Supplemental Material for:

Effect of Sterol Structure on Ordered Membrane Domain (Raft) Stability in Symmetric and Asymmetric Vesicles

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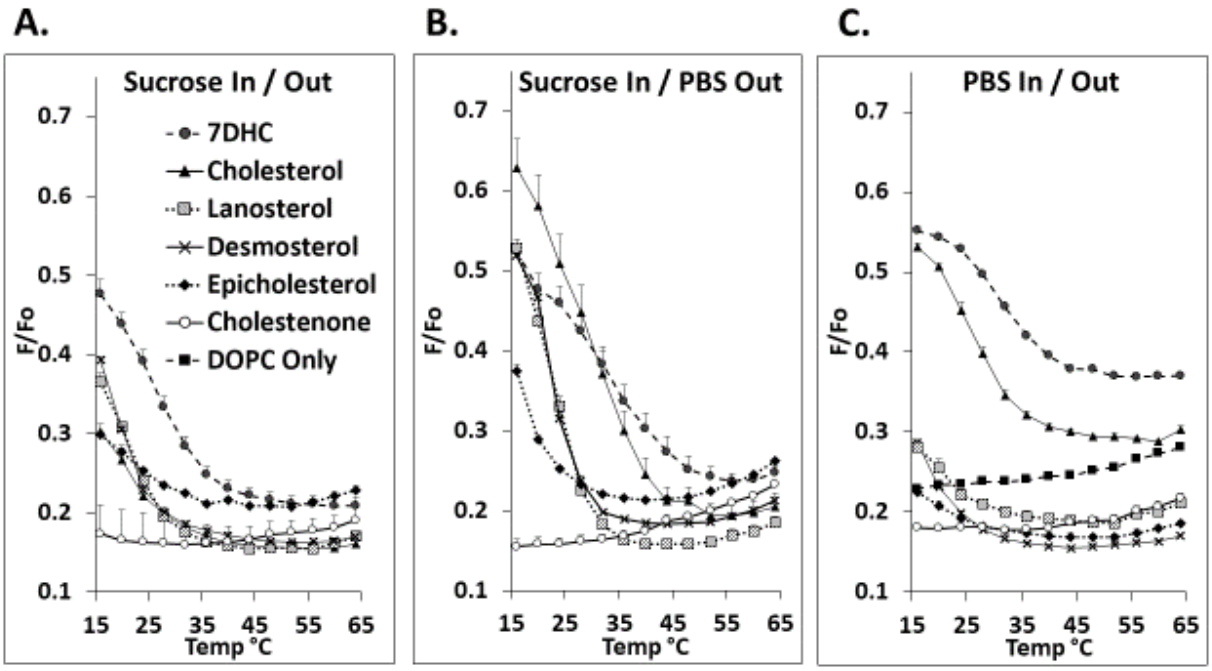


Supplemental Figure 1: Unnormalized domain melting curves for symmetric vesicles assayed by FRET. The fraction of DPH fluorescence unquenched by rhodamine lipid (F/Fo) versus temperature is shown. Samples contained LUV with 100 µM 1:1 mol:mol DOPC:SM with 25 mol% sterol. Samples also contained 0.1 µM DPH and F samples contained an additional 2 mol% rhodamine-DOPE. In panels A and B, vesicles were formed with entrapped 25% (w/w) sucrose and dispersed in either sucrose (A) or PBS (B). In C, vesicles were formed with entrapped PBS and dispersed in PBS. Mean and standard deviation from three separate experiments are shown. Symbols: Filled circles, 7DHC; triangles, cholesterol; shaded squares, lanosterol crosses, desmosterol; diamonds, epicholesterol; open circles, 4-cholesten-3-one; filled squares, no sterol.

Sterol	Symmetric LUV T_{end} - T_{mid}		
	PBS In PBS Out	**Sucrose In PBS out**	**Sucrose In Sucrose Out**
7DHC	24.6 ± 0.7	25.1 ± 1.3	34.0 ± 0.3
Cholesterol	28.7 ± 0.6	26.4 ± 0.5	30.4 ± 0.6
Desmosterol	28.2 ± 2.3	20.9 ± 1.1	38.1 ± 1.4
Lanosterol	26.5 ± 2.3	21.1 ± 0.8	22.3 ± 0.9
Epicholesterol	23.5 ± 2.4	24.7 ± 4.5	24.0 ± 1.6

