Supplemental Material for "The Dependence of Lipid Asymmetry Upon Phosphatidylcholine Acyl Chain Structure" MiJin Son and Erwin London

Supplemental Table S1. TMADPH fluorescence anisotropy in ordinary, exchange, and scrambled vesicles at 23°C.

The percent ordered state of outer leaflet was calculated from the following equation: Percent ordered = $(A - A_{100\% \text{ Ld}})/(A_{100\% \text{ ordered}} - A_{100\% \text{ Ld}})$. A is that in an exchange vesicle, $A_{100\% \text{ ordered}}$ is that in SM, and $A_{100\% \text{ Ld}}$ is that in the appropriate unsaturated lipid. This formula assumes that gel (=ordered) domain and Ld domains have A values similar to that in pure gel and Ld state vesicles, respectively. TMADPH was added to preformed vesicles at a concentration of 0.1mol % of total lipid concentration. Ordinary (symmetric) vesicles were prepared by ethanol dilution. Average (mean) values and S.D. are shown. Number of samples shown in parentheses.

Sample composition	TMADPH Anisotropy		% ordered	
	Before scrambling	After scrambling	Before scrambling	After scrambling
SM	0.344 ± 0.010 (12)	0.351 ± 0.014 (12)	≡ 100	≡ 100
di 14:1PC	0.215 ± 0.008 (3)	0.225 ± 0.002 (3)	≡ 0	≡ 0
di 16:1PC	0.242 ± 0.010 (3)	0.250 ± 0.012 (3)	≡ 0	≡ 0
di 18:1PC	0.250 ± 0.008 (3)	0.252 ± 0.005 (3)	≡ 0	≡ 0
di 20:1PC	0.263 ± 0.009 (3)	0.261 ± 0.010 (3)	≡ 0	≡ 0
di 22:1PC	0.267 ± 0.012 (3)	0.255 ± 0.005 (3)	≡ 0	≡ 0
DiphyPC	0.284 ± 0.006 (3)	0.282 ± 0.001 (3)	≡ 0	≡ 0
16:0-18:2 PC	0.245 ± 0.014 (3)	0.235 ± 0.010 (3)	≡ 0	≡ 0
16:0-20:4 PC	0.223 ± 0.007 (3)	0.222 ± 0.006 (3)	≡ 0	≡ 0
di 18:2PC	0.252 ± 0.010 (3)	0.251 ± 0.009 (3)	≡ 0	≡ 0
di 18:3PC	0.219 ± 0.006 (3)	0.220 ± 0.006 (3)	≡ 0	≡ 0
di 20:4PC	0.223 ± 0.001 (3)	0.222 ± 0.012 (3)	≡ 0	≡ 0
SMo / di 14:1PC in	0.318 ± 0.013 (7)	0.302 ± 0.008 (7)	81.4 ± 10.4	58.9 ± 6.1
SMo / di 16:1PC in	0.329 ± 0.015 (11)	0.288 ± 0.012 (11)	87.4 ± 14.7	36.4 ± 11.8
SMo / di 18:1PC in	0.339 ± 0.007 (5)	0.281 ± 0.011 (3)	97.8 ± 7.7	28.3 ± 10.7
SMo / di 20:1PC in	0.327 ± 0.005(10)	0.281 ± 0.011 (10)	82.6 ± 6.9	21.7 ± 11.8
SMo / di 22:1PC in	0.333 ± 0.016 (11)	0.281 ± 0.013 (11)	89.1 ± 17.5	25.7 ± 13.2
SMo / DiphyPC in	0.333 ± 0.013 (14)	0.298 ± 0.013 (14)	86.5 ± 22.9	22.4 ± 18.0
SMo / 16:0-18:2PC in	0.333 ± 0.016 (10)	0.305 ± 0.009 (10)	91.6 ± 16.5	58.4 ± 7.9
SMo / 16:0-20:4PC in	0.333 ± 0.016 (17)	0.302 ± 0.014 (17)	92.4 ± 14.7	59.0 ± 10.9
SMo / di 18:2PC in	0.308 ± 0.006 (14)	0.284 ± 0.011 (14)	62.8 ± 7.2	31.9 ± 10.3
SMo / di 18:3PC in	0.293 ± 0.011 (18)	0.298 ± 0.010 (18)	60.9 ± 8.6	57.4 ± 7.4
SMo / di 20:4PC in	0.303 ± 0.011 (9)	0.309 ± 0.011 (9)	67.6 ± 9.7	65.1 ± 8.5

Supplemental Table S2. DPH fluorescence anisotropy in symmetric and exchange vesicles at 23°C.

