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Supplemental Information

Lipid Interactions and Organization in Complex Bilayer Membranes

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Supplemental information: Materials and methods, 7 figures, 3 tables, and 10 references.

Lipid Interactions and Organization in Complex Bilayer Membranes

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# equal contribution

MATERIALS AND METHODS

Materials

POPC, POPE, POPS, POPC-d_{31}, POPE-d_{31}, POPS-d_{31}, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (O-lyso-PC), *N*-palmitoyl(d_{31})-D-*erythro*-sphingosylphosphorylcholine (PSM-d_{31}), D-*erythro*-sphingosyl-phosphorylcholine (lyso-SM) and egg sphingomyelin (egg SM) were purchased from Avanti Polar lipids (Alabaster, AL, U.S.A.). Reverse-phase HPLC was used to purify PSM from egg SM as previously described in (1). Cholesterol was purchased from Sigma-Aldrich (St, Louis, MO, U.S.A.). All lipids were dissolved in methanol except cholesterol and POPE which were dissolved in hexane/isopropanol (3:2 by volume) and methanol/hexane/2-propanol (10:3:2 by volume) respectively. All stock solutions were stored at -20 °C and taken to ambient temperature before use. The PL concentrations were determined with an inorganic phosphate assay as described by Rouser and co-workers (2). A surface barostat was used to determine the cholesterol concentration as described in (3). Water used in buffer preparation was purified by reverse osmosis and passage through a Millipore UF Plus water purification system to a final resistivity of 18.2 MΩcm. The solvents used were of spectroscopic grade and other inorganic and organic chemicals used were of the highest available purity.

Synthesis of trans-parinaric acid and its derivatives

tPA was synthesized in house from the methyl ester of α-linolenic acid according to the method reported by Kuklev and Smith (4). tPA was purified by crystallization from hexane at -80°C to yield 99% pure all trans tPA. *N*-tPA-sphingosylphosphorylcholine (tPA-SM) was synthesized by *N*-acylation of lyso-SM with tPA as described previously (5). 1-oleoyl-2-tPA-*sn*-3-glycero-phosphatidylcholine (O-tPA-PC) was synthesized by acylation of 1-18:1/2-OH-lyso-PC as described in (6). The tPA-containing PLs were purified using HPLC with a Discovery C18 column (Supelco, Bellefonte, PA, U.S.A.) using pure methanol as eluent. tPA and tPA derivatives were identified and confirmed pure based on ESI-MS data (Bruker Daltonics, Bremen, Germany), absorbance and fluorescence properties, and analytical HPLC analysis. Fluorophores were stored at −87 °C under argon in GC vials until the fluorophores were

dissolved in argon-purged methanol. Fluorophore concentrations were determined with absorbance using the molar extinction coefficients of 92000 $cm^{-1}M^{-1}$ at 300 nm in methanol for tPA derivatives (7). Dilute stock solutions of fluorophores were stored at −20 °C and used within a week.

^{2}H NMR sample preparation

The binary bilayers contained 14 μmol of either POPC, POPE, or POPS and 7 μmol 9',9'-d_2 -PSM . The ternary bilayers had additional 7 μmol of cholesterol. The complex bilayers contained 7 μmol PSM + 14 μmol POPC + 5.25 μmol each of POPE and POPS. PSM was perdeuterated in the *N*-palmitoyl chain (PSM-d_{31}) and the unsaturated lipids had a perdeuterated palmitoyl chain in the *sn-1* position (POPC-d_{31}, POPE-d_{31}, POPS-d_{31}). The total PL content in complex bilayers was 31.5 μmol with varying cholesterol concentration (0, 10, 18, 25, 31 mol%). The samples were mixed in glass tubes and the solvent evaporated under a constant stream of nitrogen at 40 °C and re-dissolved in 0.5 ml chloroform to give a more homogenous mixing of the lipids before they again were dried under a constant nitrogen flow at 40 °C. This was followed by overnight high vacuum. Multilamellar vesicles (MLV) were prepared by hydrating the dried lipid films with 1 ml of pure water at 65 °C followed by vigorous vortexing. Each suspension was freeze thawed three times followed by lyophilization, rehydration with deuterium depleted water until 50% hydrated (w/w) and freeze-thawed three times. Each sample was transferred to a 5 mm glass tube (Wilmad, Vineland, NJ, U.S.A.) and sealed using epoxy glue.

^{2}H NMR measurements and analysis

^{2}H measurements were recorded on a 300-MHz CMX spectrometer (Chemagnetics, Agilent, Palo Alto, CA, U.S.A.) using a 5-mm ^{2}H static probe (Otsuka Electronics, Osaka, Japan) and a quadrupole echo sequence. The 90° pulse width was 2 μs, the interpulse delay was 30 μs and the repetition rate was 0.5 s. The number of scans was approximately 100 000 and sweep width was 200 kHz. The NMR data were analyzed with TopSpin software (Bruker) and 300 Hz of line broadening was used. The first spectral moment (M_1) was calculated for each ^{2}H spectra using the following equation:

$$M_1 = \frac{\int_{-\infty}^{\infty} |\omega| f(\omega) d\omega}{\int_{-\infty}^{\infty} f(\omega) d\omega} \quad (1)$$

where ω is the frequency with respect to the central Larmor frequency ω_0, $f(\omega)$ is the line shape, and n is the order of the spectral moment (8). The chosen spectra were fast Fourier transform dePaked to enhance the resolution and give spectra similar to a planar membrane of single alignment (9).