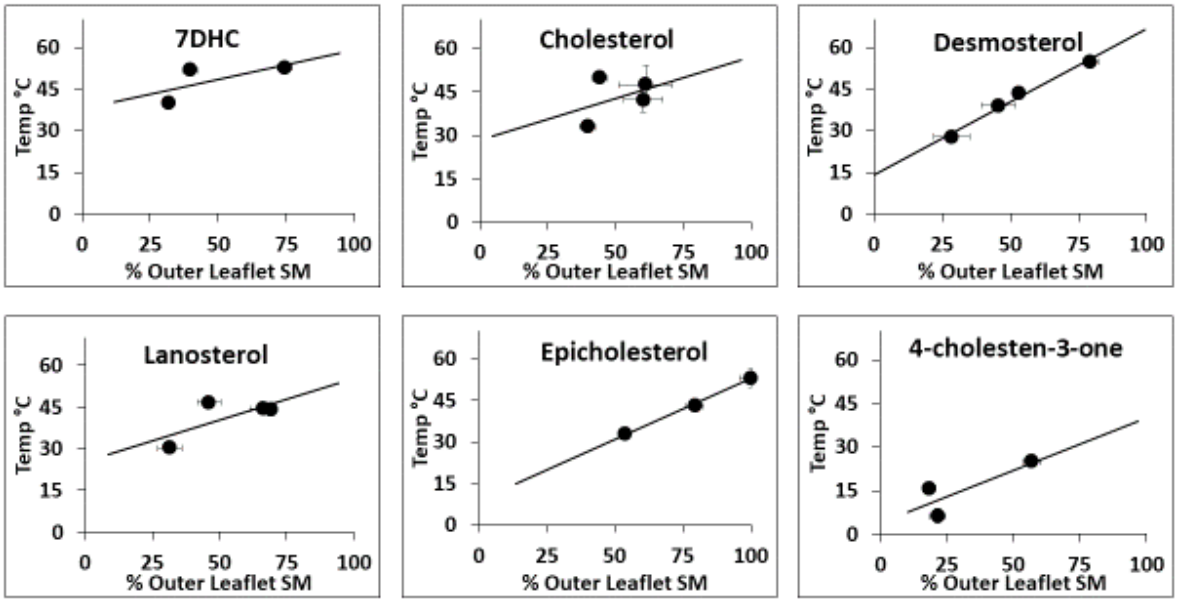
Supplemental Table 1. Difference between T_{mid} and T_{end} values for symmetric LUV. Values were derived from data shown in Figure 3. T_{mid} is the point of maximal slope of a sigmoidal fit of the F/Fo curve. T_{end} is the minimum value of a polynomial fit applied to normalized F/Fo. See Methods for details. Mean and standard deviation from three separate vesicle preparations are shown.

Sterol	% SM in Donor Lipid	% SM in Outer Leaflet
7DHC	25	31.8 ± 1.1
	50	39.7 ± 2.8
	75	74.4 ± 2.5
Cholesterol	50	39.7 ± 2.8
	59	44.3 ± 2.7
	67	61.1 ± 9.5
	85	60.2 ± 7.2
Desmosterol	25	28.5 ± 6.6
	50	45.7 ± 6.2
	67	53.1 ± 2.0
	67	79.4 ± 2.8
Lanosterol	25	31.5 ± 4.7
	50	46.1 ± 4.3
	75	69.1 ± 4.8
	85	66.2 ± 5.0
Epicholesterol	67	53.8 ± 0.7
	75	79.4 ± 3.3
	100	100 ± 4.2
4-cholesten-3-one	25	18.4 ± 2.5
	50	21.7 ± 2.9
	67	56.1 ± 3.3

Supplemental Table 2. Relationship between % SM in donor lipids and % outer leaflet SM in F samples (samples with rhodamine-DOPE). The % outer leaflet SM (as a percent of SM+DOPC in the outer leaflet) = [(SM/(SM+DOPC))/0.52) x 100%]. This is calculated from the total lipid composition of AUV assuming that SM transferred into the outer leaflet, and that the outer leaflet contains 52% of the total AUV lipid. % SM in donor = (SM/(SM+DOPC)) x 100%. Mean and standard deviation from three separate vesicle preparations shown.

