Supplemental Data

Preparation and Properties of Asymmetric Vesicles That Mimic Cell Membranes: Effect Upon Lipid Raft Formation and Transmembrane Helix Orientation

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Supplemental Methods

Extraction of TMADPH from SM MLV by MβCD— TMADPH (0.05mol% of total lipid concentration) was added to each of 2 tubes of 200μM SM MLV. After incubating at room temperature for 5 min, the initial TMADPH fluorescence of each sample was measured. Then, 10mM MβCD (16μl from 625mM stock solution dissolved in PBS) was added into one of the SM MLV tubes. In the control sample, an equivalent volume of PBS was added. After 5 min incubation at room temperature TMADPH fluorescence intensity was re-measured. Both samples were then centrifuged at 49,000 x g for 5 min using an air-driven microultracentrifuge (Beckman Airfuge, Fullerton, CA). For each sample, the supernatant was transferred to a new glass tube and the pellet was then re-suspended with PBS. Optical density at 450nm and TMADPH fluorescence of the supernatants and pellets were measured as described in Experimental Procedures.

Ca^{2+}-induced vesicle aggregation—To measure Ca^{2+}-induced vesicle aggregation ordinary SUV and exchange SUV were made as described above except that 137 mM NaCl, 20 mM Tris-Cl pH 7.4 (Tris buffer) was substituted for PBS. Optical density at 450 nm was measured and then the samples were titrated with aliquots from a 200 mM solution of $CaCl_2$ dissolved in water. Samples were mixed by pipetting immediately after adding $CaCl_2$ and (to avoid vesicle settling) again immediately before optical density measurements. One minute after the addition of each aliquot optical density was measured on a Beckman 640 spectrophotometer. The change in optical density was calculated after correcting for the Tris buffer background by subtracting the value of optical density in the absence of $CaCl_2$. Ordinary vesicles containing SM and/or cholesterol were prepared by ethanol dilution, while ordinary vesicles lacking both SM and cholesterol were prepared by sonication. Control experiments in which ethanol was added to sonicated preparations showed ethanol had no significant effect upon Ca^{2+}-induced aggregation (data not shown).

Supplemental Results

Confirming asymmetry using Ca^{2+}-induced vesicle aggregation— A second asymmetry assay involved detection of the amount of PS on the outside of exchange vesicles via Ca^{2+}-induced vesicle aggregation. Vesicles containing the anionic lipid PS in their outer leaflet aggregate and fuse extensively in the presence of externally added Ca^{2+} (1). This process can be detected by an increase in light scattering (optical density). If PS is restricted to the inner leaflet of vesicles should be

