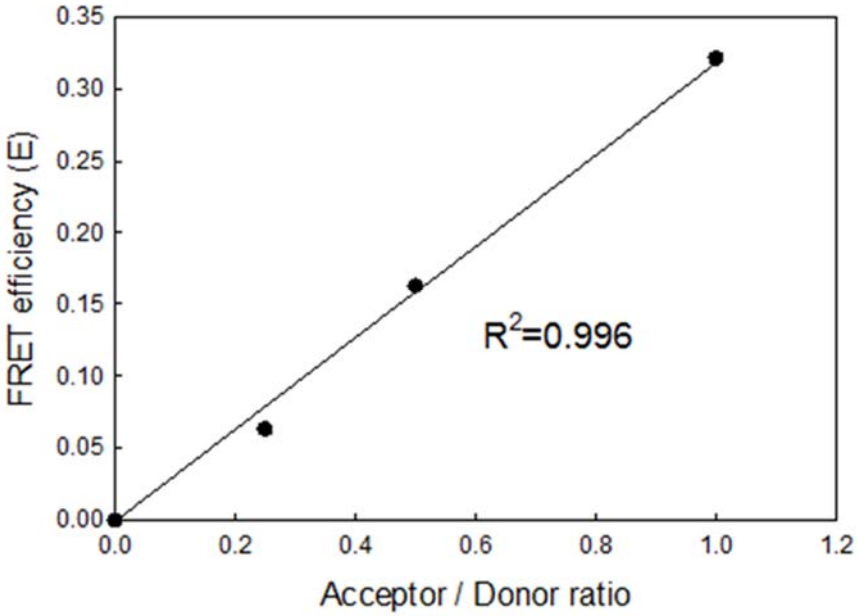

Figure S4. The FRET efficiency had a linear dependency on the acceptor/donor ratio in the concentration range used in the experiments. The FRET efficiency between DPH-PC and TopFluor-cholesterol was determined in POPC LUVs at 37 ºC at different acceptor/donor ratios with a constant DPH-PC concentration. The data in this figure is representative.

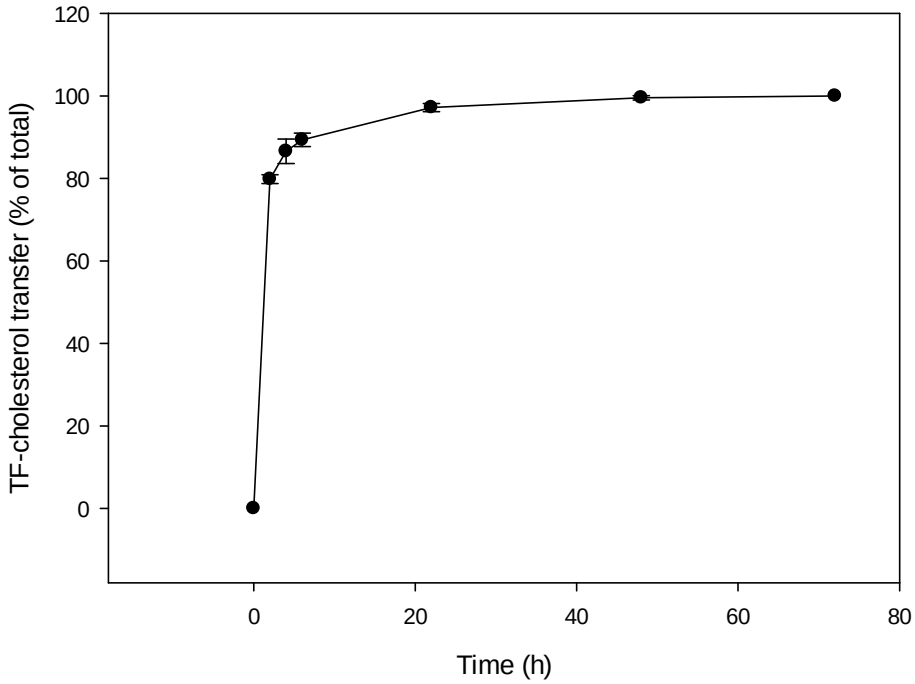

Figure S5. The time-dependence of TF-cholesterol transfer between donor POPC and POPC/PSM (80:20) acceptor LUVs. Donor and acceptor LUVs were mixed 1:1 and incubated at 37 ºC. The FRET efficiency between DPH-PC and TF-cholesterol was measured as a function of time, and transfer percentage was calculated from this data.

