Supplementary Information

Membrane Cholesterol Regulates the Oligomerization and Fusogenicity of SARS-CoV Fusion Peptide: Implications in Viral Entry

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Methods

Peptide Synthesis

The sequence of SARS CoV fusion peptide is MWKTPTLKYFGGFNFSQIL which is chemically synthesized and purified by GL-Biochem (China) with a purity of >98%. Small aliquots of peptide stock solutions, prepared in DMSO, were added to the vesicle suspensions. The amount of DMSO was always less than 1% (v/v), and this amount of DMSO had no detectable effect on either fusion or membrane structure.[1]

Preparation of Vesicles

Vesicles were prepared from either a mixture of DOPC/DOPE/DOPG (50/30/20 mol%) or DOPC/DOPE/DOPG/CH (40/30/20/10 mol%), or DOPC/DOPE/DOPG/CH (30/30/20/20 mol%) using the sonication method.[2] Lipids at this appropriate molar ratio in chloroform were dried overnight in vacuum desiccator. The dried film was hydrated and vortexed in assay buffers for 1 hour. The column and experimental buffer contained 10 mM TES, 100 mM NaCl,

1 mM EDTA, 1mM $CaCl_2$ at pH 7.4. Then small unilamellar vesicles were prepared using probe sonication as documented previously.[3, 4] All lipid mixing, contents mixing and leakage experiments were performed with 200μM lipid.

Small aliquots of peptides and probes were added from their respective stock solutions prepared in DMSO to prepare the working solutions. The DMSO content was always less than 1% (v/v), and it has been found that this small quantity of DMSO had no detectable effect on membrane structure and peptide interaction with the membrane.[5]

Lipid Mixing Assay

The lipid transfer during PEG-mediated vesicle fusion was monitored using the change in FRET efficiency between NBD-PE (donor) and Rh-PE (acceptor).[6] In order to measure kinetics of lipid transfer (mixing) FRET dilution was measured as a function of time as discussed earlier.[7] In short, we prepared a set of vesicles that contain FRET pair probes in equal concentration, and hence this condition shows maximum FRET. These probe-containing vesicles were mixed with probe-free vesicles at a ratio of 1:9.[8] 6% (w/w) PEG or PEG and peptide were added to induce lipid mixing and was measured by monitoring the reduction in FRET via the change in donor fluorescence intensity. The emission intensities of the donor were monitored in Hitachi F-7000 (Japan), spectrofluorometer. The excitation and the emission wavelength for donor (NBD-PE) are 460 nm and 530 nm, respectively. A minimum slit of 5 nm were used in both the excitation and emission side throughout the experiment. Each experiment was repeated at least three times. Percentage of lipid mixing was calculated using following equation,[9]

$$\% \, LipidMixing = \left(\frac{F_t - F_0}{F_\infty - F_0}\right) * 100 \qquad \textbf{(1)}$$

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 as proposed by Wilschut *et al.* using Tb^{3+} and DPA.[10, 11] Vesicles were prepared either in 80 mM DPA or 8 mM $TbCl_3$. The un-trapped DPA and $TbCl_3$ were removed from the external buffer of the 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). 2 µM peptides were added to a 200µM mixture (1:1) of Tb^{3+} and DPA-containing vesicles 10 mins before addition of PEG to monitor the content mixing. The content mixing was measured in terms of an increase in fluorescence intensity due to the formation of Tb/DPA complex with time. The excitation and the emission wavelength for content mixing assay were used as 278 nm and 490 nm, respectively. A minimum slit width of 5 nm in both excitation and emission side was used for all measurements. Each experiment was repeated at least three times. The percentage of content mixing was calculated in the following way:

$$\%\ ContentMixing = \left(\frac{F_t - F_0}{F_\infty - F_d}\right) * 100 \qquad \textbf{(2)}$$

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

Leakage assay

The leakage assay was carried out by monitoring decrease in fluorescence intensity of the vesicles containing both $TbCl_3$ and DPA in presence of PEG or PEG and peptide.[12] 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 the vesicles, and the external $TbCl_3$ and DPA were removed using 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).[13] The maximum leakage (100%) was characterized by the fluorescence intensity of a co-encapsulated $TbCl_3$/DPA vesicle treated with 0.1% (w/v) Triton X-100.The excitation and the emission wavelength were fixed at 278 nm and 490 nm, respectively. Slits of 5 nm were used in both the excitation and emission sides throughout the experiment. Each experiment was repeated at least three times. Percentage of content leakage was calculated in the following way:

$$\%\, ContentLeakage = \left(\frac{F_0 - F_t}{F_0 - F_d}\right) * 100 \qquad \textbf{(3)}$$

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

Steady State Fluorescence Measurements

Steady state fluorescence measurements were carried out at 37 °C with Hitachi F-7000 (Japan), spectrofluorometer using 1cm path length quartz cuvettes. Peptides were excited at 295 nm and fluorescence emission was monitored from 310 to 450 nm. Excitation and emission slits with a nominal band pass of 5 nm were used for all measurements. Background (peptide free) intensities of samples were subtracted from each sample spectrum to eliminate the contribution of solvent Raman peak and other scattering artefacts.