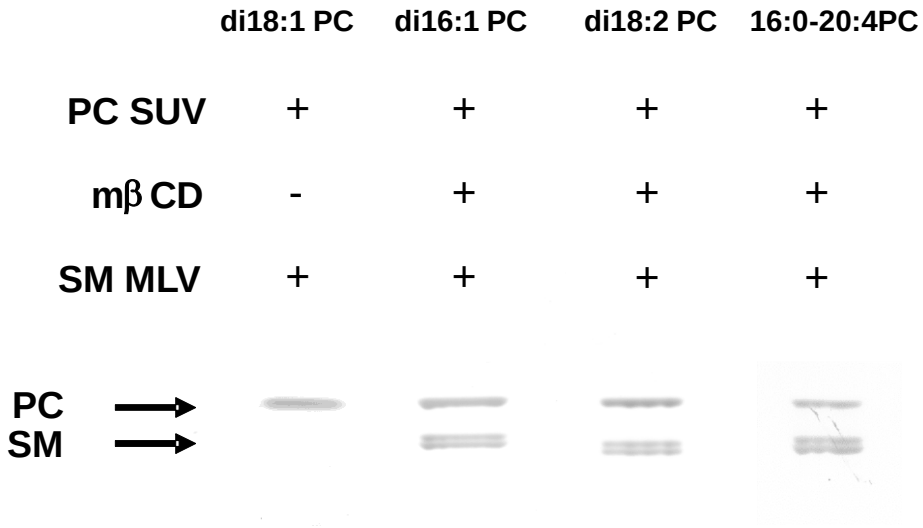
The percent ordered state of outer leaflet was estimated from the following equation: Percent ordered = $(A - A_{100\% \text{ Ld}})/(A_{100\% \text{ ordered}} - A_{100\% \text{ Ld}})$. A is that in an exchange vesicle, $A_{100\% \text{ ordered}}$ is that in SM, and $A_{100\% \text{ Ld}}$ is that in the appropriate unsaturated lipid. This formula assumes that gel (=ordered) domain and Ld domains have A values similar to that in pure gel and Ld state vesicles, respectively. DPH was added to preformed vesicles at a concentration of 0.1mol % of total lipid concentration. Ordinary (symmetric) vesicles were prepared by ethanol dilution. Average (mean) values and S.D. are shown. Number of samples shown in parentheses.

Samples	DPH Anisotropy	% ordered
SM	0.311 ± 0.002 (5)	≡ 100
di 14:1PC	0.088 ± 0.001 (3)	≡ 0
di 16:1PC	0.094 ± 0.008 (3)	≡ 0
di 18:1PC	0.107 ± 0.005 (3)	≡ 0
di 20:1PC	0.107 ± 0.004 (3)	≡ 0
di 22:1PC	0.121 ± 0.002 (3)	≡ 0
diphyPC	0.150 ± 0.003 (3)	≡ 0
16:0-18:2 PC	0.096 ± 0.004 (3)	≡ 0
16:0-20:4 PC	0.085 ± 0.002 (3)	≡ 0
di 18:2PC	0.086 ± 0.002 (3)	≡ 0
di 18:3PC	0.063 ± 0.002 (3)	≡ 0
di 20:4PC	0.058 ± 0.006 (3)	≡ 0
SMo / di 14:1PC in	0.278 ± 0.007 (6)	85.3 ± 4.9
SMo / di 16:1PC in	0.246 ± 0.008 (7)	70.1 ± 6.6
SMo / di 18:1PC in	0.206 ± 0.000 (4)	48.5 ± 3.6
SMo / di 20:1PC in	0.214 ± 0.007 (6)	52.5 ± 4.7
SMo / di 22:1PC in	0.236 ± 0.002 (6)	60.5 ± 3.5
SMo / diphyPC in	0.294 ± 0.013 (7)	89.6 ± 10.1
SMo / 16:0-18:2PC in	0.231 ± 0.025 (10)	62.7 ± 12.1
SMo / 16:0-20:4PC in	0.248 ± 0.021 (10)	71.9 ± 9.8
SMo / di 18:2PC in	0.209 ± 0.004 (7)	54.5 ± 3.0
SMo / di 18:3PC in	0.247 ± 0.0010 (7)	74.2 ± 5.2
SMo / di 20:4PC in	0.258 ± 0.009 (11)	79.2 ± 5.6