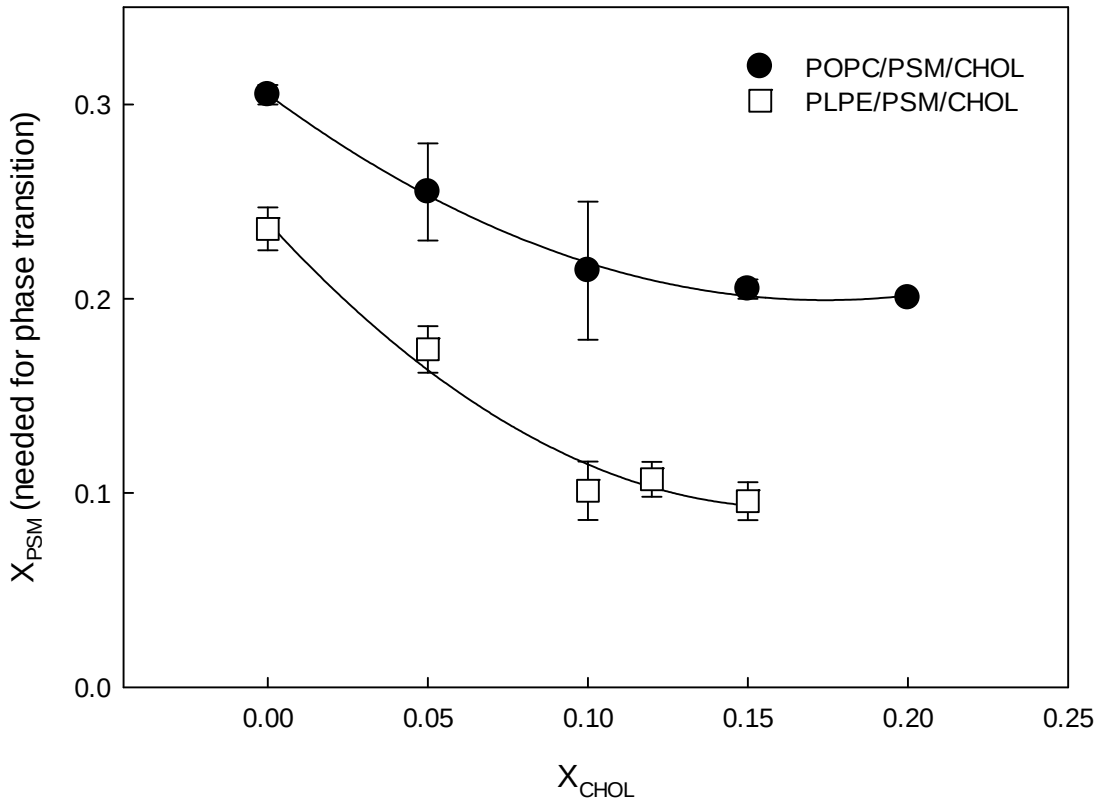

Figure S6. The effect of cholesterol on lateral segregation in PLPE/PSM/Cholesterol bilayers. The formation of gel or l_o domains was determined from tPA lifetimes measured at 23°C. For comparison, the data for the POPC/PSM/cholesterol systems is included. The values are averages $\pm$ SD with n = 3.