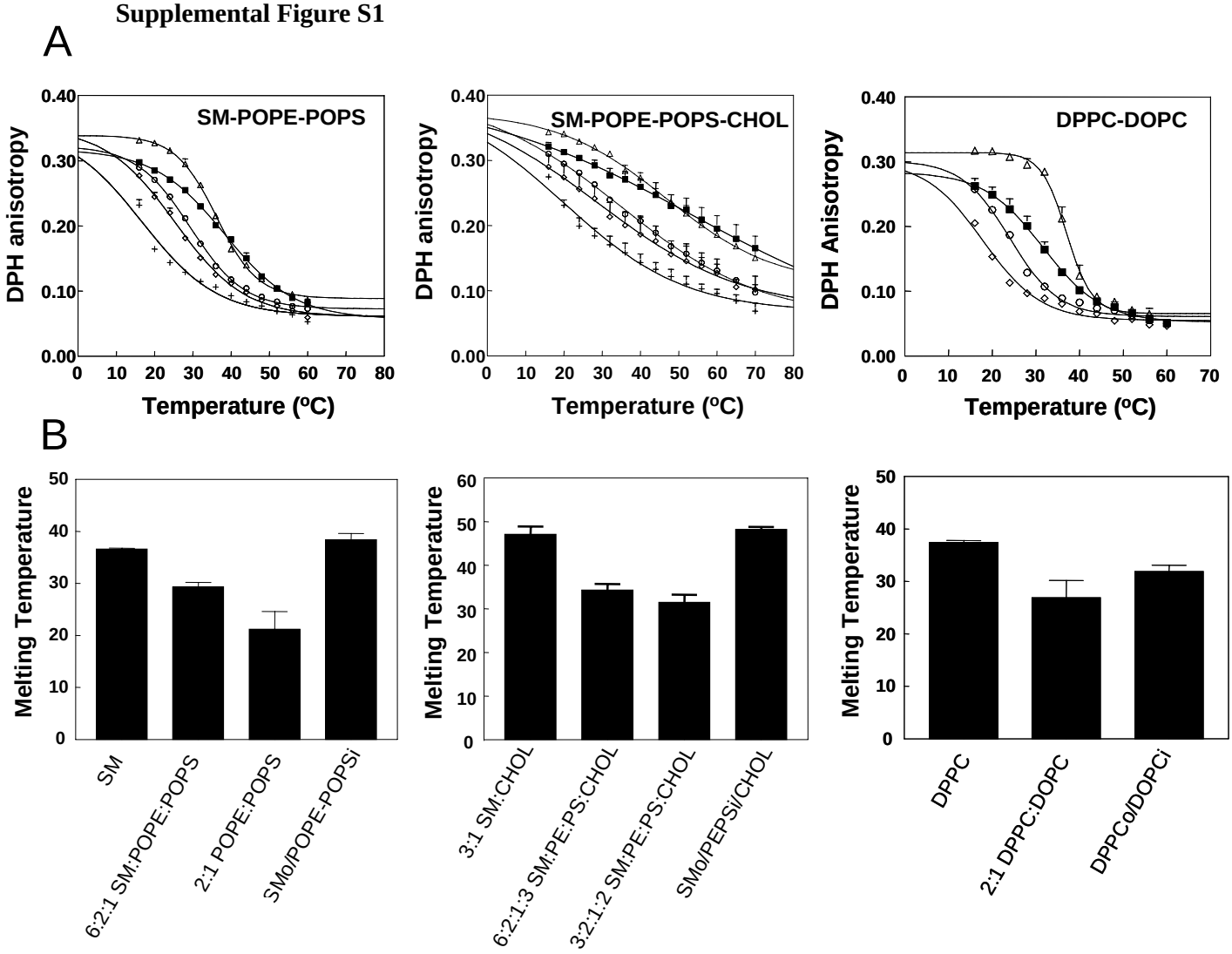
inaccessible to Ca^{2+}, and should not contribute to Ca^{2+}-induced vesicle aggregation.

The behavior of ordinary and exchange vesicles containing POPS in the presence of Ca^{2+} was consistent with the predictions that the exchange vesicles were asymmetric. As shown in Fig. S4 *A*, SM vesicles did not aggregate in the presence of Ca^{2+}, while ordinary 1:1 mol:mol POPE:POPS vesicles extensively aggregated. POPE:POPS vesicles mixed with SM vesicles aggregated to a similar degree as POPE:POPS vesicles in the absence of SM vesicles, indicating that SM vesicles do not participate in, or interfere with, the aggregation of POPE:POPS vesicles. In contrast SMo/POPE:POPSi exchange vesicles with a POPE and POPS content comparable to that in the mixture of separate SM and POPE:POPS vesicle populations showed very little aggregation, inconsistent with the hypothesis that the exchange preparation consisted of separate POPE:POPS SUV and SM SUV populations, but consistent with an asymmetric lipid distribution with POPS being largely restricted to the inner leaflet. This conclusion was supported by the observation that ordinary vesicles composed of 2:1:1 or 6:1:1 SM:POPE:POPS aggregate to a greater degree (increase in optical density of 0.039 and 0.009, respectively at 18 mM $CaCl_2$, respectively) than the MβCD exchange vesicles (increase in optical density of 0.0008 at 18 mM $CaCl_2$) even though the ordinary vesicles contained a lower amount of POPS than the exchange vesicles.

The small degree of Ca^{2+}-induced aggregation in the ordinary vesicles presumably reflects a strong dependence of aggregation upon POPS density in the outer leaflet, but the presence of POPE complicates interpretation. To rule out complications due to POPE, analogous experiments were carried out upon SM-POPS vesicles (Fig. S4 *B*). Again, the level of aggregation in a mixture of SM and POPS vesicles was much higher than in SMo/POPSi exchange vesicles. Furthermore, the differences between ordinary SM:POPS and SMo/POPSi exchange vesicles were analogous to those observed in the SM-POPE-POPS vesicles, but larger (Fig. S4, *A* and *B*). This confirmed the exchange vesicles do not consist of a mixture of unexchanged vesicle populations and are asymmetric, with SM in the outer leaflet and the unsaturated glycerophospholipid in the inner leaflet.