Sterol	% SM in Donor Lipid	% SM in Outer Leaflet
7DHC	25	34.3. ± 0.7
	50	40.6 ± 4.2
	75	61.9 ± 1.1
Cholesterol	50	40.6 ± 4.2
	59	35.6 ± 2.8
	67	66.6 ± 11.6
	85	45.9 ± 3.4
Desmosterol	25	31.7 ± 2.7
	50	50.0 ± 3.4
	67	37.9 ± 1.5
	67	76.4 ± 4.2
Lanosterol	25	32.2 ± 2.9
	50	43.7 ± 5.0
	75	55.2 ± 1.9
	85	41.4 ± 1.4
Epicholesterol	67	61.2 ± 7.2
	75	56.6 ± 6.0
	100	74.8 ± 4.8
4-cholesten-3-one	25	21.7 ± 3.4
	50	25.4 ± 0.8
	67	54.1 ± 4.0

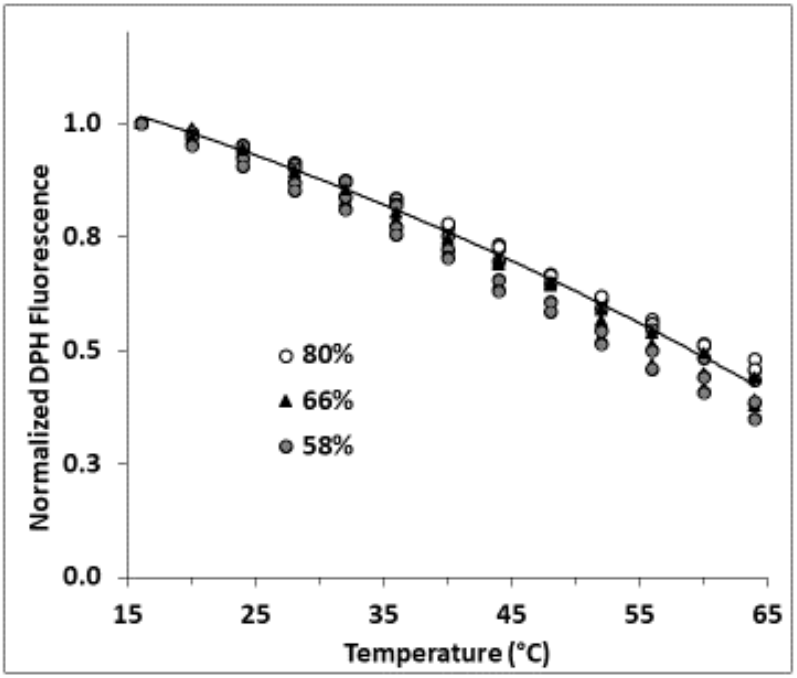
Supplemental Table 3. Relationship between % SM in donor lipids and % outer leaflet SM in Fo samples (without rhodamine-DOPE). The % outer leaflet SM (as a percent of SM+DOPC in the outer leaflet) = [(SM/(SM+DOPC))/0.52) x 100%]. This is calculated from the total lipid composition of AUV assuming all SM transferred into the AUV outer leaflet, and that the outer leaflet contains 52% of the total AUV lipid. % SM in donor lipid = (SM/(SM+DOPC)) x 100%. Mean and standard deviation from three separate vesicle preparations are shown.