Fluorescence experiments

For time resolved fluorescence experiments MLVs were prepared in Tris buffer (50 mM Tris pH 7.4, 140 mM NaCl) to the final PL concentration of 0.1 mM for the binary bilayer and 0.2 mM for the ternary bilayers. Cholesterol was added in a concentration of 31 mol% for the ternary and 47 mol% for the binary bilayers. A maximum of 1 mol% of O-tPA-PC or tPA-SM fluorophores were added to glass tubes. The solvent was evaporated under nitrogen flow at 40 °C. The fully dried lipid films were hydrated in pre-warmed argon purged Tris buffer at 55 °C for 30 min and then vortexed and sonicated at 55 °C for 5 min in a FinnSonic M3 Bath

Sonicator (FinnSonic Oy, Lahti, Finland). The samples were cooled to room temperature before measurements were performed at 20, 25, 30, 40 and 50 °C using a FluoTime 200 instrument (PicoQuant Gmb, Berlin, Germany). The fluorophores were excited with a PLS LED laser with a maximum signal at 298 nm, and the emission was collected at 405 nm. The fluorescence decays were analyzed using FluoFit Pro software (PicoQuant Gmb).

DATA GRAPHS AND TABLES

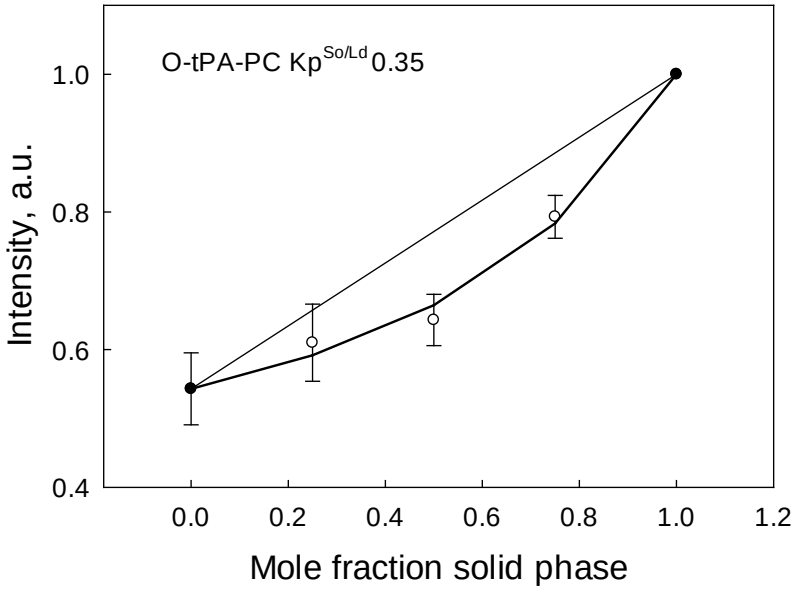

Figure S1. Partitioning of O-tPA-PC between gel and fluid phase at 23°C. The steady-state intensity of O-tPA measured in POPC/PSM bilayers. The lipid compositions was selected based on the binary POPC/PSM phase diagram (10) so that the fraction of gel phase in the samples was increased from 0 to 100. The partition coefficients ($K_p^{So/ld}$) were obtained by fitting the data with equation 1.

$$I = \frac{\mathrm{K}(\varepsilon_g \phi_g K_p X_g + \varepsilon_f \phi_f X_f}{(K_p X_g + X_f)} \quad (1)$$

the suffix *g* indicates results obtained in the gel phase and *f* indicates the fluid phase. *K* is the factor used to normalize the fluorescence intensity values, ε is the molar extinction coefficient, $\phi$is the quantum yield and *X* denotes the fraction of each phase present as determined from the binary phase-diagram. K_p was obtained by fitting the equations to the experimental data. The obtained $K_p^{So/ld}$ value is shown in the figure legends. The simulated data is shown as a line connecting the dots and compared with an ideal 1:1 partitioning. Similar results were obtained with anisotropy and lifetime data (data not shown). n = 5.

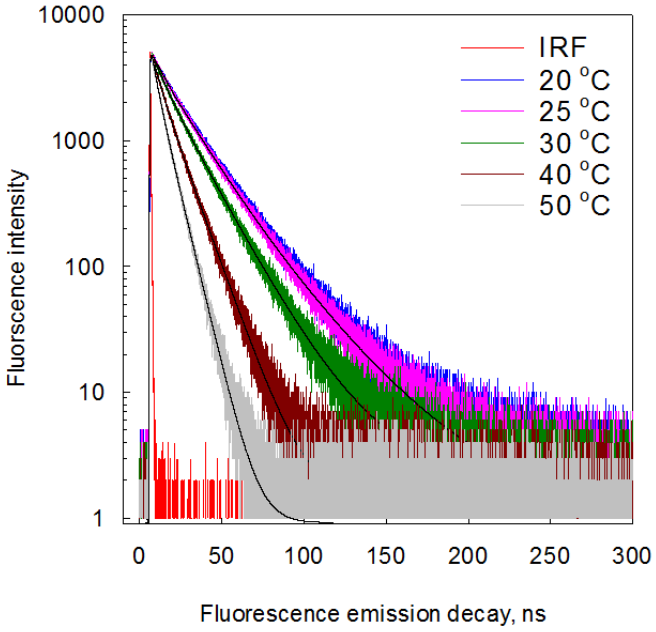

Figure S2. Decays of tPA-SM in POPC/PSM/Chol (34.5: 34.5: 30 mol%) multilamellar vesicles as a function of temperature. IRF= instrument background factor. Note the logarithmic scale.

