Supplementary Information

Effect of Polyunsaturated Free Fatty Acids on Membrane Fusion Mechanism

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Methods

Preparation of Vesicles

We have prepared SUVs from either a mixture of DOPC/DOPE (70/30mol%), DOPC/DOPE/LA (65/30/5 mol%), or DOPC/DOPE/ALA (65/30/5 mol%) using the sonication method.[1, 2] Lipids dissolved in chloroform and ethanolic solutions to make a thin film, and, were dried overnight in a vacuum desiccator for complete evaporation of the solvents. The dried films were hydrated and vortexed in assay buffer for 1 hr for uniform dispersion of lipids. The buffer passed through the Sephadex G-75 column as well as the experimental buffer contained 10 mM TES, 100 mM NaCl, 1 mM EDTA, and 1mM $CaCl_2$ at pH 7.4. The hydrated lipids were sonicated to prepare SUVs using the Hielscher Ultrasonics GmbH model UP100H (Germany) probe sonicator, as documented previously.[3] The average hydrodynamic radii of vesicles were measured utilizing dynamic light scattering (Malvern Zetasizer Nano ZS-90), and were found to be 50-60 nm, with a polydispersity index less than 0.2. Lipid mixing, content mixing, and content leakage experiments were done taking 200 μM lipid. The concentration of the probes was kept constant at 2 μM. Small aliquots of the probe were added from their respective stock solutions prepared in DMSO into working solutions. The DMSO content was always less than 1% (v/v), and this small quantity of DMSO was established not to produce any detectable effect on membrane structure.[4]

Steady-state Fluorescence Anisotropy Measurements

Steady-state fluorescence anisotropy measurements were performed using Hitachi F-7000 (Japan) spectrofluorometer with a 1-cm path length quartz cuvette. DPH and TMA-DPH were excited at 360 nm, and were monitored at 428 nm. Excitation and emission slits with a nominal bandpass of 5 nm were used in all measurements. Each experiment was repeated at least thrice. All measurements were conducted in a buffer containing 10mM TES, 100 mM NaCl, 1 mM $CaCl_2$, and 1 mM EDTA, pH 7.4 at 37 °C. Anisotropy values were calculated using the following equation:[5]

$$r = \frac{I_{VV} - G \times I_{VH}}{I_{VV} + 2G \times I_{VH}} \quad \textbf{(1)}$$

where, $G=I_{HV}/I_{HH,}$ (grating correction or G-factor), I_{VV} and I_{VH} are the measured fluorescence intensities with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively.

Lipid Mixing Assay

NBD-PE (donor) and Rh-PE (acceptor) were used for measuring the transfer of lipids (Lipid Mixing) during PEG-mediated vesicle fusion and was monitored based on the change in FRET efficiency between FRET lipid pairs.[6] FRET dilution as a function of time is considered a marker to measure the kinetics of lipid transfer (mixing) between two vesicles.[1] We prepared a set of vesicles containing FRET lipid pairs in equal concentration (0.8 mol%) and showing maximum FRET. The probe-containing vesicles were mixed with probe-free vesicles at a ratio of 1:9.[7] The lipid mixing process was induced by 3% (w/w) PEG and the process was measured by monitoring the reduction in FRET efficiency via increased in donor intensity. Emission intensity of the donor (NBD-PE) was monitored with Hitachi F-7000 (Japan) spectrofluorometer, keeping the excitation and emission wavelengths fixed at 460 nm and 530 nm, respectively. A minimum slit of 5 nm was used in both the excitation and emission sides, throughout the experiment. Each experiment was repeated at least thrice. The percentage of lipid mixing was calculated using the following equation:

$$\% \, Lipid \, Mixing = \left(\frac{F_t - F_0}{F_\infty - F_0}\right) * 100 \quad \textbf{(2)}$$

where 'F_0', 'F_t', F_∞, are the fluorescence intensities at zeroth time, time = t and time = ∞, respectively. F_∞ has been measured in presence of TX-100, which is considered as the complete mixing of lipids.

Content Mixing Assay

We have monitored the content mixing using the well-established Tb^{3+} and DPA assay proposed by Wilschut *et al.*[8, 9] Vesicles were prepared either in 80 mM DPA or 8 mM $TbCl_3$ and the untrapped DPA and $TbCl_3$were removed from the external buffer of vesicles using a Sephadex G-75 column equilibrated with assay buffer (10 mM TES, 100 mM NaCl, 1 mM EDTA,1 mM $CaCl_2$ at pH 7.4). Content mixing within a mixture of 1:1 $Tb^{3+}$and DPA-containing vesicles was monitored by measuring the increase in fluorescence intensity due to the formation of Tb/DPA complex with time (3% (w/w) PEG was added to initiate the mixing process). The excitation and emission wavelengths of the Tb/DPA complex were 278 nm and 490 nm, respectively. Slits of 5 nm width were used in both excitation and emission sides throughout the experiment. The percentage of content mixing was calculated using the following equation:

$$\% \, Content \, Mixing = \left(\frac{F_t - F_0}{F_\infty - F_d}\right) * 100 \quad \textbf{(3)}$$

where 'F_0' and 'F_t' are the fluorescence intensities at zeroth time and time = t, respectively. 'F_∞' and 'F_d' have been calculated from the fluorescence intensity of the leakage sample at zeroth time and in presence of detergent, respectively.

