Electronic Supplementary Information for:

Controllable engineering of asymmetric phosphatidylserine-containing lipid vesicles using calcium cations

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Materials and methods

Sample preparation. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) (DOPS), (1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) (DPPS), 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphoserine (ammonium salt) (NBD-PS) and 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3- phosphocholine (NBD-PC) were purchased from Avanti Polar Lipids. NBD-PS and NBD-PC were bought in the 1 mg/mL chloroform solution form. DPPC and DOPS were dissolved in chloroform for stock solutions while DPPS was dissolved in chloroform–methanol mixed solvent. Certain amounts of DPPC and DOPS (or DPPS) were mixed to prepare DPPC–DOPS and DPPC–DPPS mixtures, where the molar percentages of DOPS are 5% and 20% for DPPC–DOPS mixtures and 20% only for DPPC–DPPS mixture. Lipid mixtures were dried under a stream of nitrogen and then kept in a vacuum for more than 12 h to remove the residual solvent. For fluorescence experiments, 0.5% molar percentage of NBD-PS or NBD-PC was added. The mixtures were suspended in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, and recycled between 0 and 70 ^{o}C for 6 times along with vortex to make homogenous dispersions. Ca^{2+}-free MES buffer consisted of 10 mM MES and 100 mM NaCl with a pH value of 7.0, while MES buffer containing 0.5 and 1.0 mM Ca^{2+} was prepared by the addition of proper amount of $CaCl_2$ into Ca^{2+}-free MES buffer. Unilamellar vesicles (ULVs) with a diameter of 100 and 200 nm were prepared by extrusion. The concentration of total lipids was 1 mg/mL for nano differential scanning calorimetry (nanoDSC) experiments and 0.02 mg/mL for fluorescence experiments. Three kinds of vesicle solutions were prepared: vesicles without Ca^{2+}, vesicles with 0.5 mM Ca^{2+} on both sides and vesicles with 0.5 mM Ca^{2+} only on the outer side. The first two kinds of vesicles were prepared directly by

extruding MLVs suspended in the respective buffer solutions at 70 ^{o}C; the third vesicles were obtained by adding an equivalent volume of MES buffer containing 1.0 mM Ca^{2+} into the solution of vesicles without Ca^{2+} at 25 ^{o}C.

NanoDSC. All the calorimetric data were obtained using a CSC Model 6300 Nano III differential scanning calorimeter (Calorimetry Sciences Corp., Lindon, UT) with a capillary cell volume of 0.3 mL. The heating and cooling scans were performed at a constant scan rate of 1 ^{o}C/min.

Fluorescence quenching assay. The fluorescence spectra were recorded by an LS 55 spectrofluorometer (Perkin-Elmer). We used a fluorescence quenching assay method proposed by McIntyre and Sleight to measure the asymmetric degrees of vesicles.[1] The fluorescence emission of the vesicle solutions was measured before and after the addition of a quenching solution of sodium hydrosulfite (1 M $Na_2S_2O_4$ in 100 mM Tris buffer at pH = 10). Sodium hydrosulfite can change the fluorophore 7-nitro-2-1,3-benzoxadiazol-4-yl (NBD) into a non-fluorescent 7-amino-2,1,3-benzoxadiazol-4-yl (ABD) group. The important feature is that sodium hydrosulfite cannot diffuse across lipid bilayer and can only quench NBD groups in the outer leaflet of the vesicles, and thus the relative intensity of fluorescence after adding excess sodium hydrosulfite reflects the percentage of remaining NBD groups in the inner leaflet. To quench the NBD groups in the inner leaflet, Triton X–100 was added to destroy the bilayer structure. If the relative intensity of fluorescence reduced to 0, it means that the added amount of sodium hydrosulfite is in excess. The excitation wavelength for these measurements was set at 460 nm, and the emission spectrum was collected from 480 to 605 nm.