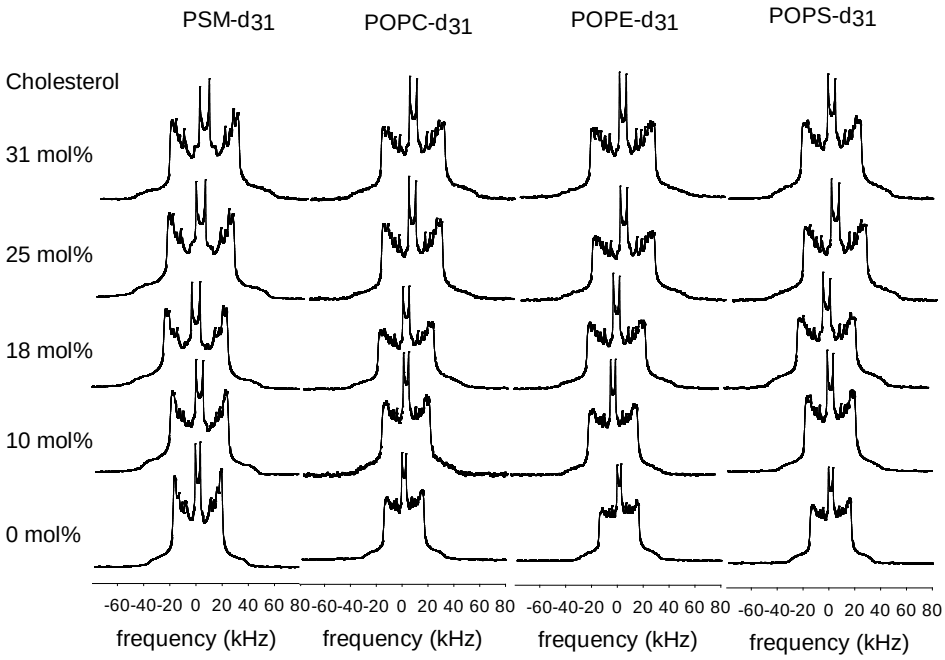

Figure S3. ^{2}H Spectra of complex bilayers at 20 °C as a cholesterol gradient. Multilamellar vesicles were prepared to 50 % hydration and the bilayers contained PSM/POPC/POPE/POPS 22:44:17:17 in the sample without cholesterol.

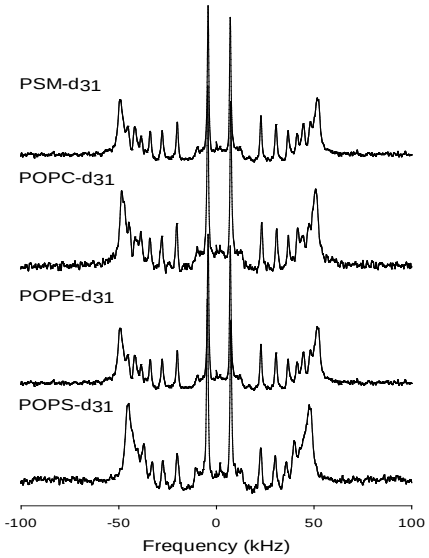

Figure S4. No double Pake peaks were observed in each perdeuterated lipid in the complex bilayers. The spectra were dePaked to show if double Pake peaks are observed. The spectra is at 20 °C at the highest cholesterol concentration (31 mol%) measured but the same results were obtained at all temperatures and cholesterol concentrations. Multilamellar vesicles were prepared to 50 % hydration with the composition PSM/POPC/POPE/POPS 22:44:17:17 + 31 mol% cholesterol.