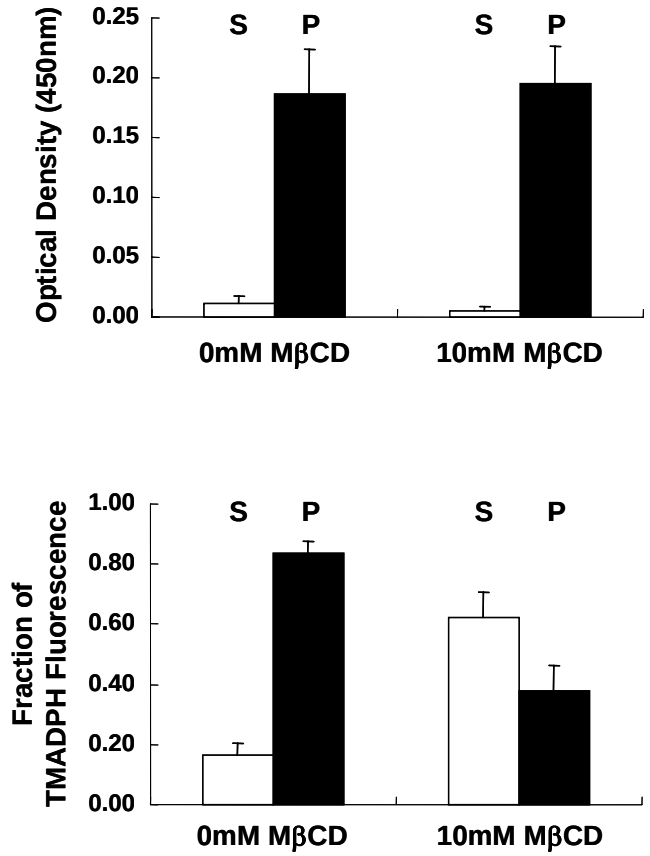
Similar results were found for analogous ordinary and exchange vesicles containing cholesterol (Fig. S4 *C*), indicating that the introduction of cholesterol did not disrupt asymmetry.

Supplemental Figure S1



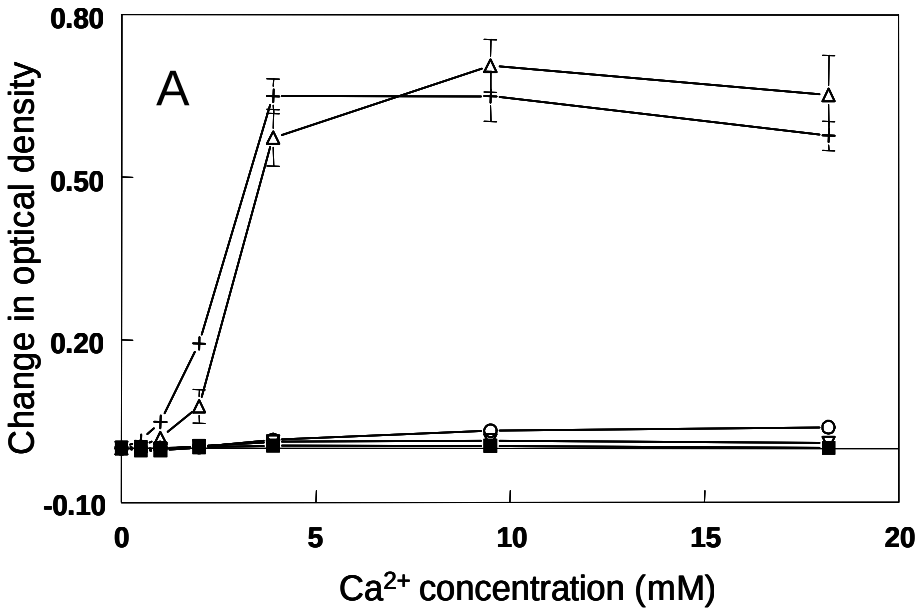
Measurement of ordered domain thermal stability in SM-POPE-POPS, SM-POPE-POPS-cholesterol, and DPPC-DOPC vesicles. (A) Temperature dependence of DPH anisotropy in ordinary and exchange vesicles. Left panel: (Δ) SM vesicles; (o) ordinary 6:2:1 SM:POPE:POPS vesicles; (◊) ordinary 3:2:1 SM:POPE:POPS vesicles; (■) SMo/2:1 POPE:POPSi vesicles; (+) 2:1 POPE:POPS vesicles. Middle panel: (Δ) 3:1 SM:CHOL vesicles; (o) ordinary 6:2:1:3 SM:POPE:POPS:CHOL vesicles; (◊) ordinary 3:2:1:2 SM:POPE:POPS:CHOL vesicles; (■) SMo/2:1 POPE:POPSi/CHOL vesicles with ~25 mol% CHOL; (+) 2:1:1 POPE:POPS:CHOL vesicles. Right panel: (Δ) DPPC vesicles; (o) ordinary 2:1 DPPC:DOPC vesicles; (◊) ordinary 1:1 DPPC:DOPC vesicles; (■) DPPCo/DOPCi vesicles. PBS-dispersed samples contained ~50 μM total lipid with 0.1% DPH. Lipid ratios given for exchange vesicles are approximate. Average anisotropy values from triplicate experiments and standard deviations are shown. (B) Tm values for curves shown in (A). The average Tm derived from each sample and the standard deviation in Tm values is shown. X-axis labels give vesicle lipid compositions.