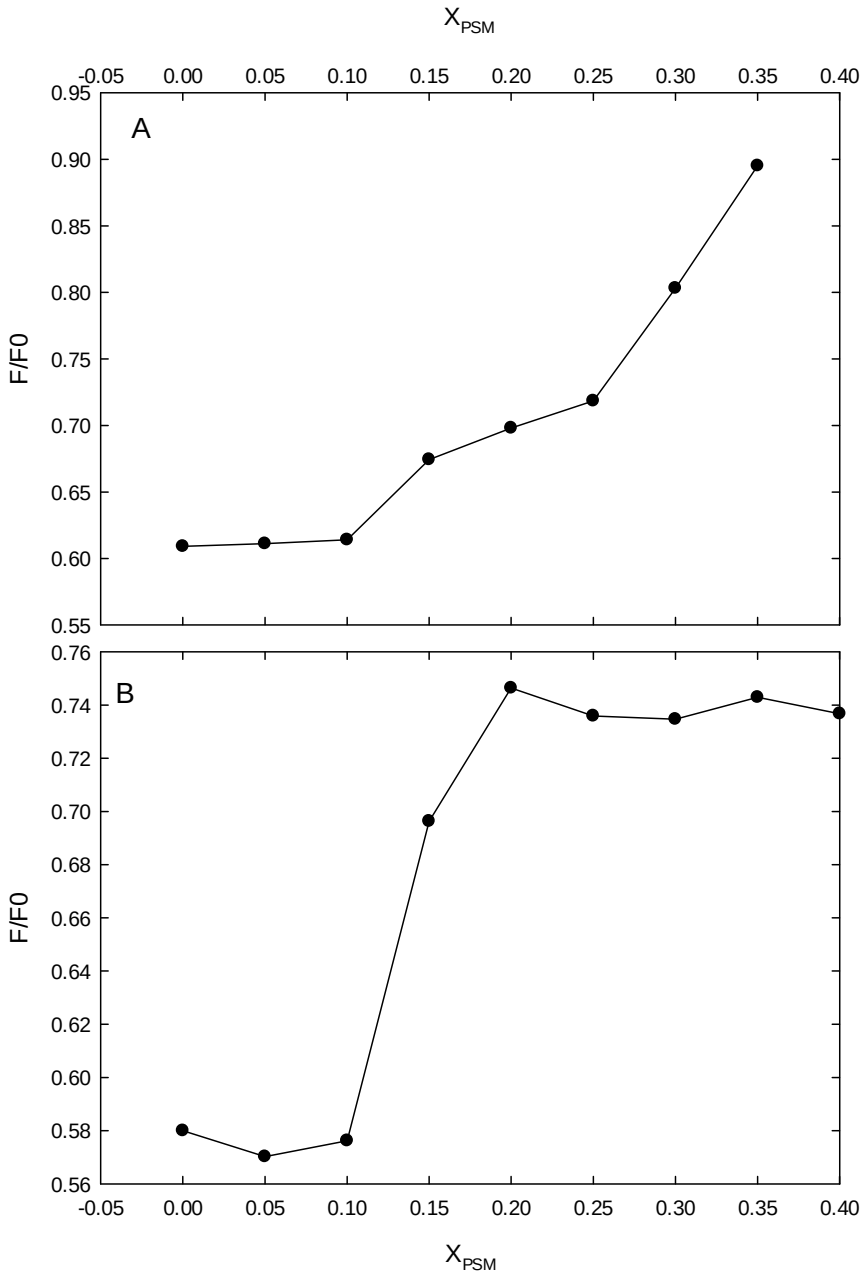

Figure S7. The formation of l_o domains in PLPE/PSM/CHOL bilayers as determined by FRET at 23 ^oC. Using the FRET pair DPH (0.1 mol%) and rhodamine-DOPE (0.5 mol%) the formation of nanodomains can be determined (4). In the PLPE/PSM/CHOL bilayers with both 15 (panel A) and 20 mol% cholesterol (panel B), F/F_0 was more or less unchanged up to 10 mol% PSM, after which it clearly increased. This decrease in FRET efficiency was likely due to the formation of l_o domains, into which only DPH partitioned, which in turn led to a partial separation of DPH and rhodamine-PE in the bilayers. We therefore interpret the data to show that the l_o domains started to form at this ($X_{PSM} = 0.10$) PSM content. The data is representative data from two separate experiments.