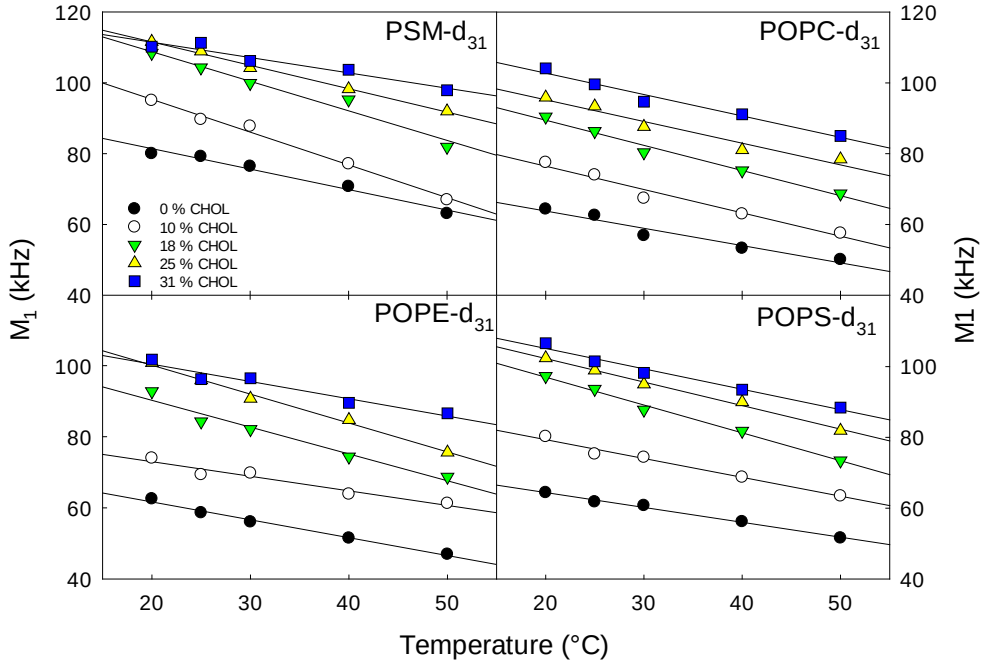

Figure S5. Cholesterol induced ordering of perdeuterated lipids in complex bilayers as a function of temperature. Ordering was measured as the first spectral moment (M_1). The data is shown for each perdeuterated lipid in each panel, as a cholesterol gradient. Multilamellar vesicles were prepared to 50 % hydration with the composition PSM/POPC/POPE/POPS 22:44:17:17 plus addition of 10-31 mol % cholesterol. The lines are only a guide to the eye.

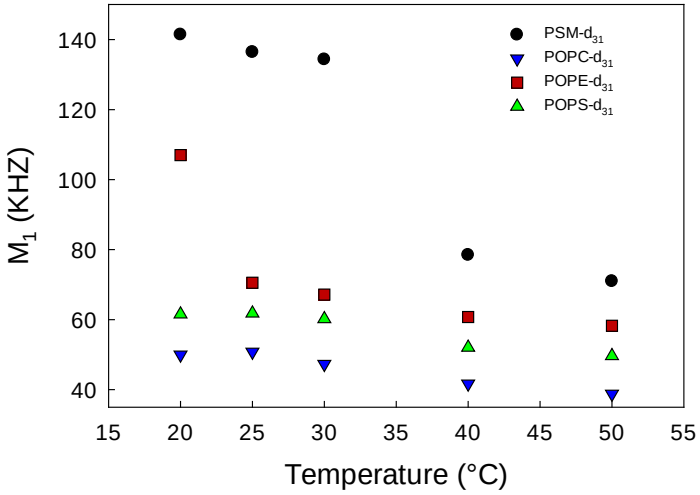

Figure S6. Pure perdeuterated lipid as a function of temperature. Ordering was measured as the first spectral moment (M_1). Multilamellar vesicles were prepared to 50 % hydration.

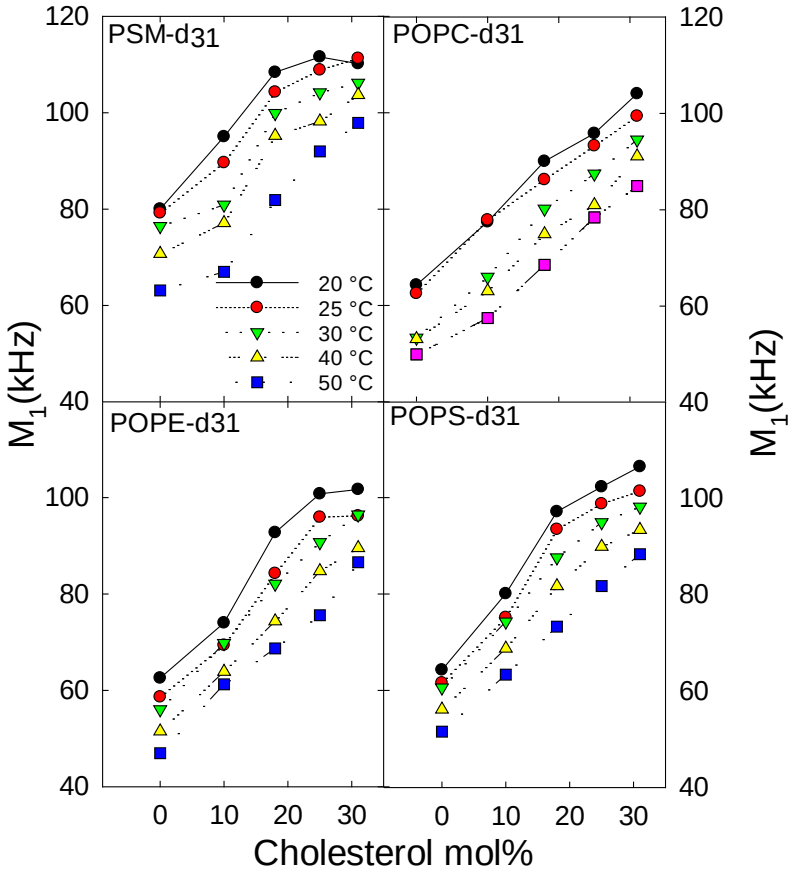

Figure S7. Acyl chain order of perdeuterated lipids in complex bilayers at different temperatures as a function of cholesterol concentration. Ordering was measured as the first spectral moment (M_1).Panel A) PSM-d_{31} B) POPC-d_{31}, C) POPE-d_{31} D) POPS-d_{31}. Multilamellar vesicles were prepared to 50 % hydration.

Table S1: Comparison of time resolved fluorescence decays reported by 1 mol% of O-tPA-PC and tPA-SM in identical bilayers at 20 °C.