Supplemental Table S3. The percent ordered state of leaflets in the exchange vesicles
Values of percent of the outer leaflet in an ordered state, and the per cent of the entire bilayer in an ordered state were from Table S1 and Table S2 The % of the inner leaflet in an ordered state was calculated using the following equation: Inner leaflet % ordered = (% total bilayer ordered – % outer leaflet ordered*0.67) / 0.33. This uses the estimate that the % of total vesicle surface area (and thus lipid) is ~67% in the outer leaflet ~33% in the inner leaflet. Average (mean) values and S.D. are shown.

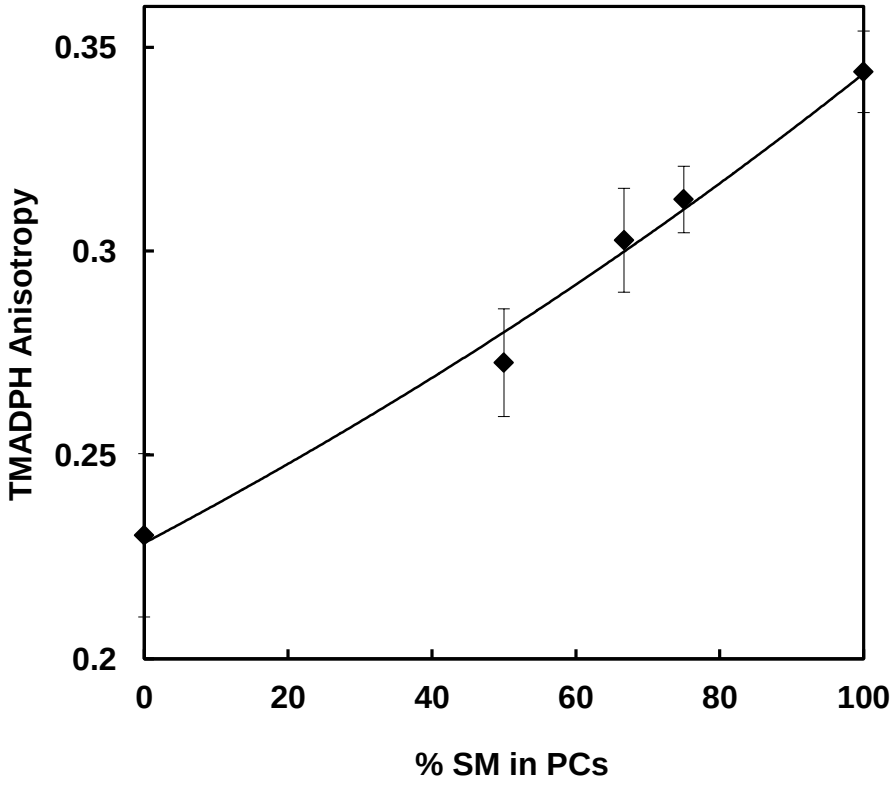
Sample composition	% Ordered		
	Both leaflets	**Outer leaflet**	**Inner leaflet**
SMo / di 14:1PC in	85.3 ± 4.9	81.4 ± 10.4	93.4 ± 25.5
SMo / di 16:1PC in	70.1 ± 6.6	87.4 ± 14.7	35.7 ± 35.5
SMo / di 18:1PC in	48.5 ± 3.6	97.8 ± 7.7	-50.0 ± 18.8
SMo / di 20:1PC in	52.5 ± 4.7	82.6 ± 6.9	-7.5 ± 19.7
SMo / di 22:1PC in	60.5 ± 3.5	89.1 ± 17.5	3.5 ± 36.5
SMo / diphyPC in	89.6 ± 10.1	86.5 ± 22.9	96.1 ± 54.9
SMo / 16:0-18:2PC in	62.7 ± 12.1	91.6 ± 16.5	5.1 ± 49.1
SMo / 16:0-20:4PC in	71.9 ± 9.8	92.4 ± 14.7	31.1 ± 41.6
SMo / di 18:2PC in	54.5 ± 3.0	62.8 ± 7.2	38.1 ± 17.0
SMo / di 18:3PC in	74.2 ± 5.2	60.9 ± 8.6	101.0 ± 23.2
SMo / di 20:4PC in	79.2 ± 5.6	67.6 ± 9.7	102.6 ± 25.7

Supplemental Figure S1. Representative HP-TLC profile of SUV fraction from Sepharose CL-2B chromatography after lipid exchange. Note that SM runs as two closely spaced bands.