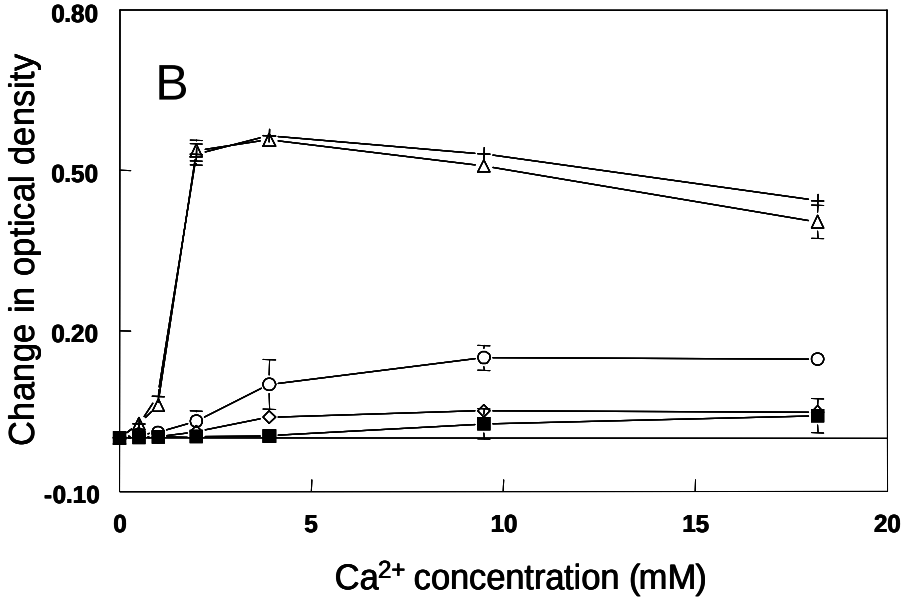
Supplemental Figure S2

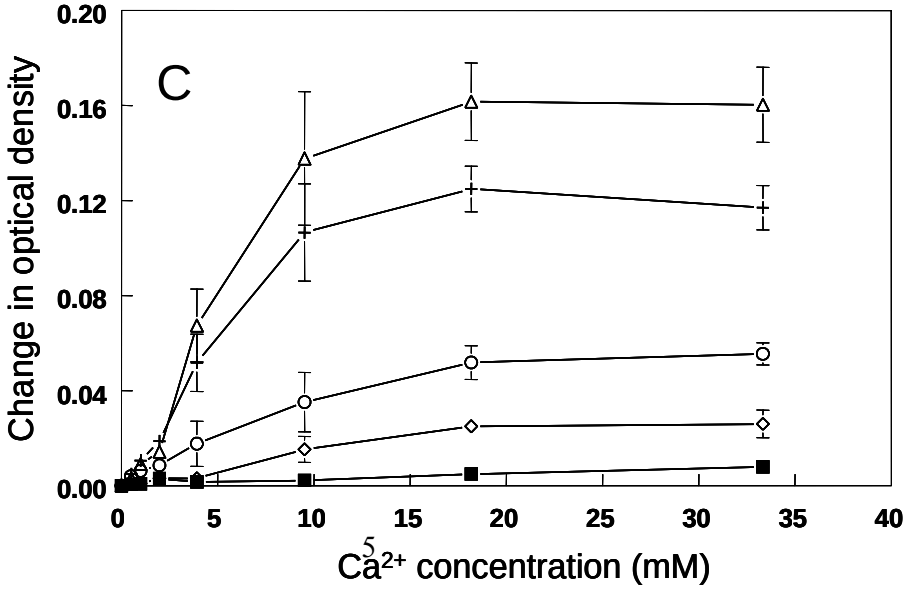


MβCD-induced extraction of TMADPH from SM MLV assayed by centrifugation. Upper panel: Optical density at 450nm of supernatant (S) and pellet (P) from 200μM SM MLV treated with or without 10mM MβCD. Lower panel: TMADPH fluorescence of supernatant (S) and pellet (P) from 200μM SM MLV treated with or without 10mM MβCD. Relative TMADPH fluorescence of pellet or supernatant was normalized to the sum of TMADPH fluorescence from supernatant and pellet in each sample. Notice that due to the decrease in TMADPH fluorescence upon binding to MβCD the fraction of TMADPH bound to MβCD is much higher than the fraction of fluorescence of TMADPH bound to MβCD. In both panels, samples contained 0.05 mol% TMADPH in the outer leaflet of the MLV. Average values from triplicate experiments and standard deviations are shown.