Probe tests

Sample	Probe	I τAV			τ1			τ 2			τ 3			α1			α2			α3		
PSM	**tPA-SM**	**50.1**	±	*3.12*	**67.0**	±	*8.10*	**43.6**	±	*5.37*	**3.4**	±	*2.25*	**0.21**	±	*0.07*	**0.65**	±	*0.02*	**0.13**	±	*0.05*
PSM	**O-tPA-PC**	**40.2**	±	*0.37*	**51.3**	±	*2.14*	**23.0**	±	*0.42*	**3.2**	±	*0.01*	**0.26**	±	*0.03*	**0.22**	±	*0.03*	**0.52**	±	*0.00*
POPC	**tPA-SM**	**7.0**	±	*0.55*				**7.5**	±	*0.54*	**3.2**	±	*0.04*				**0.73**	±	*0.02*	**0.27**	±	*0.02*
POPC	**O-tPA-PC**	**5.5**	±	*0.33*				**6.3**	±	*0.27*	**2.9**	±	*0.49*				**0.59**	±	*0.04*	**0.41**	±	*0.04*
PSM/Chol	**tPA-SM**	**29.6**	±	*0.25*	**45.9**	±	*5.37*	**24.7**	±	*2.50*	**6.2**	±	*2.54*	**0.16**	±	*0.08*	**0.70**	±	*0.04*	**0.14**	±	*0.04*
PSM/Chol	**O-tPA-PC**	**19.5**	±	*0.85*	**39.2**	±	*1.11*	**18.8**	±	*0.61*	**6.3**	±	*1.25*	**0.05**	±	*0.02*	**0.63**	±	*0.03*	**0.32**	±	*0.01*
POPC/Chol	**tPA-SM**	**17.8**	±	*0.97*				**19.3**	±	*0.56*	**8.2**	±	*2.59*				**0.73**	±	*0.08*	**0.27**	±	*0.08*
POPC/Chol	**O-tPA-PC**	**13.1**	±	*0.27*				**14.3**	±	*0.73*	**6.5**	±	*3.10*				**0.69**	±	*0.08*	**0.31**	±	*0.08*

Multilamellar vesicles were prepared with 1 mol % probe to a composition of pure phospholipid or phospholipid: cholesterol (53; 47 mol%) in the binary bilayers and measured at 20 °C. I τAV, intensity weighted average lifetime (ns); α, fractional amplitudes. All samples were measured at 20 °C.

Table S2: Detection of membrane order by time resolved fluorescence decays of domain selective probes in binary and ternary multi-lamellar vesicles as function of temperature.

POPC O-tPA-PC

temp	I τAV			τ1			τ 2			α1			α2		
20	**5.7**	±	*0.07*	**7.1**	±	*0.22*	**4.1**	±	*0.09*	**0.38**	±	*0.01*	**0.62**	±	*0.01*
25	**4.7**	±	*0.03*	**6.5**	±	*0.07*	**3.7**	±	*0.18*	**0.25**	±	*0.04*	**0.75**	±	*0.04*
30	**3.9**	±	*0.01*	**6.3**	±	*0.28*	**3.3**	±	*0.15*	**0.13**	±	*0.04*	**0.87**	±	*0.04*
40	**3.0**	±	*0.05*	**6.0**	±	*0.31*	**2.6**	±	*0.02*	**0.05**	±	*0.02*	**0.95**	±	*0.02*
50	**2.3**	±	*0.02*	**4.9**	±	*0.03*	**2.0**	±	*0.02*	**0.06**	±	*0.01*	**0.94**	±	*0.01*

POPC/Chol O-tPA-PC

temp	I τAV			τ1			τ 2			α1			α2		
20	**13.2**	±	*0.08*	**14.8**	±	*0.02*	**8.7**	±	*0.69*	**0.63**	±	*0.04*	**0.37**	±	*0.04*
25	**10.8**	±	*0.28*	**11.9**	±	*0.23*	**5.6**	±	*0.28*	**0.71**	±	*0.02*	**0.29**	±	*0.02*
30	**9.0**	±	*0.09*	**10.1**	±	*0.12*	**5.5**	±	*0.18*	**0.64**	±	*0.01*	**0.36**	±	*0.01*
40	**6.5**	±	*0.07*	**8.4**	±	*0.24*	**5.6**	±	*0.16*	**0.24**	±	*0.07*	**0.76**	±	*0.07*
50	**4.7**	±	*0.15*	**7.1**	±	*0.77*	**3.9**	±	*0.31*	**0.15**	±	*0.06*	**0.85**	±	*0.06*

POPC/PSM/Chol O-tPA-PC

temp	I τAV			τ1			τ 2			α1			α2		
20	**16.0**	±	*0.85*	**22.6**	±	*2.93*	**11.0**	±	*3.06*	**0.28**	±	*0.15*	**0.72**	±	*0.15*
25	**12.9**	±	*0.19*	**16.4**	±	*0.62*	**7.3**	±	*0.46*	**0.42**	±	*0.04*	**0.58**	±	*0.04*
30	**10.0**	±	*0.13*	**12.5**	±	*0.45*	**5.7**	±	*0.24*	**0.43**	±	*0.04*	**0.57**	±	*0.04*
40	**6.4**	±	*0.50*	**8.3**	±	*0.33*	**4.1**	±	*0.36*	**0.38**	±	*0.16*	**0.62**	±	*0.16*
50	**4.5**	±	*0.06*	**6.0**	±	*0.16*	**3.1**	±	*0.14*	**0.33**	±	*0.02*	**0.67**	±	*0.02*