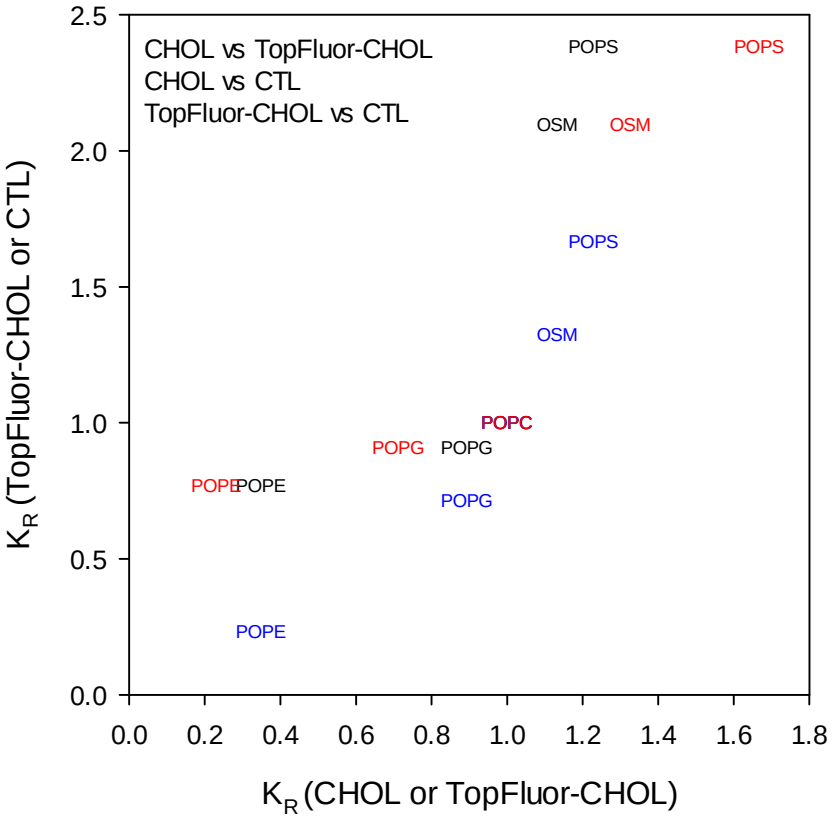

Figure **S8.** Comparison of the partitioning behavior of TopFluor-cholesterol, CTL and cholesterol. To compare the data obtained with the three sterols the K_R determined with one sterol were plotted against another. The K_R values for the sterol first mentioned is shown on the x-axis and values for the second sterol is shown on the y-axis. The values are average values.