Leakage Assay

The leakage assay was performed by monitoring the reduction in fluorescence intensity of vesicles containing both $TbCl_3$ and DPA using 3% (w/w) PEG;[8, 9] 8 mM $TbCl_3$ (prepared in 10 mM TES and 100 mM NaCl, pH 7.4) and 80 mM DPA (prepared in 10 mM TES, pH 7.4)

were co-encapsulated in vesicles and the externalTb^{3+}/DPA probe was eliminated by running through a Sephadex G-75 column, equilibrated with the assay buffer (10 mM TES, 100 mM NaCl, 1 mM EDTA, 1 mM $CaCl_2$, pH 7.4).[10]When co-encapsulated Tb^{3+}/DPA complex was discharged from the vesicles, the fluorescence intensity dropped with time due to quenching of Tb^{3+} by EDTA present in the external buffer. Minimum leakage (0%) was characterized by the fluorescence intensity of co-encapsulated Tb/DPA in buffer at zeroth time. The maximum content leakage (100% leakage) was characterized by fluorescence intensity of co-encapsulated $TbCl_3$/DPA vesicle treated with 0.1% (w/v) Triton X-100. The Tb/DPA probe was excited at 278 nm and emission intensity was measured at 490 nm. A minimum slit of 5 nm was used in both the excitation and emission sides, throughout the experiment. Each experiment was repeated at least thrice. The percentage of content leakage was calculated using the following equation:

$$\%\ Content\ Leakage = \left(\frac{F_0 - F_t}{F_0 - F_d}\right) * 100 \qquad \textbf{(4)}$$

where 'F_0' and 'F_t' and 'F_d' are the fluorescence intensities at zeroth time, time = t and in presence of detergent, respectively.

Time-resolved Fluorescence Measurements

Fluorescence lifetimes were calculated from time-resolved fluorescence intensity decays using the IBH 5000F Nano LED equipment (Horiba, Edison, NJ) with Data Station software in time-correlated single photon counting (TCSPC) mode, as mentioned earlier.[11] A pulsed light-emitting diode (LED) was used as the excitation source. The LED generated an optical pulse at 340 nm (for exciting DPH and TMA-DPH), with a pulse duration less than 1.0 ns, and was run at a repetition rate of 1 MHz. Instrument Response Function (IRF) was measured at the respective excitation wavelength using Ludox (colloidal silica) as a scatterer. Ten thousand photon counts were collected in the peak channel to optimize the signal-to-noise ratio. All experiments were performed using emission slits with 8-nm bandpass for both DPH and TMA-DPH. Data were stored and analyzed using DAS 6.2 software (Horiba, Edison,

NJ). Fluorescence intensity decay curves were deconvoluted with the instrument response function and analyzed as a sum of exponential terms as follows:

$$F(t) = \sum_{i}^{n} \alpha_i exp(- t/\tau_i) \quad \textbf{(5)}$$

A considerable plot was obtained with a random deviation around zero with a maximum χ^2 value of 1.2 or less. Intensity-averaged mean lifetimes τ_{avg} for n-exponential decays of fluorescence were calculated from the decay times and pre-exponential factors using the following equation:[5]

$$\tau_{avg} = \frac{\sum_{i=1}^{n} \alpha_i \tau_i^2}{\sum_{i=1}^{n} \alpha_i \tau_i} \quad \textbf{(6)}$$

where, α_i is the fraction that shows lifetimeτ_i.

Calculation of Apparent Rotational Correlation Time

Apparent rotational correlation time provides information about the ease of rotational motion of fluorophores during its lifetime, which is affected by the rigidity of its immediate microenvironment. Therefore, we can obtain information about membrane viscosity in the interfacial as well as hydrophobic region by monitoring the apparent rotational correlation time (θ_c) of TMA-DPH and DPH from steady-state anisotropy and the average lifetime values using Perrin's Equation[5] as follows:

$$\theta_c = \frac{\tau \times r}{r_0 - r} \quad \textbf{(7)}$$

where, 'τ' is the average lifetime of the probe and r is anisotropy. The constant r_0 value was considered as 0.4 for both DPH and TMA-DPH.[5]

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