PSM tPA-SM

temp	τAV			τ1			τ 2			τ 3			α1			α2			α3		
20	**46.9**	±	*0.05*	**53.3**	±	*0.04*	**29.3**	±	*0.03*				**0.60**	±	*0.01*	**0.40**	±	*0.01*			
25	**42.3**	±	*0.01*	**49.7**	±	*0.35*	**28.0**	±	*0.58*				**0.52**	±	*0.02*	**0.48**	±	*0.02*			
30	**37.1**	±	*0.07*	**45.0**	±	*0.93*	**25.5**	±	*0.95*				**0.45**	±	*0.05*	**0.55**	±	*0.05*			
40	**25.1**	±	*0.24*	**42.6**	±	*16.4*	**19.7**	±	*6.61*	**6.2**			**0.24**	±	*0.29*	**0.61**	±	*0.07*	**0.15**	±	*0.05*
50	**3.8**	±	*0.19*				**5.4**	±	*0.50*	**3.0**	±	*0.50*				**0.22**	±	*0.09*	**0.78**	±	*0.09*

PSM/Chol tPA-SM

temp	τAV			τ1			τ 2			τ 3			α1			α2			α3		
20	**26.5**	±	*0.68*	**41.1**	±	*1.50*	**21.3**	±	*0.69*	**3.6**	±	*0.78*	**0.15**	±	*0.05*	**0.61**	±	*0.12*	**0.23**	±	*0.16*
25	**23.5**	±	*0.29*	**39.3**	±	*2.61*	**20.5**	±	*0.93*	**4.6**	±	*2.37*	**0.10**	±	*0.03*	**0.66**	±	*0.04*	**0.24**	±	*0.04*
30	**20.0**	±	*0.42*	**36.9**	±	*3.89*	**18.5**	±	*0.95*	**5.3**	±	*2.14*	**0.07**	±	*0.04*	**0.69**	±	*0.03*	**0.24**	±	*0.04*
40	**14.5**	±	*0.37*	**27.5**	±	*1.83*	**14.3**	±	*0.35*	**3.5**	±	*0.44*	**0.03**	±	*0.01*	**0.75**	±	*0.01*	**0.22**	±	*0.01*
50	**10.2**	±	*0.04*				**11.0**	±	*0.08*	**4.7**	±	*0.01*				**0.74**	±	*0.01*	**0.26**	±	*0.01*

POPC/PSM/Chol tPA-SM

temp	τAV			τ1			τ 2			τ 3			α1			α2			α3		
20	**27.1**	±	*0.96*	**44.1**	±	*3.10*	**23.5**	±	*0.90*	**5.3**	±	*2.58*	**0.11**	±	*0.03*	**0.77**	±	*0.05*	**0.12**	±	*0.06*
25	**22.2**	±	*0.68*	**35.3**	±	*3.02*	**18.8**	±	*1.55*	**4.1**	±	*1.97*	**0.13**	±	*0.05*	**0.73**	±	*0.09*	**0.14**	±	*0.05*
30	**17.6**	±	*0.37*	**34.7**	±	*2.83*	**17.0**	±	*0.41*	**5.9**	±	*0.58*	**0.04**	±	*0.01*	**0.77**	±	*0.01*	**0.20**	±	*0.02*
40	**11.0**	±	*0.26*				**12.2**	±	*0.20*	**5.7**	±	*0.23*				**0.67**	±	*0.06*	**0.33**	±	*0.06*
50	**7.2**	±	*0.03*				**8.1**	±	*0.03*	**3.9**	±	*0.09*				**0.66**	±	*0.01*	**0.34**	±	*0.01*

POPE O-tPA-PC

temp	τAV			τ1			τ 2			α1			α2		
20	**25.9**	±	*0.36*	**31.8**	±	*0.10*	**8.9**	±	*2.55*	**0.44**	±	*0.01*	**0.56**	±	*0.01*
25	**8.1**	±	*1.29*	**16.1**	±	*7.97*	**6.1**	±	*1.23*	**0.14**	±	*0.13*	**0.86**	±	*0.13*
30	**5.4**	±	*0.24*	**6.3**	±	*0.14*	**2.6**	±	*0.27*	**0.59**	±	*0.04*	**0.41**	±	*0.04*
40	**4.0**	±	*0.19*	**5.6**	±	*1.01*	**2.2**	±	*0.54*	**0.32**	±	*0.12*	**0.68**	±	*0.12*
50	**3.3**	±	*0.66*	**5.5**	±	*0.26*	**1.8**	±	*0.65*	**0.17**	±	*0.11*	**0.83**	±	*0.11*

POPE/Chol O-tPA-PC

temp	τAV			τ1			τ 2			α1			α2		
20	**12.1**	±	*0.43*	**13.8**	±	*0.30*	**6.1**	±	*1.06*	**0.61**	±	*0.13*	**0.39**	±	*0.13*
25	**10.6**	±	*0.46*	**11.6**	±	*0.07*	**4.6**	±	*0.65*	**0.71**	±	*0.11*	**0.29**	±	*0.11*
30	**8.7**	±	*0.31*	**9.6**	±	*0.03*	**3.1**	±	*0.52*	**0.65**	±	*0.11*	**0.35**	±	*0.11*
40	**6.2**	±	*1.28*	**7.2**	±	*0.81*	**2.7**	±	*1.89*	**0.52**	±	*0.23*	**0.48**	±	*0.23*
50	**4.6**	±	*0.40*	**5.6**	±	*0.23*	**2.5**	±	*1.15*	**0.46**	±	*0.06*	**0.54**	±	*0.06*