Supplementary results and discussion

Flip-flop model. Flip-flop process of lipids is a common feature of biomembranes. Here the flip process means that lipids move from outer leaflet to inner leaflet and the flop process is the reverse process. For our work with DPPC and DOPS mixed lipids, the percentage of PS molecules in the inner leaflet (r_{in}) over the total amount of PS molecules can be expressed by the following equation:

$$r_{in} = \frac{c_{in}}{c_{in} + c_{out}} \tag{1}$$

where c_{in} and c_{out} are the concentrations of PS in the inner and outer leaflets of the vesicles, respectively. The flip-flop of lipids is usually considered as a first order chemical reaction.[2] The flip-flop of PS can be expressed in the following form:

$$\mathrm{Lipid_{in}} \underset{k_{in}}{\overset{k_{out}}{\rightleftharpoons}} \mathrm{Lipid_{out}} \tag{2}$$

where $\mathrm{Lipid_{in}}$ and $\mathrm{Lipid_{out}}$ represent lipids in the inner and outer leaflets of the vesicles, respectively. Assuming that k_{in} and k_{out} are the rate constants of the flip and flop process, respectively, at a given temperature, the kinetic equation of the isothermal flip-flop process is readily expressed as follows:

$$\frac{\mathrm{d}c_{in}}{\mathrm{d}t} = k_{in}c_{out} - k_{out}c_{in} \tag{3}$$

By dividing ($c_{in} + c_{out}$), we have:

$$\frac{\mathrm{d}r_{in}}{\mathrm{d}t} = k_{in}(1 - r_{in}) - k_{out}r_{in} \tag{4}$$

In equation (4), when the equilibrium state is reached, $\frac{\mathrm{d}r_{in}}{\mathrm{d}t}$ equals to 0. Then we have,

$$r_{\text{in}}^{\infty} = \frac{k_{\text{in}}}{k_{\text{in}} + k_{\text{out}}} \tag{5}$$

The solution of equation (4) is as follows:

$$r_{\text{in}} = \frac{k_{\text{in}} - Ae^{-(k_{\text{in}} + k_{\text{out}})t}}{k_{\text{in}} + k_{\text{out}}} \tag{6}$$

where A is a constant for a given system with inherent flip-flop process.

NBD-PC fluorescence quenching assay

In NBD-PC fluorescence quenching assay, the relative fluorescence intensity of the PS-containing vesicles after quenching treatment (using $Na_2S_2O_4$) showed an obvious decrease after incubation with Ca^{2+} for 6 h, which agreed well with our prediction.

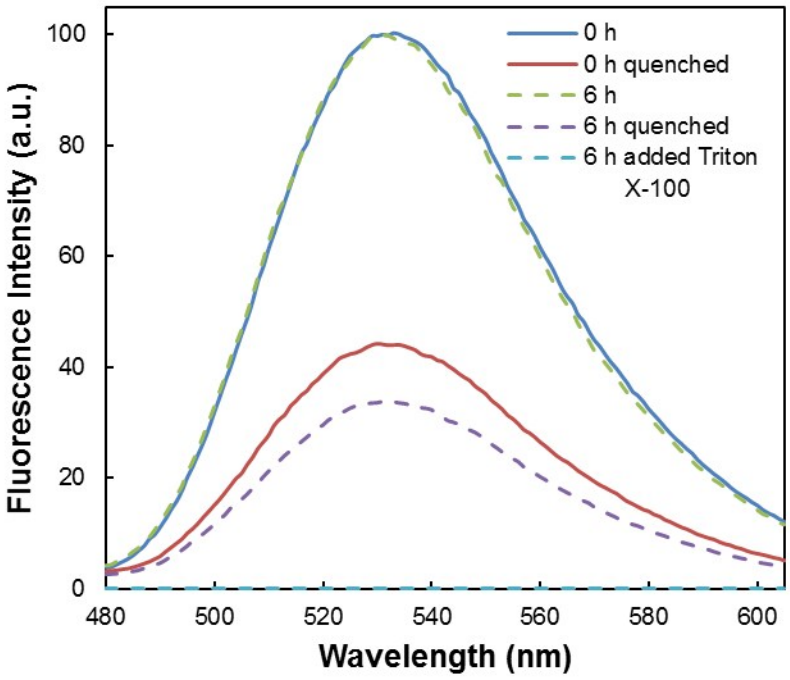

Fig. S1 NBD-PC fluorescence quenching assay. The 0 h spectra (solid lines) were recorded using DPPC–DOPS–NBD-PC (94.5/5/0.5 mol/mol/mol) liposome (*m*(lipid) = 1

mg/mL) prepared in Ca^{2+}-free MES buffer (10 mM MES, 100 mM NaCl, pH = 7.0). The 6 h spectra (broken lines) were obtained using the liposome solution after incubation at 70 ^{o}C for 6 h in the presence of Ca^{2+} ($c(Ca^{2+})$ = 1 mM). The Ca^{2+}-containing liposome solution was prepared by diluting the Ca^{2+}-free liposome solution with equal volume of the buffer containing an extra 2 mM $CaCl_2$.