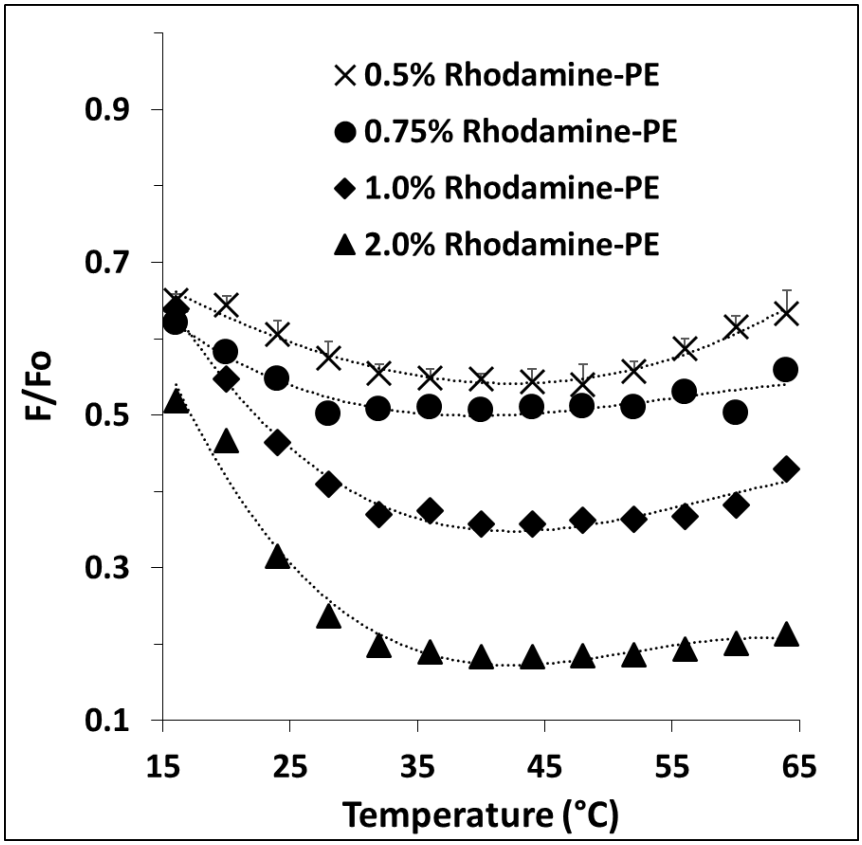
Supplemental Figure 3. Effect of SM amount in AUV upon T_{end} for AUV dispersed in sucrose. T_{end} vs. % outer leaflet SM is shown. For each sterol, three or four sets of AUV with varying % outer leaflet SM were prepared. Mean T_{end}, mean % outer leaflet SM, standard deviation for both T_{end} (y-axis error bars), and standard deviation for % outer leaflet SM (x axis error bars) from three separate vesicle preparations are shown. Note: error bars are only visible when larger than symbol size.

Sterol	Asymmetric LUV F/Fo 64°C
7DHC	0.55 ± 0.14
Cholesterol	0.64 ± 0.22
Desmosterol	0.54 ± 0.11
Lanosterol	0.71 ± 0.17
Epicholesterol	0.31 ± 0.16
4-cholesten-3-one	0.62 ± 0.06

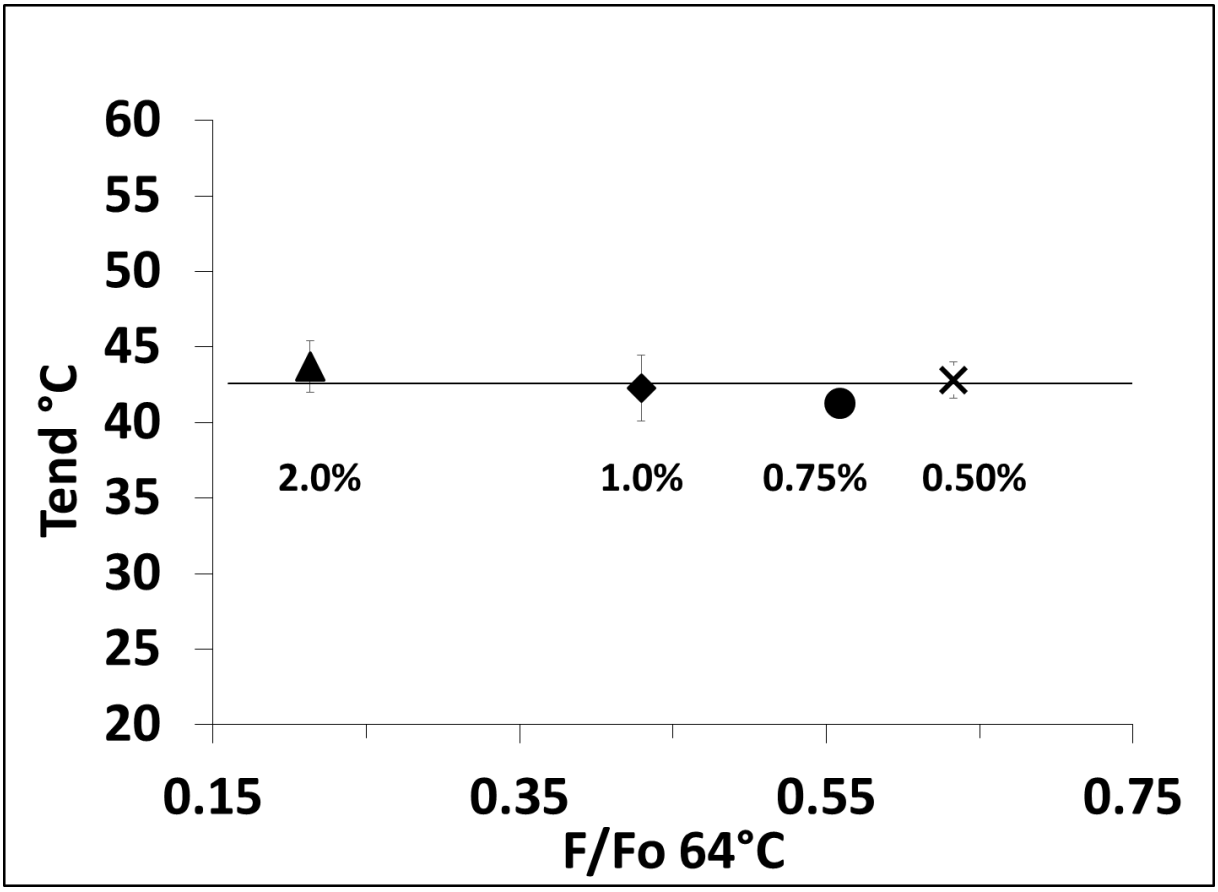
Supplemental Table 4. Mean high temperature F/Fo values in AUV with various sterols. The F/Fo value at 64°C is shown, averaging data for AUV dispersed in sucrose and PBS at various outer leaflet SM levels. Mean values and the standard deviation from 9 to 12 separate vesicle preparations are shown.