POPE/PSM/Chol O-tPA-PC

temp	τAV			τ1			τ 2			α1			α2		
20	**18.6**	±	*0.31*	**27.8**	±	*0.16*	**15.8**	±	*0.19*	**0.15**	±	*0.01*	**0.85**	±	*0.01*
25	**15.0**	±	*0.40*	**20.1**	±	*2.77*	**11.1**	±	*3.43*	**0.30**	±	*0.23*	**0.70**	±	*0.23*
30	**11.9**	±	*0.08*	**13.4**	±	*0.05*	**6.2**	±	*0.10*	**0.63**	±	*0.02*	**0.37**	±	*0.02*
40	**7.8**	±	*0.03*	**8.7**	±	*0.09*	**3.9**	±	*0.14*	**0.67**	±	*0.04*	**0.33**	±	*0.04*
50	**5.2**	±	*0.04*	**6.3**	±	*0.04*	**3.5**	±	*0.01*	**0.46**	±	*0.01*	**0.54**	±	*0.01*

POPE/PSM/Chol tPA-SM

temp	I τAV			τ1			τ 2			τ 3			α1			α2			α3		
20	**28.7**	±	*1.90*	**43.7**	±	*0.53*	**24.3**	±	*0.39*	**6.7**	±	*1.75*	**0.14**	±	*0.05*	**0.74**	±	*0.08*	**0.17**	±	*0.05*
25	**23.9**	±	*0.97*	**39.6**	±	*1.87*	**21.4**	±	*0.46*	**5.7**	±	*0.18*	**0.09**	±	*0.03*	**0.80**	±	*0.08*	**0.16**	±	*0.10*
30	**19.1**	±	*0.49*	**25.4**	±	*1.75*	**15.5**	±	*0.93*	**3.8**	±	*0.60*	**0.26**	±	*0.07*	**0.62**	±	*0.07*	**0.12**	±	*0.05*
40	**12.4**	±	*0.41*				**13.4**	±	*0.17*	**5.0**	±	*1.89*				**0.73**	±	*0.11*	**0.27**	±	*0.11*
50	**8.3**	±	*0.09*				**9.0**	±	*0.25*	**4.4**	±	*1.16*				**0.72**	±	*0.06*	**0.28**	±	*0.06*

POPS O-tPA-PC

temp	I τAV			τ1			τ 2			α1			α2		
20	**6.1**	±	*0.03*	**7.6**	±	*0.27*	**3.7**	±	*0.16*	**0.45**	±	*0.07*	**0.55**	±	*0.07*
25	**5.0**	±	*0.06*	**7.0**	±	*0.69*	**3.7**	±	*0.19*	**0.27**	±	*0.09*	**0.73**	±	*0.09*
30	**4.2**	±	*0.07*	**7.6**	±	*0.42*	**3.6**	±	*0.09*	**0.08**	±	*0.01*	**0.92**	±	*0.01*
40	**3.1**	±	*0.01*	**7.4**	±	*1.08*	**2.7**	±	*0.01*	**0.03**	±	*0.01*	**0.97**	±	*0.01*
50	**2.4**	±	*0.01*	**6.7**	±	*0.99*	**2.1**	±	*0.01*	**0.03**	±	*0.01*	**0.97**	±	*0.01*

POPS/Chol O-tPA-PC

temp	I τAV			τ1			τ 2			α1			α2		
20	**10.4**	±	*0.36*	**12.3**	±	*0.03*	**5.2**	±	*0.08*	**0.54**	±	*0.06*	**0.46**	±	*0.06*
25	**9.0**	±	*0.16*	**10.5**	±	*0.02*	**4.5**	±	*0.14*	**0.57**	±	*0.03*	**0.43**	±	*0.03*
30	**7.5**	±	*0.04*	**9.1**	±	*0.01*	**4.3**	±	*0.01*	**0.50**	±	*0.01*	**0.50**	±	*0.01*
40	**5.6**	±	*0.08*	**7.1**	±	*0.11*	**3.6**	±	*0.27*	**0.39**	±	*0.06*	**0.61**	±	*0.06*
50	**4.3**	±	*0.01*	**5.8**	±	*0.08*	**2.9**	±	*0.02*	**0.33**	±	*0.01*	**0.67**	±	*0.01*

POPS/PSM/Chol O-tPA-PC

temp	I τAV			τ1			τ 2			α1			α2		
20	**17.7**	±	*0.09*	**24.2**	±	*0.36*	**8.9**	±	*0.14*	**0.33**	±	*0.02*	**0.67**	±	*0.02*
25	**13.8**	±	*1.04*	**20.3**	±	*0.89*	**8.7**	±	*1.81*	**0.25**	±	*0.13*	**0.75**	±	*0.13*
30	**11.6**	±	*0.21*	**15.5**	±	*0.04*	**6.2**	±	*0.04*	**0.35**	±	*0.02*	**0.65**	±	*0.02*
40	**8.1**	±	*0.07*	**10.0**	±	*0.25*	**4.0**	±	*0.36*	**0.46**	±	*0.02*	**0.54**	±	*0.02*
50	**5.6**	±	*0.21*	**7.1**	±	*0.11*	**3.0**	±	*0.01*	**0.43**	±	*0.03*	**0.57**	±	*0.03*