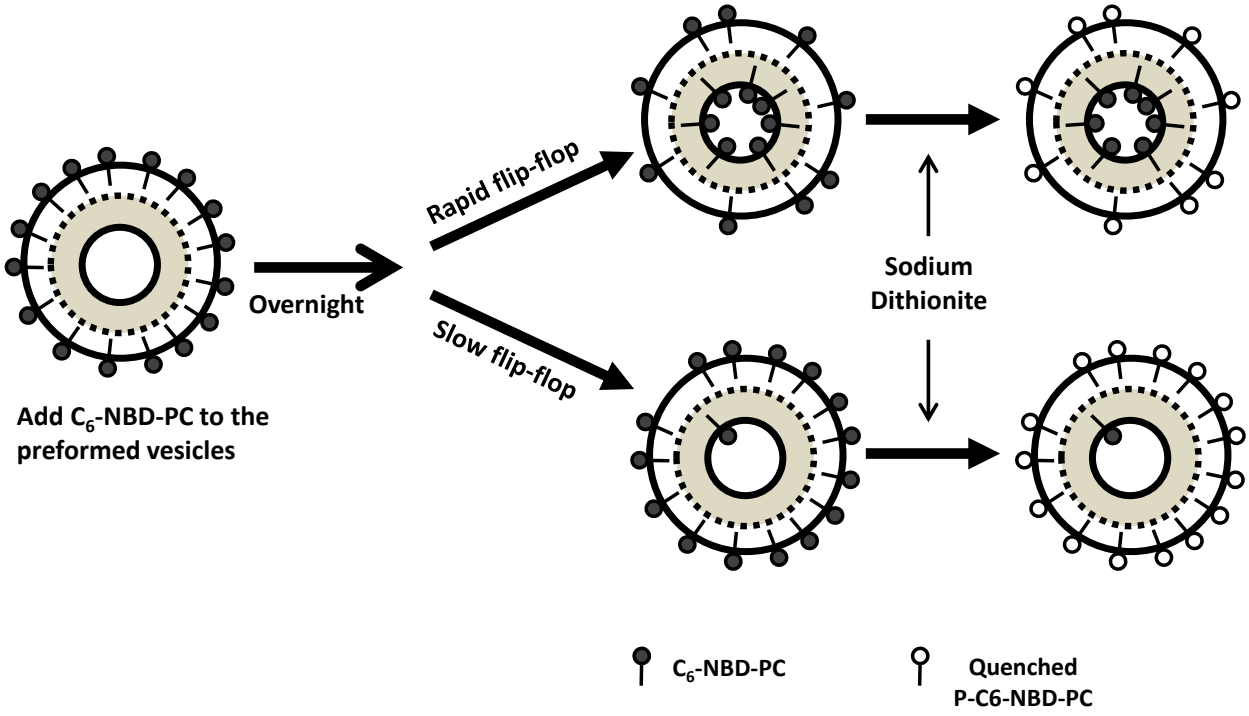


Supplemental Figure S2. Standard curve to estimate the % SM in exchange vesicle outer leaflets at 23°C.

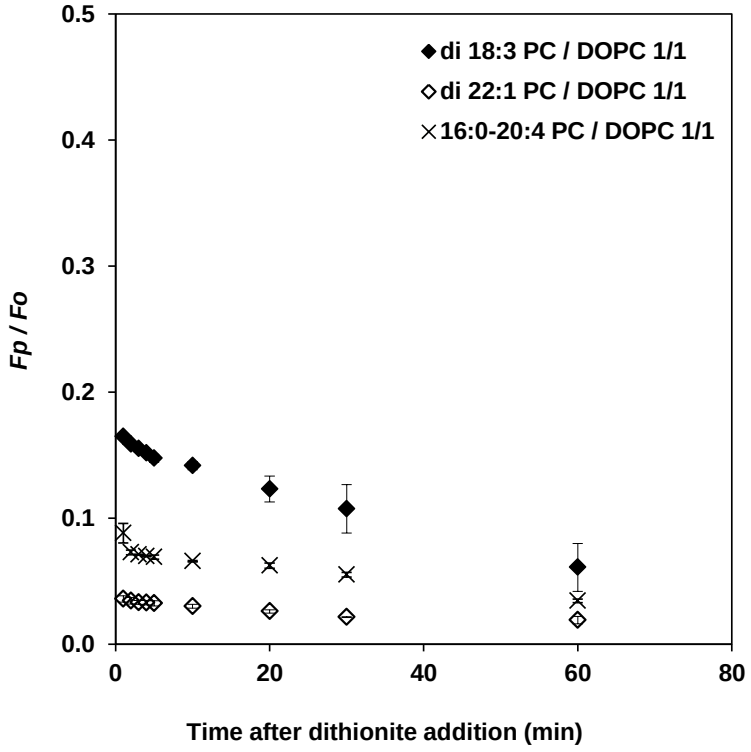
This curve was generated by adding TMADPH (1 mol%) to preformed symmetric vesicles composed of SM/16:0-20:4PC, di18:3PC, or di20:4PC mixtures at varying proportions. These vesicles all gave very similar curves, and the average of the values for these three lipids and S.D. is shown. Similar curves were generated for other binary mixtures of SM with various PC. The symmetric SUVs were made by ethanol dilution. The percent of SM in the outer leaflet was estimated using standard curve fitting (SigmaPlot software, Jandel Scientific, Ercrath, Germany).