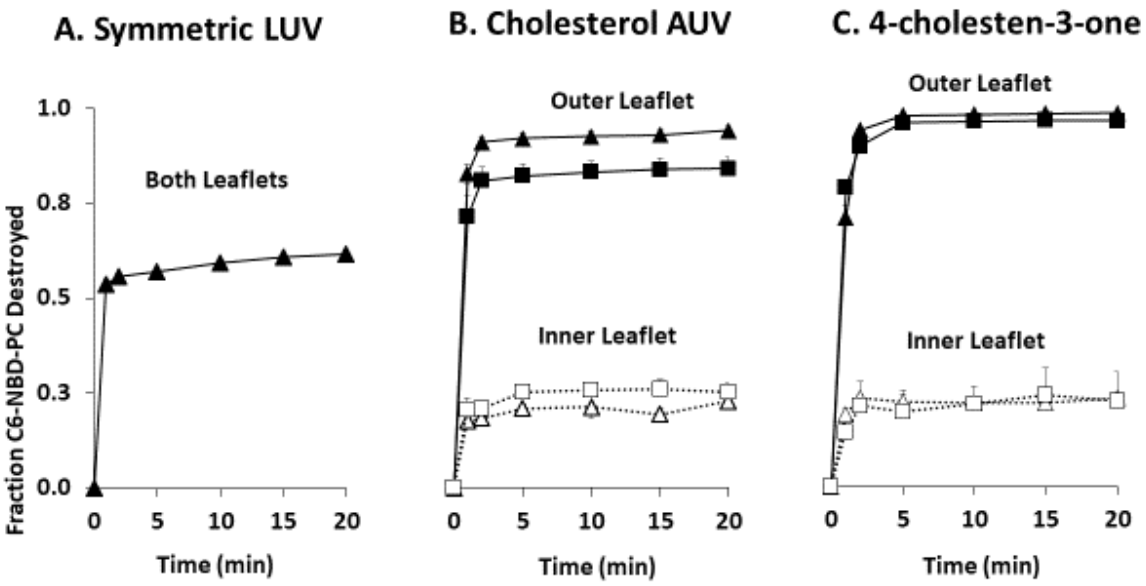
Supplemental Figure 4. Effect of outer leaflet SM content on fluorescence in Fo samples (samples with FRET donor, but no FRET acceptor). Fraction of DPH fluorescence relative to that at 16^{o}C is shown. AUV contained: (open circles) 80%, (triangles) 66%, or (shaded circles) 58% outer leaflet SM, inner leaflets containing DOPC, and 25 mol% epicholesterol. 0.1 µM DPH was added to AUV dispersed in PBS.