POPS/PSM/Chol tPA-SM

temp	I τAV			τ1			τ 2			τ 3			α1			α2			α3		
20	**30.8**	±	*1.18*	**51.5**	±	*4.50*	**27.1**	±	*1.49*	**9.4**	±	*1.55*	**0.11**	±	*0.04*	**0.63**	±	*0.02*	**0.26**	±	*0.07*
25	**26.0**	±	*0.77*	**48.0**	±	*5.24*	**24.2**	±	*0.98*	**8.6**	±	*0.76*	**0.07**	±	*0.03*	**0.63**	±	*0.04*	**0.29**	±	*0.06*
30	**21.2**	±	*0.59*	**42.8**	±	*0.63*	**20.7**	±	*0.53*	**7.2**	±	*1.06*	**0.04**	±	*0.01*	**0.63**	±	*0.03*	**0.32**	±	*0.02*
40	**13.9**	±	*0.23*				**16.8**	±	*0.27*	**7.0**	±	*0.57*				**0.49**	±	*0.01*	**0.51**	±	*0.01*
50	**9.7**	±	*0.13*				**10.8**	±	*0.03*	**3.8**	±	*0.42*				**0.65**	±	*0.02*	**0.35**	±	*0.02*

I τAVG, intensity weighted average lifetime (ns); α, fractional amplitudes. 1 mol% of the fluorescent probes tPA-SM (partitions to ordered domains) or O-tPA-PC (partition to disordered domains) were included in the bilayer.

Table S3: Detection of membrane order by time resolved fluorescence decays of phase selective probes in complex five-component bilayers as function of temperature.

Complex bilayer O-tPA-PC

temp	I τAV	τ1	τ 2	α1	α2
20	**7.0** ± *0.04*	**9.1** ± *0.70*	**3.5** ± *1.04*	**0.39** ± *0.09*	**0.61** ± *0.09*
25	**5.6** ± *0.07*	**7.4** ± *0.34*	**3.2** ± *0.85*	**0.35** ± *0.09*	**0.65** ± *0.09*
30	**4.7** ± *0.16*	**6.7** ± *0.34*	**3.0** ± *0.80*	**0.27** ± *0.12*	**0.73** ± *0.12*
40	**3.6** ± *0.49*	**7.1** ± *0.28*	**2.6** ± *0.33*	**0.09** ± *0.06*	**0.91** ± *0.06*
50	**3.1** ± *0.92*	**7.2** ± *0.08*	**2.0** ± *0.22*	**0.07** ± *0.07*	**0.93** ± *0.07*

Complex bilayer/Chol O-tPA-PC

temp	I τAV	τ1	τ 2	α1	α2
20	**13.9** ± *0.30*	**16.0** ± *0.68*	**5.9** ± *2.13*	**0.57** ± *0.02*	**0.43** ± *0.02*
25	**11.3** ± *0.41*	**12.6** ± *0.35*	**4.1** ± *1.02*	**0.64** ± *0.04*	**0.36** ± *0.04*
30	**9.0** ± *0.20*	**10.1** ± *0.21*	**3.8** ± *1.02*	**0.71** ± *0.14*	**0.29** ± *0.14*
40	**6.0** ± *0.04*	**7.2** ± *0.36*	**3.3** ± *0.94*	**0.51** ± *0.09*	**0.49** ± *0.09*
50	**4.3** ± *0.09*	**5.8** ± *0.71*	**2.8** ± *0.80*	**0.32** ± *0.21*	**0.68** ± *0.21*

Complex bilayer tPA-SM

temp	I τAV	τ1	τ 2	τ 3	α1	α2	α3
20	**13.0** ± *1.60*	**25.0** ± *8.42*	**11.2** ± *2.21*	**3.9** ± *1.53*	**0.09** ± *0.06*	**0.65** ± *0.03*	**0.25** ± *0.10*
25	**8.7** ± *0.51*	**14.1** ± *2.90*	**9.8** ± *0.34*	**4.7** ± *0.01*	**0.03** ± *0.01*	**0.54** ± *0.05*	**0.43** ± *0.04*
30	**6.5** ± *0.18*		**7.4** ± *0.21*	**3.5** ± *0.05*		**0.59** ± *0.00*	**0.41** ± *0.00*
40	**4.2** ± *0.05*		**5.6** ± *0.42*	**3.1** ± *0.31*		**0.31** ± *0.10*	**0.69** ± *0.10*
50	**3.0** ± *0.05*		**5.3** ± *0.77*	**2.5** ± *0.18*		**0.10** ± *0.06*	**0.90** ± *0.06*

Complex bilayer/Chol tPA-SM

temp	I τAV	τ1	τ 2	τ 3	α1	α2	α3
20	**21.7** ± *1.12*	**26.9** ± *1.65*	**13.2** ± *4.35*		**0.43** ± *0.05*	**0.57** ± *0.05*	
25	**17.8** ± *0.38*	**25.4** ± *3.06*	**15.8** ± *1.09*	**3.34** ± *0.94*	**0.17** ± *0.09*	**0.66** ± *0.18*	**0.18** ± *0.09*
30	**14.2** ± *0.08*		**15.4** ± *0.31*	**5.63** ± *2.74*		**0.71** ± *0.01*	**0.29** ± *0.01*
40	**9.28** ± *0.08*		**10.1** ± *0.29*	**3.94** ± *0.72*		**0.7** ± *0.06*	**0.3** ± *0.06*
50	**6.36** ± *0.14*		**7.44** ± *0.49*	**3.66** ± *0.28*		**0.56** ± *0.08*	**0.44** ± *0.08*

Temp, temperature, I τAV, intensity weighted average lifetime (ns); α, fractional amplitudes. 1 mol% of the fluorescent probes tPA-SM (partitions to ordered domains) or O-tPA-PC (partition to disordered domains) was included in the bilayers. Multilamellar vesicles were prepared with the composition PSM/POPC/POPE/POPS 22:44:17:17 with and without 31 mol% cholesterol.

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