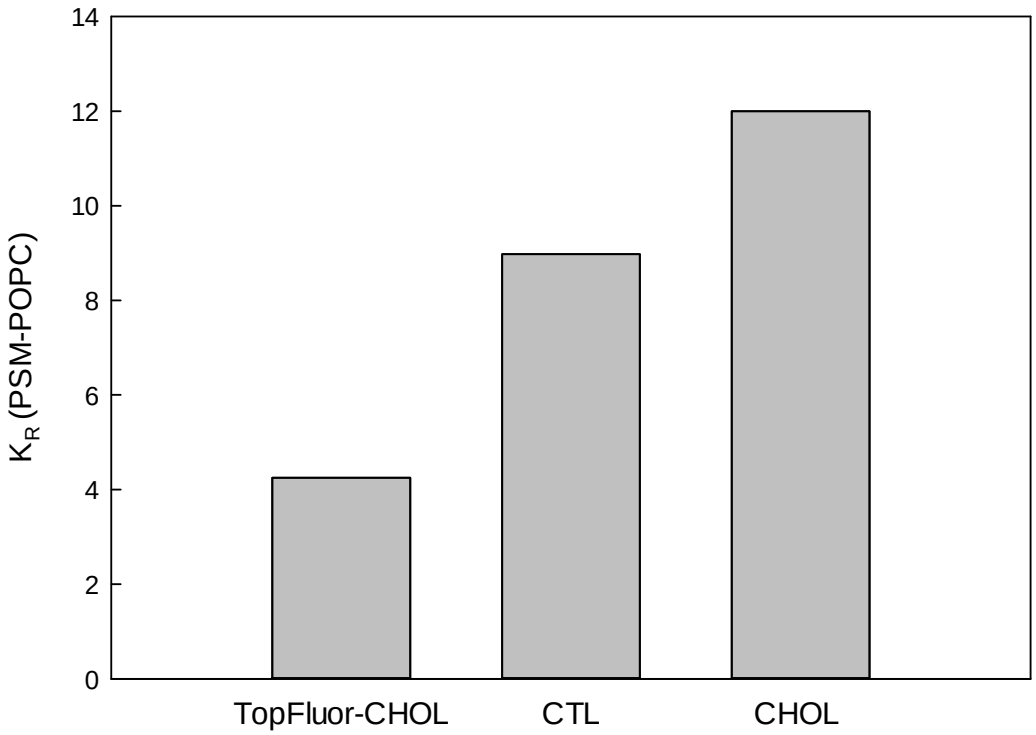

Figure S9. The relative partitioning coefficients determined for the partitioning of different sterols between POPC and PSM LUVs. TopFluor-cholesterol partitioning results obtained at 50 ºC are from (5), the CTL data is from the this work (at 37 ºC) and the PSM data (50 ºC) from (5, 6). The K_R for cholesterol at 50 ºC was obtained from (7). For CTL we were not able to determine the K_R at 50 ºC due to the low fluorescence at this temperature. The values were calculated from two average values.

Table S1. Gibbs free energies for transfer of sterols from POPC to other phospholipids.[a]

Phospholipid	**ΔG (kcal/mol)**		
	CTL	**TF-cholesterol**	**Cholesterol**
POPE	1.08	3.80	2.72
POPG	0.25	0.88	0.29
OSM	-1.91	-0.72	-0.32
POPS	-2.18	-1.32	-0.53
POPC/DPPE	0.32		0.17
POPC/DPPG	-0.41		-0.03
POPC/DPPS	-0.80		-0.06
POPC/DPPC	-1.35		-0.14
POPC/PSM	-1.95		-0.35

[a] All Gibbs free energies where calculated from the K_R values obtains experimentally at 37 ^{o}C with the different sterols. Gibbs free energies for the transfer of sterol from the POPC to bilayers composed of other phospholipids were calculated using the following equation: $\Delta G = -RT \ln K$.

Table S2. Estimated distribution of sterols between the inner and outer leaflets [a]

Average membranes [b]	Cholestatrienol	Cholesterol	TopFluor-cholesterol
Average K (inner)	2.26	2.40	1.28
Average K (outer)	3.48	4.43	1.96
K (outer/inner)	1.54	1.84	1.53
ΔG (kcal/mol)	-1.11	-1.57	-1.09
Fraction of sterol (inner)	0.39	0.35	0.40
Fraction of sterol (outer)	0.61	0.65	0.60

Brain membranes [b]	Cholestatrienol	Cholesterol	TopFluor-cholesterol
Average K (inner)	1.51	0.95	1.29
Average K (outer)	2.51	1.46	3.06
K (outer/inner)	1.67	1.54	1.54
ΔG (kcal/mol)	-1.32	-2.23	-1.54
Fraction of sterol (inner)	0.38	0.30	0.39
Fraction of sterol (outer)	0.62	0.70	0.61

[a] The distribution of cholestatrienol, cholesterol and TopFluor-cholesterol between the inner and outer leaflet of the plasma membranes was calculated using the partitioning coefficients determined for the different sterols between different phospholipid bilayers. First, the Average K for the inner and outer leaflets was calculated by combining the partitioning coefficients of all phospholipid types in the leaflet and normalizing them according to the fraction of each lipid class present in the leaflet. Then K (outer/inner) was calculated by dividing Average K (outer) with Average K (inner). This parameter was then used to calculate the fraction of the different sterols in the two leaflets. Gibbs free energies for the transfer of sterol from the inner to the outer leaflet were calculated from the K (outer/inner) using the following equation: $\Delta G = -RT \ln K$.

[b] The lipid composition in the bilayers that the calculations were based on was based on the composition used in (8).

Supporting References

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