Supplemental Figure 5. Effect of rhodamine lipid levels on FRET. The fraction of DPH fluorescence unquenched by FRET to rhodamine-DOPE versus temperature (F/Fo) curves are shown. Samples consist of 100 µM lipid sucrose-entrapped symmetric LUV containing 1:1 DOPC:SM LUV with 25 mol% desmosterol, and dispersed in PBS. F samples were prepared with rhodamine-DOPE. Fo samples were prepared without rhodamine-DOPE. Samples contained 0.1 µM DPH added to preformed LUV. Mean and standard deviation from three separate experiments are shown. Symbols for mol% rhodamine-DOPE: crosses, 0.5%; circles, 0.75%; diamonds, 1.0%; triangles, 2.0%.



Supplemental Figure 6. Effect of rhodamine lipid content on T_{end}. T_{end} values were derived from data shown in Supplemental Figure 5. T_{end} was calculated as described in Methods. Mean T_{end} and standard deviation from three separate vesicle preparations are shown. The standard deviation in F/Fo values is not shown, but averaged 4.8% of F/Fo value and never exceeded 10% of F/Fo. Symbols for mol% rhodamine-DOPE: cross, 0.5%; circle,, 0.75%'; diamond, 1.0%; triangle, 2.0%. Horizontal line shows mean T_{end} which was 42.6 ± 1.4°C.



Supplemental Figure 7. Evaluation of stability of lipid asymmetry from C6-NBD-PC leaflet localization. The fraction of C6-NBD-PC bleached vs. time after addition of sodium dithionite (NaDt) to preformed vesicles is shown. A. Symmetric sucrose-entrapped LUV composed of 1:1 DOPC:SM with 25 mol% cholesterol and 0.25 mol% C6-NBD-PC. C6-NBD-PC was introduced to both leaflets by adding it to lipid mixtures before LUV formation. External sucrose was removed by pelleting, and vesicles were dispersed to about 100 µM in 1M Tris base pH 10. B. and C. AUV with (B.) cholesterol or (C.) 4-cholesten-3-one were prepared with C6-NBD PC primarily in the outer or inner leaflet. To prepare AUV with C6-NBD-PC in the outer leaflet, 10 mol% C6-NBD-PC was added to 3:2 DOPC:SM donor lipid mixtures. To prepare AUV with C6-NBD-PC primarily in the inner leaflet 0.25 mol% C6-NBD-PC was added to acceptor LUV lipid mixtures prior to vesicle formation. Outer leaflet C6-NBD-PC was then removed during lipid exchange. AUV were dispersed to approximately 2 mM in Tris base, pH 10 and preincubated covered in foil for either 1 h or 48 h at room temperature. AUV were further diluted to approximately 100 µM using 1M Tris base pH 10 just prior to the addition of NaDt to a final concentration of 5 mM. NBD fluorescence was measured for a period of 20 min. Symbols: triangles, 1 h preincubation; squares, 48 h preincubation. In B. and C. filled symbols, outer leaflet labeled; open symbols, inner leaflet labeled. Mean and standard deviation from three separate vesicle preparations are shown.

In symmetric LUV, NBD fluorescence was reduced to approximately half of the original value after NaDt addition (i.e. half of the fluorescence was destroyed), indicating the C6-NBD-PC was distributed nearly equally in the inner and outer leaflets, as expected. When NaDt was added to AUV containing cholesterol with C6-NBD-PC primarily in the outer leaflet, fluorescence intensity was reduced by approximately 90%. Residual fluorescence may reflect incomplete reaction with NaDt, a small amount of contaminating donor vesicles, or fluorescence arising from a species other than NBD, e.g. residual fluorescence from the product of reduction of NBD by NaDt. The small increase in % fluorescence protected from NaDt after 48h preincubation may reflect a small amount of flip of C6-NBD-PC into the inner leaflet. Similar results were observed in AUV with 4-cholesten-3-one, but lipid flip was, if anything, slower than in AUV

containing cholesterol. For both cholesterol and 4-cholesten-3-one complementary results were obtained from AUV with C6-NBD-PC mainly in the inner leaflet. Most of the NBD lipid was protected from NaDt, and no preincubation time dependence of reaction was observed, indicating very little lipid flip. In inner leaflet labeled samples the residual ~ 20% C6-NBD-PC in the outer leaflet likely reflects incomplete lipid exchange. Exchange commonly replaces about 75-90% of the outer leaflet phospholipid [1-3].