Zeta potential experiments

In zeta potential experiments, an increase in the zeta potential value was observed when the PS-containing vesicles were exposed to Ca^{2+} (Fig. S2A). This can be explained by the increased positive surface charges due to the attraction interaction between Ca^{2+} and the negatively charged vesicle surface. On the other hand, upon incubation of this sample at 70^{o}C for a different period of time, a decrease of zeta potential was observed (Fig. S2B). This can be explained by the decrease of negative charges in the outer leaflet of vesicles due to the flop of PS lipids into the inner leaflet of the vesicles. The relocation of PS molecules caused the detachment of Ca^{2+} from the vesicles, leading to the decrease in the positive charges in Stern layer.

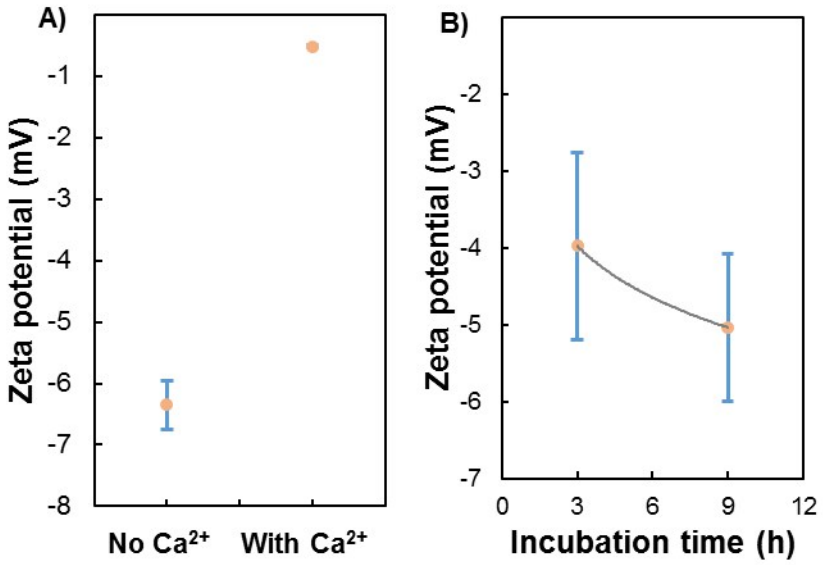

Fig. S2 A) Zeta potential change of DPPC–DOPS (95/5 mol/mol) vesicles before and after the addition of Ca^{2+}. Buffer = HEPES, pH = 7, $c(Ca^{2+})$ = 1 mM, m(lipid) = 1 mg/mL. B) Time-dependence of the zeta potential of the vesicles after incubation with Ca^{2+} at 70°C. Buffer = MES, pH = 7, $c(Ca^{2+})$ = 1 mM, m(lipid) = 1 mg/mL.

Stability of asymmetric degree

The asymmetric degree (r_{in}) can be maintained by lowering the storage temperature, at which the flip-flop rate constants are reduced significantly. This method can keep the asymmetric vesicles stable, as evidenced by the following nanoDSC results.

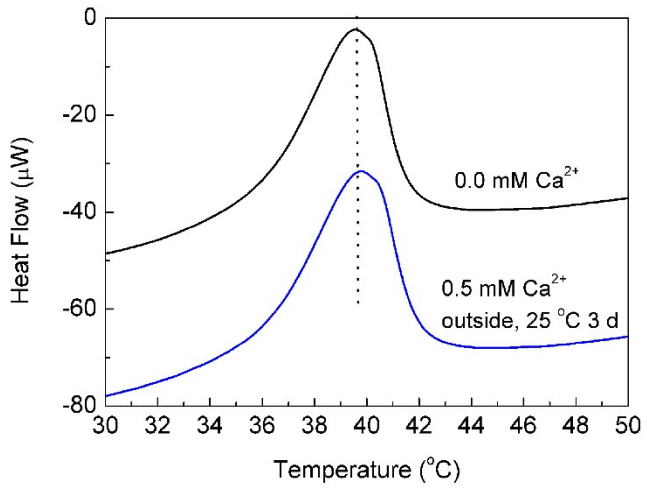

Fig. S3 NanoDSC thermograms of DPPC–DOPS (95/5 mol/mol) vesicles with 0.5 mM Ca^{2+} outside the vesicles after incubation at 25 °C for 3 d (blue line), in comparison with symmetric vesicles in the absence of Ca^{2+} (black line, 25 °C). Note: The nanoDSC curves are vertically shifted for better presentation.