Supplemental Figure S3. Schematic representation of transverse diffusion/flip-flop assay. NBD lipids indicated by circles with line attached. Intact (fluorescent) NBD lipids have filled headgroups, chemically quenching (dithionite reduced) NBD lipids shown with unfilled headgroups. Not shown is the case in which flip-flop is so fast that the NBD lipids in the inner leaflet flip to the outer leaflet after dithionite is added. This case was ruled out by preparing vesicles with NBD lipids in both leaflets and showing that the inner leaflet NBD lipid was inaccessible to dithionite (data not shown).

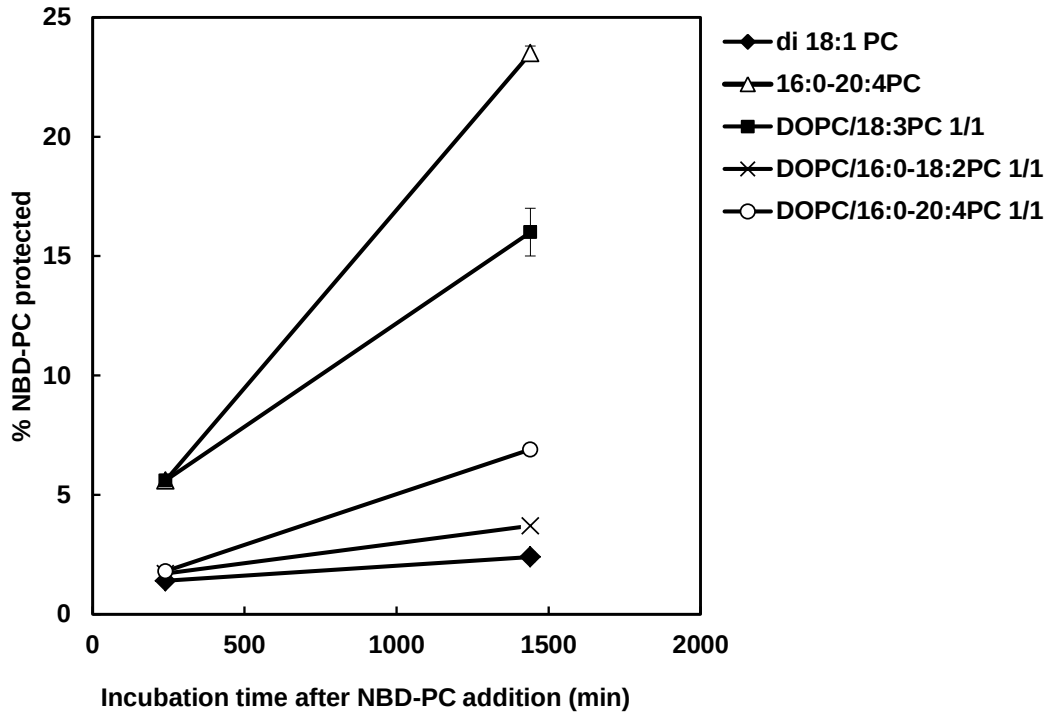


Supplemental Figure S4. Examples of lipid transverse diffusion measurement.
The fraction of C_6-NBD-PC protected (*Fp/Fo*) is the ratio the C_6-NBD-PC fluorescence protected (*Fp*) from external dithionite to initial NBD fluorescence emission (*Fo*) before sodium dithionite addition. The fluorescence emission intensity was recorded for 60 minutes after dithionite addition. Average (mean) values and range derived from duplicate samples are shown. Similar curves were generated for other lipid mixtures**.** Experimental protocol is given in the Methods section. *Fp/Fo*=1 at time zero (not shown due to being off-scale).