Supplemental Figure S3

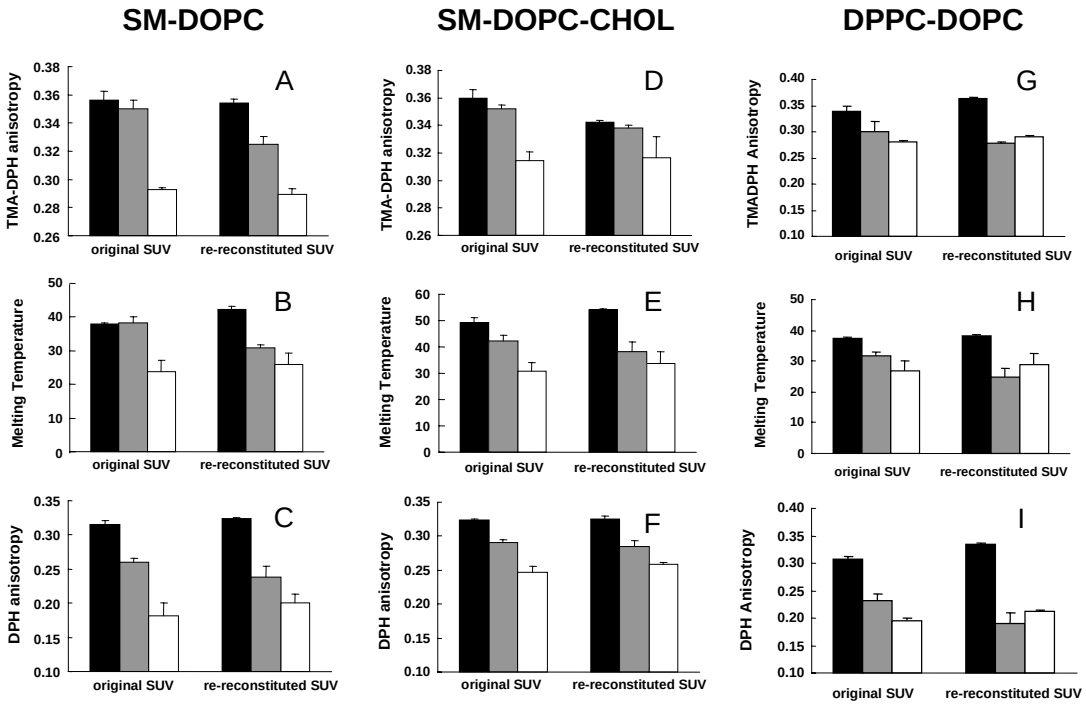






Comparison of the sensitivity of ordinary and exchange vesicles to Ca^{2+}-induced aggregation. The effect of aliquots of 200 mM $CaCl_2$ titrated into the samples at room temperature upon optical density at 450 nm was measured for: (A) SM-POPE-POPS vesicles: (Δ) ordinary vesicles composed of 200 μM 1:1 mol:mol POPE:POPS; (+) mixture of ordinary vesicles composed of 200 μM 1:1 mol:mol POPE:POPS plus vesicles composed of 220 μM SM; (o) ordinary vesicles composed of 200 μM 2:1:1 mol:mol SM:POPE:POPS; (◊) ordinary vesicles composed of 200 μM 6:1:1 mol:mol SM:POPE:POPS; (■) exchange vesicles composed of 333 μM SMo/113 μM POPE:130 μM POPSi (average of three preparations). Values shown are the average and standard deviation for 3-4 preparations. (B) SM-POPS vesicles: (Δ) ordinary vesicles composed of 150 μM POPS SUV; (+) mixture of ordinary vesicles composed of 150 μM POPS plus vesicles composed of 200 μM SM; (o) 200 μM ordinary vesicles composed of 1:1 mol:mol SM:POPS; (◊) ordinary vesicles composed of 200 μM 3:1 mol:mol SM:POPS; and (■) exchange vesicles composed of 230 μM SMo/177 μM POPSi (average of two preparations). Values shown are the average and standard deviation for 2-4 preparations. (C) SM-POPE-POPS-CHOL vesicles: (Δ) ordinary vesicles composed of 100 μM 3:3:2 mol:mol POPE:POPS:CHOL; (+) mixture of ordinary vesicles composed of 100 μM 3:3:2 mol:mol POPE:POPS:CHOL plus vesicles composed of 100 μM 3:1 SM:CHOL; (o) ordinary vesicles composed of 200 μM 6:3:3:4 mol:mol SM:POPE:POPS:CHOL; (◊) ordinary vesicles composed of 200 μM 18:3:3:8 mol:mol