Supplementary material

Determination of phase fractions from fluorescence anisotropy and mean fluorescence lifetime of t-PnA

Gel and liquid ordered phase fractions, X_G and X_{lo}, were calculated according to the following expressions (1):

i) from mean fluorescence lifetime, $<\tau>$,

$$<\tau>=\frac{<\tau>_1 K_p X_1 + \bar{\tau}_2/\bar{\tau}_1 <\tau>_2 X_2}{K_p X_1 + \bar{\tau}_2/\bar{\tau}_1 X_2} \qquad \textbf{Eq. S1}$$

ii) from steady-state fluorescence anisotropy, $<r>$,

$$<r>=\frac{\varepsilon_1\phi_1 r_1 K_p X_1 + \varepsilon_2\phi_2 r_2 X_2}{\varepsilon_1\phi_1 K_p X_1 + \varepsilon_2\phi_2 X_2} \qquad \textbf{Eq. S2}$$

where 1 = l_o or gel and 2 = l_d or fluid, ε_i is the molar absorption coefficient, ϕ_i the quantum yield, $<\tau>_i$, $\bar{\tau}_i$ and r_i are the mean fluorescence lifetime, the lifetime-weighted quantum yield and steady-state fluorescence anisotropy of the probe in phase *i*, respectively. The partition coefficient of the t-PnA between l_o and l_d, $K_p^{lo/ld}$, and between gel and fluid phases, $K_p^{g/f}$ are 0.88±0.05 and 4.50±0.60, respectively (1). $<\tau>_i$, $\bar{\tau}_i$ and r_i were taken from (1,2) for PCer-enriched gel and PSM/Chol-enriched l_o phases, respectively.

Determination of phase fractions POPC/PSM/PCer ternary phase diagram

Knowing the amount of PCer formed upon 2h of hydrolysis of PSM in the binary POPC/PSM mixtures (Figure 1), the amount and composition of gel phase formed in the resultant ternary POPC/PSM/PCer mixtures is directly determined from the tie-lines in the respective phase diagram (3). The mixtures are plotted in the ternary phase diagram in Figure 1B. For the mixtures that lie within the tie-triangle, the fraction and composition of each of the phases were determined as explained in (3).

The expected amount of gel phase formed upon SMase action in POPC/PSM/Chol, assuming that Chol is not interfering with the ability of PCer to interact with PSM and segregate into a gel phase, was also determined from this phase diagram. For this situation, the ratio between POPC, PSM-remaining and PCer-formed was calculated based on the hydrolysis data (Figure 2). The resultant mixtures are plotted in Figure 1B, and the fraction and composition of the phases were calculated as for the binary POPC/PSM mixtures.

For the ternary mixture with lowest Chol content (T2), X_G formed upon PSM hydrolysis is ~ 22%, similar to that obtained by the fluorescence parameters of t-PnA (Eq. S1 and S2), showing that Chol is not strongly affecting PCer/PSM-gel phase formation. For this mixture, the composition of the gel and fluid phase are POPC/PSM/PCer 16:30:54 and 87:11:2, respectively, as defined by the extreme of the tie-line (the direction of the tie-lines is shown in Figure 6 in (3)). This composition was taken into account for the determination of the area involved in formation of the gel phase. To this end, an area per molecule of 66.4 Å^2 for POPC (4), 47.8 Å^2 for PSM (5), 37.7 Å^2 for Chol (6) and 40 Å^2 for PCer (7) was used, resulting in ~10.2 Å^2 involved in gel phase.

Models and parameters used in determination of the amount of, and total area covered by the gel phase fraction and the size of the domains

FRET data obtained with a t-PnA/NBD-DPPE D/A pair was used to determine the size of PCer/PSM-gel domains. The model applied is the same as described in the Appendix section of (1). Briefly, for t-PnA and NBD-DPPE cromophores that are located in the bilayer center and membrane surface respectively, the model that describes out-of-plane (*trans*) FRET with randomly distributed D and A molecules, assuming a radius of exclusion of acceptors (R_e) around the donor, is given by:

$$\rho_{trans}(t) = \exp\left\{-\frac{2c}{\Gamma(2/3)\cdot b}\int_0^{w/\sqrt{w^2+R_e^2}}\left[1-\exp(-t\,b^3\,\alpha^6)\right]\alpha^{-3}\,d\alpha\right\} \qquad \textbf{Eq. S3}$$

where

$$c = \Gamma(2/3)\cdot n\cdot\pi\cdot R_0^{\,2}\cdot\bar{\tau}^{\,-1/3} \qquad \textbf{Eq. S4}$$

In this equation, n is the surface density of acceptors, R_0 is the Förster radius, R_e is the exclusion radius, Γ is the complete gamma function, $b = (R_0/w)^2/\ \bar{\tau}^{\,1/3}$, and w is the interplanar donor-acceptor distance. In the calculation of the surface density of acceptors, the area per molecule of each of the lipids is considered (6). For this D/A pair R_0 in the l_d, l_o and gel phases are 25 Å, 29 Å and 31 Å, respectively. These are not small compared to the membrane thickness and transfer to the two leaflets occurs. In this situation, the donor decay in the presence of acceptor is given by:

$$i_{DA}(t) = i_D(t)\rho_{trans1}(t)\rho_{trans2}(t) \qquad \textbf{Eq. S5}$$

FRET efficiency, E, is computed numerically using the relationship $E = 1-\bar{\tau}_{DA}/\bar{\tau}_D$.