[1] Q. Wang, E. London, Lipid Structure and Composition Control Consequences of Interleaflet Coupling in Asymmetric Vesicles, Biophys. J. (2018) 115, 664-678.
[2] Q. Lin, E. London, Preparation of artificial plasma membrane mimicking vesicles with lipid asymmetry, PLoS One (2014) 9, e87903.
[3] Q. Lin, E. London, Ordered raft domains induced by outer leaflet sphingomyelin in cholesterol-rich asymmetric vesicles, Biophys. J. (2015) 108, 2212-2222.

Sterol	T_{mid} PBS In / PBS Out					T_{end} PBS In / PBS Out				
	7DHC	Chol	Desm	Lan	Epi	7DHC	Chol	Desm	Lan	Epi
7DHC										
Chol	0.0001					0.17				
Desm	0.0035	0.016				0.0001	0.0001			
Lan	0.0034	0.037	0.19			0.0016	0.0018	0.028		
Epi	0.0091	0.072	0.26	0.90		0.0001	0.0001	0.55	0.027	
4-chol	nc	nc	nc	nc	nc	0.0001	0.0001	0.0001	0.0002	0.0002

Sterol	T_{mid} Sucrose In / PBS Out					T_{end} Sucrose In / PBS Out				
	7DHC	Chol	Desm	Lan	Epi	7DHC	Chol	Desm	Lan	Epi
7DHC										
Chol	0.021					0.25				
Desm	0.0002	0.0002				0.0007	0.0003			
Lan	0.0002	0.0003	0.51			0.0003	0.0001	0.92		
Epi	0.057	0.093	0.31	0.33		0.0001	0.0001	0.061	0.016	
4-chol	nc	nc	nc	nc	nc	0.0001	0.0001	0.0001	0.0001	0.0001

Sterol	T_{mid} Sucrose In / Sucrose Out					T_{end} Sucrose In / Sucrose Out				
	7DHC	Chol	Desm	Lan	Epi	7DHC	Chol	Desm	Lan	Epi
7DHC						xx				
Chol	0.0004					0.0003				
Desm	0.0016	0.0039				0.0001	0.13			
Lan	0.0006	0.0082	0.014			0.0001	0.0002	0.0001		
Epi	0.072	0.39	0.028	0.61		0.0001	0.0002	0.0001	0.015	
4-chol	nc	nc	nc	nc	nc	0.0001	0.0001	0.0001	0.0001	0.0001

Supplemental Table 5. Significance of differences in mean T mid and T_{end} values from Table 1 for symmetric LUV with different sterols. Students T-test was applied using mean T_{mid} or T_{end}, standard deviation and number of replicates to compute two-tailed P values for each sterol vs. all others. nc = not computed. Values shown in red correspond to differences not considered significant. Sterol abbreviations: 7DHC (7-deydrocholesterol), Chol (cholesterol), Desm (desmosterol), Lan (lanosterol), Epi (epicholesterol), 4-chol (4-cholesten-3-one). Analysis utility used at www.graphpad.com/quickcalcs/ttest2/.

Sterol	Asymmetric LUV Sucrose In / Sucrose Out				
	7DHC	Chol	Desm	Lan	Epi
7DHC					
Chol	0.14				
Desm	0.013	0.28			
Lan	0.0076	0.17	0.33		
Epi	0.0005	0.0035	0.0003	0.0001	
4-chol	0.0002	0.0006	0.0001	0.0001	0.0014

Sterol	Asymmetric LUV Sucrose In / PBS Out				
	7DHC	Chol	Desm	Lan	Epi
7DHC					
Chol	0.0083				
Desm	0.0046	0.13			
Lan	0.0042	0.031	0.10		
Epi	0.0001	0.0001	0.0002	0.021	
4-chol	0.0001	0.0001	0.0001	0.0006	0.0003

Supplemental Table 3. Significance of differences in mean T_{end} values for AUV with different sterols and different solution conditions shown in Table 2. Students T-test was applied using mean T_{end}, standard deviation and number of replicates to compute two-tailed P values for T_{end} at 50% outer leaflet SM for each sterol vs. all others. Values shown in red correspond to differences not considered significant. Sterol abbreviations: 7DHC (7-deydrocholesterol), Chol (cholesterol), Desm (desmosterol), Lan (lanosterol), Epi (epicholesterol), 4-chol (4-cholesten-3-one). Analysis utility used at www.graphpad.com/quickcalcs/ttest2/.