SM:POPE:POPS:CHOL; (■) exchange vesicles composed of 142 μM SMo/44 μM POPE:60 μM POPSi/56 μM CHOL. Lipid concentrations in the exchange vesicles in these experiments were estimated by HP-TLC. Values shown are the average and range for two to three preparations, except in the case of the exchange vesicles where a single preparation was used. In all cases above vesicles were prepared in 137 mM NaCl/20 mM Tris-Cl buffer pH 7.4 instead of PBS. The addition of $CaCl_2$ to solution by itself has no effect upon optical density.

Supplemental Figure S4



Effect of re-reconstitution upon the level and thermal stability of ordered domains in SM-DOPC, SM-DOPC-cholesterol, and DPPC-DOPC vesicles. Panels A-C: SM-DOPC vesicles. Panels D-F: SM-DOPC-CHOL vesicles. Panel G-I: DPPC-DOPC vesicles. (A), (D) and (G): TMADPH anisotropy at room temperature. (B), (E) and (H): Ordered domain Tm. (C), (F) and (I): DPH anisotropy at room temperature. Black bars: SM vesicles (panel A-C), 3:1 SM:CHOL vesicles (panel D-F), or DPPC vesicles (panel G-I). Gray bars: Exchange SMo/DOPCi vesicles (panels A-C), SMo/DOPCi vesicles with ~25 mol% cholesterol (panels D-F), or DPPCo/DOPCi vesicles (panel G-I). Unfilled bars: Ordinary 1:1 SM:DOPC vesicles (panels A-C), ordinary 3:3:2 SM:DOPC:CHOL vesicles (panels D-F), or 2:1 DPPC:DOPC vesicles (panel G-I). Original SUV= SUV before re-reconstitution. Re-reconstituted SUV = SUV after re-reconstitution. Average values from triplicate experiments and standard deviations are shown.

Supplemental Reference

1. Portis, A., Newton, C., Pangborn, W., and Papahadjopoulos, D. (1979) *Biochemistry* **18**, 780-790