Liposome size

The liposomes were prepared with an extruder (100 nm membrane). The liposome sizes were measured by using dynamic light scattering (DLS) after calcium ion and 70 °C (or 50 °C) treatments as shown in the following figure.

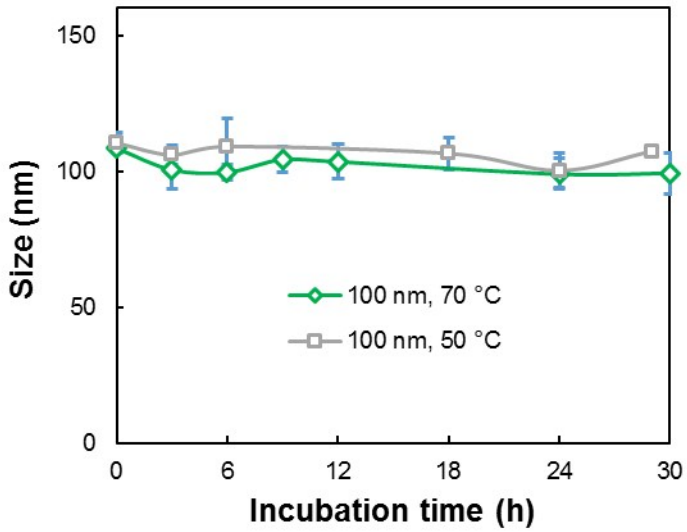

Fig. S4 Change of DPPC–DOPS (95/5 mol/mol) liposome size (measured by DLS) with incubation time. The liposomes were incubated with Ca^{2+} (outside) at 50 or 70 °C.

NanoDSC results of other PS-containing vesicles

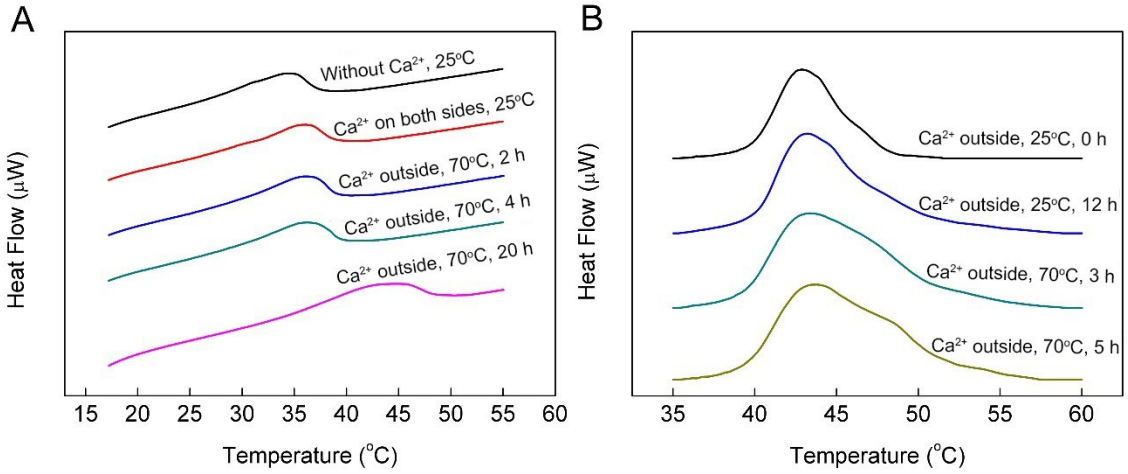

Fig. S5 A) NanoDSC results of DPPC–DOPS (80/20 mol/mol) vesicles with 0.5 mM Ca^{2+} outside the vesicles after incubation at 70 °C for different periods of time, in comparison with vesicles at 25 °C with (red line) and without (black line) 0.5 mM Ca^{2+} on both sides. B) NanoDSC results of DPPC–DPPS (80/20 mol/mol) vesicles with 0.5

mM Ca^{2+} outside the vesicles after incubation at 25 or 70 ^{o}C for different time periods. Note: The nanoDSC curves are vertically shifted for better presentation.

References

(1) McIntyre, J. C.; Sleight, R. G. *Biochemistry* **1991**, *30*, 11819.

(2) Marsh, D. *CRC Handbook of Lipid Bilayers*; CRC Press: Boca Raton, 1990.