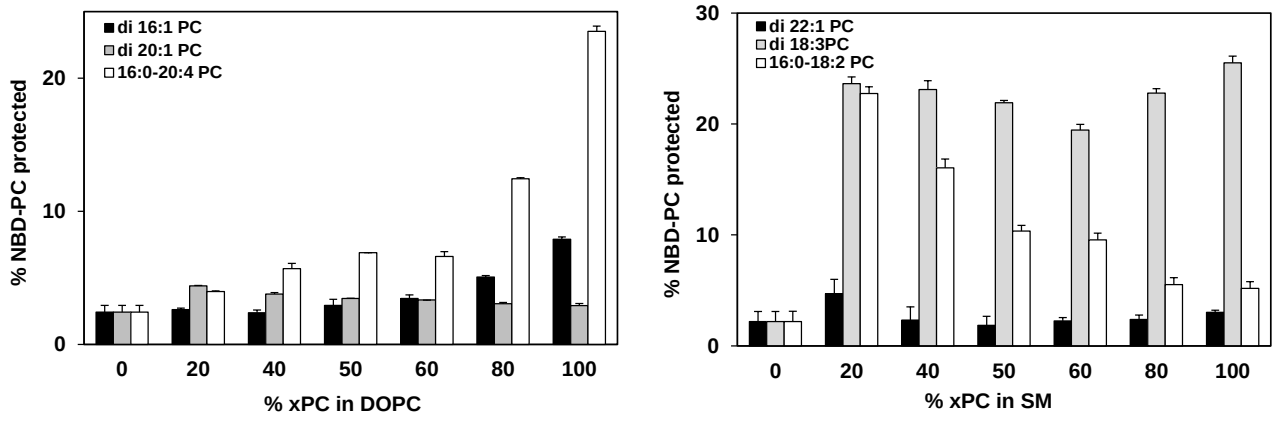
Supplemental Figure S5. Effect of incubation time on protection of C_6-NBD-PC from dithionite in symmetric SUVs

The % NBD protection (which is equal to the % NBD lipid in the inner leaflet) after a 4 h or ~24 h incubation time is shown. Average values and range derived from duplicate samples are shown.



Supplemental Figure S6. Transverse diffusion of C_6-NBD-PC in symmetric SUVs containing different mixtures of PC and SM.
Symmetric SUVs composed of di16:1PC, di20:1PC, di22:1PC, di18:3PC, 16:0-18:2PC, or 16:0-20:4PC mixed with DOPC or SM were used to examine the extent of transverse diffusion as a function of lipid composition. Analogous curves were generated for the other PC combinations with DOPC and SM. Average values and range derived from duplicate samples are shown.

Notice that in mixtures of DOPC (which allows only slow transverse diffusion) with different PC species that showed faster transverse diffusion, the extent of transverse diffusion tended to increase as the fraction of DOPC decreased. However, the effect was not linear in the fraction of DOPC in the membrane. The effect of SM concentration upon transverse diffusion in mixtures of SM with PC was more complex, with maximal C_6-NBD-PC transverse diffusion often occurring at intermediate SM contents. This probably reflects de-mixing of unlabeled PC and SM into co-existing SM-rich gel and PC-rich liquid disordered domains in these vesicles. C_6-NBD-PC would locate in the PC-rich domains, which would result in it having significant transverse diffusion if the unlabeled PC species present tends to support fast transverse diffusion. Fast transverse diffusion may also occur in such mixtures at the boundaries between the gel and Ld domains.



Supplemental Figure S7. Transverse diffusion rates of C_6-NBD-PC in symmetric LUVs and SUVs

The effect of curvature on transverse diffusion as estimated from protection from was dithionite was assayed as described above. Left: Large unilamellar vesicles (LUV). Right: Small unilamellar vesicles (SUV). Symmetric LUVs were prepared by freeze/thaw and extrusion of MLVs through polycarbonate filters with a pore size of 100 nm as described in Methods. Symmetric SUVs were prepared by ethanol dilution. Average values and range derived from duplicate samples are shown.