If phase separation occurs, the donor decay in the absence of acceptors is given by:

$$i_D(t) = x_1\, i_{D1}(t) + x_2\, i_{D2}(t) \qquad \textbf{Eq. S6}$$

where x_i is the mole fraction and $i_{Di}(t)$ the fluorescence decay of the donor in phase i = 1,2. When the domains are big enough to prevent significant FRET from donor in one phase to acceptors in a different phase, the donor decay in the presence of acceptor is given by:

$$i_{DA}(t) = x_1 i_{D1}(t)\rho_{trans1,1}(t)\rho_{trans2,1}(t) + x_2 i_{D2}(t)\rho_{trans1,2}(t)\rho_{trans2,2}(t) \qquad \textbf{Eq. S7}$$

where $\rho_{trans,i}(t)$ are calculated as for the one phase situation but taking into account the parameters of each phase.

Equations S6 and S7 were used to determine the size of gel domains that are formed upon PSM hydrolysis. For the calculation of the area covered by each of the phases, the composition of the mixture resulting from the hydrolysis was taken into account. For 22% gel phase the size of the domains is given by the R_e that is required to obtain the experimental E ~ 12% (because PCer-rich domains exclude the acceptors), resulting in R_e ~ 8.5 nm.

The FRET model for random distribution of the NBD-DPPE/Rho-DOPE Förster pair was already described in detail (1). Since both cromophores are located in the bilayer surface, both in-plane (*cis*) and out-of-plane (*trans*) energy transfer occur, and the donor decay in the presence of acceptor is given by:

$$i_{DA}(t) = i_D(t)\rho_{cis}(t)\rho_{trans}(t) \qquad \textbf{Eq. S8}$$

where,

$$\rho_{cis}(t) = \exp\left\{-\pi R_0^2 n\gamma\left[\frac{2}{3},\left(\frac{R_0}{R_e}\right)^6(t/\bar{\tau})\right](t/\bar{\tau})^{1/3} + \pi R_e^2 n\left(1-\exp\left[-\left(\frac{R_0}{R_e}\right)^6(t/\bar{\tau})\right]\right)\right\} \qquad \textbf{Eq. S9}$$

and,

$$\gamma(x,y) = \int_0^y z^{x-1}\exp(-z)dz \qquad \textbf{Eq. S10}$$

is the incomplete Gamma function.

When 22% gel phase is formed (corresponding to a reduction of 10.2 $Å^2$ per molecule), gel domains that exclude the probes are formed. The calculated *E* value assuming the reduction in the available area for probe distribution (~ 65.7%) is similar to the experimental one (~ 66.5%), confirming that the formation of PCer/PSM-gel phase is not significantly affected by the presence of low Chol amounts.

Supplementary Figures Legend

Figure S1 – Variation in the fraction of PSM during hydrolysis with SMase in (○) 30% and (▽) 40% PSM containing binary mixtures and in POPC/PSM/Chol ternary mixtures containing (▲) 20, (△) 23, (◆) 26, (□) 30, (■) 33, (▼) 35 and (●) 37% PSM, respectively.
Figure S2 – Variation of the gel phase fraction formed 2h after enzyme reaction with the ratio between PCer and Chol present in the mixtures.

Figure S1

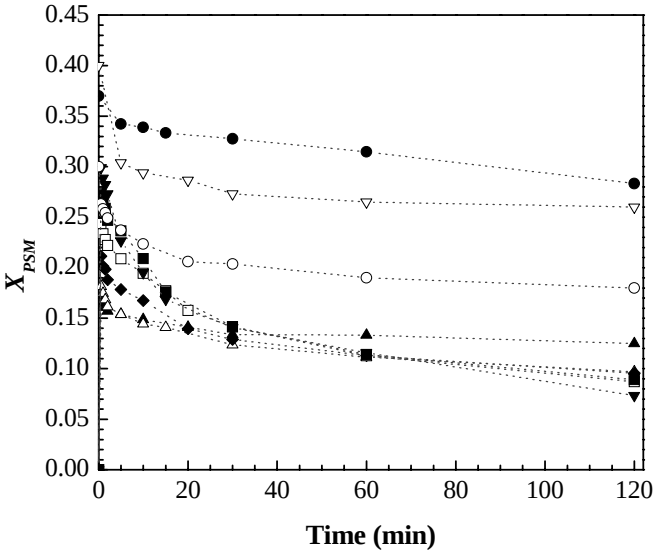
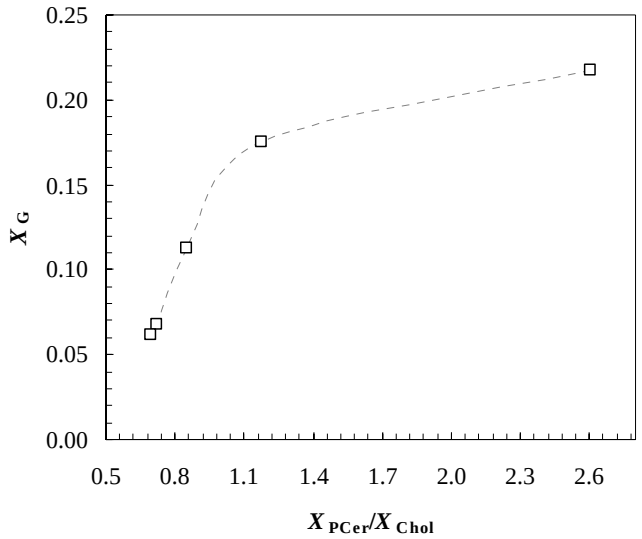

0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
X_{PSM}
0
20
40
60
80
100
120
Time (min)

Figure S2



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