Fluorescence anisotropy measurements of DPH and TMA-DPH were performed using the same instrument keeping excitation wavelength at 360 nm and emission was monitored at 428 nm. Excitation and emission slits with a nominal band pass of 5 nm were used for fluorescence anisotropy measurements of DPH and TMA-DPH. Fluorescence anisotropy measurement of Tryptophan was performed using the same instrument keeping excitation wavelength at 295 nm and emission was monitored at 350 nm. Excitation and emission slits with a nominal band pass of 10 nm were used for fluorescence anisotropy measurements of Tryptophan. Background (peptide free) intensities of samples were subtracted from each sample spectrum to eliminate the contribution of solvent Raman peak and other scattering artefacts in case of fluorescence anisotropy of Tryptophan. Anisotropy values were calculated using the following equation:[14]

$$r = \frac{I_{VV} - G \times I_{VH}}{I_{VV} + 2G \times I_{VH}} \quad \textbf{(4)}$$

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.

Time-resolved Fluorescence Measurements

Fluorescence lifetimes were calculated from time-resolved fluorescence intensity decays using IBH 5000F Nano LED equipment (Horiba, Edison, NJ) with Data Station software in the time-correlated single photon counting (TCSPC) mode as mentioned earlier.[5, 15] A pulsed light-emitting diode (LED) was used as the excitation source. This LED generates optical pulse at 340 nm (for exciting DPH and TMA-DPH) with pulse duration 1.2 ns and are run at 1 MHz repetition rate. The Instrument Response Function (IRF) was measured at the respective excitation wavelength using Ludox (colloidal silica) as scatterer. To optimize the signal-to-

noise ratio, 10,000 photon counts were collected in the peak channel. The lifetime of DPH and TMA-DPH were performed using emission slits with band pass of 8 nm. 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:

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

A considerable plot was obtained with random deviation about zero with a maximum χ^2 value of 1.2 or less. Intensity averaged mean lifetimes τ_{avg} for tri-exponential decays of fluorescence were calculated from the decay times and pre-exponential factors using the following equation.[14]

$$\tau_{avg} = \frac{\left(\alpha_1 \tau_1^2 + \alpha_2 \tau_2^2 + \alpha_3 \tau_3^2\right)}{(\alpha_1 \tau_1 + \alpha_2 \tau_2 + \alpha_3 \tau_3)}$$

(5)

where, α_i is the fraction that shows τ_i lifetime.

Measurement of location of tryptophan in the membrane

The locations of tryptophan in SARS-CoV fusion peptide can be predicted by monitoring the ratio of fluorescence resonance energy transfer (FRET) efficiency of tryptophan between TMA-DPH and DPH in three different compositions of membranes (DOPC/DOPE/DOPG (50/30/20 mol%) DOPC/DOPE/DOPG/CH (40/30/20/10 mol%) and DOPC/DOPE/DOPG/CH (30/30/20/20 mol%)). DPH and TMA-DPH are known to locate at hydrophobic and interfacial region of the bilayer respectively. DPH is located at an average distance of ~7.8 Å from the centre of the bilayer whereas TMA-DPH locates at the interfacial region because of its polar trimethylammonium group with an average distance of ~10.9 Å from the centre of the

bilayer.[16] We have utilized the distance dependence of FRET efficiency,[17] and measured the FRET efficiency between tryptophan and TMA-DPH and tryptophan and DPH separately. The fluorescence intensity of donor (F_D, tryptophan) in absence and in presence of the acceptor (F_{DA}, TMA-DPH or DPH) were measured and FRET efficiency was calculated using the following equation:[14]

$$E_T = 1 - \left(\frac{F_{DA}}{F_D}\right) \quad \textbf{(6)}$$

Finally, we have calculated the ratio of FRET efficiency ($E_{T,TMA\text{-}DPH}/E_{T,DPH}$) of tryptophan between TMA-DPH and DPH. The location of tryptophan could be speculated from the ratio of the FRET efficiency, higher ratio indicates the closeness of tryptophan to TMA-DPH (shallow penetration) whereas, lower value of ratio is indicative of closeness of tryptophan to DPH (deeper penetration).[18]

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