Electronic Supplementary Information (ESI)

Cholesterol-dependent interaction of SARS-CoV internal and N-terminal fusion peptides: Implications in membrane fusion

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MATERIALS AND METHODS

Materials

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (sodium salt) (DOPG), and Cholesterol (CH), were purchased from Avanti Polar Lipids (Alabaster, AL). N-(7-nitro-2-1,3-benzoxadiazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (NBD-PE), and lissamine rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (Rh-PE), were procured from Thermo Fisher Scientific (Waltham, MA). We have purchased N-[tris(hydroxymethyl)-methyl]2-2-aminoethane sulfonic acid (TES) from Alfa Aesar (Haverhill, MA). Triton X-100 (TX-100), 1,6-diphenyl-1,3,5-hexatriene (DPH), trimethylammonium derivative of 1,6-diphenyl-1,3,5-hexatriene (TMA-DPH), and poly (ethylene glycol) of molecular weight 7000–9000 (PEG 8000) were obtained from Sigma Aldrich (St. Louis, MO). Calcium chloride and ethylenediaminetetraacetic acid (TES) were obtained from Merck (India), while sodium chloride was obtained from Fisher Scientific (India), respectively. UV-grade solvents such as chloroform, DMSO, and methanol were purchased from Spectrochem (India). All the chemicals used in this work are of more than 99% purity. Water was purified through the Adrona Crystal (Latvia) water purification system.

Methods

Peptide Synthesis

The fusion peptide (FP), ^{770}MWKTPTLKYFGGFNFSQIL^{788}, and the internal fusion peptide 1 (IFP1), ^{873}GAALQIPFAMQMAYRF^{888}, were chemically synthesized and purified by BioChain Incorporated (New Delhi, India). Peptides were synthesized using Fmoc chemistry in solid-phase as described elsewhere.[1-3] The HPLC-purified peptide has been characterized by mass spectrometry. Peptide stock solutions were prepared in DMSO, and small aliquots were added to vesicle suspensions. The amount of DMSO was always less than 1.2% (v/v), and this amount of DMSO had no detectable effect on either fusion or membrane structure.[4]

Preparation of Vesicles

Small unilamellar vesicles (SUVs) were prepared from either a mixture of DOPC/DOPE/DOPG (60/30/10 mol%), DOPC/DOPE/DOPG/CH (50/30/10/10 mol%), DOPC/DOPE/DOPG/CH (40/30/10/20 mol%), or DOPC/DOPE/DOPG/CH (30/30/10/30 mol%) using the sonication method as documented earlier.[5, 6] The vesicles have an average hydrodynamic diameter of 50-60 nm, with a polydispersity index of less than 0.2. The vesicular size has been measured using an Anton Paar dynamic light scattering instrument, model Litesizer DLS 500. Lipids at the appropriate molar ratios from chloroform stock solution were dried overnight in a vacuum desiccator, and the dried film was hydrated and vortexed in assay buffers for 1 hour. The experimental buffer contained 10 mM TES, 100 mM NaCl, 1 mM EDTA, and 1 mM $CaCl_2$ at pH 7.4. All lipid mixing experiments were performed with 200 μM lipids. Small aliquots of peptide and probes were added from their respective stock solutions prepared in DMSO to prepare the working solutions.

Measurement of FP-IFP1 Interaction in the Membrane

The FP-IFP1 interaction in the membrane milieu has been evaluated by measuring the fluorescence intensity of the tryptophan residue present in the FP. The fluorescence intensity of the tryptophan changes upon the addition of IFP1 as the formation of the FP-IFP1 complex changes the microenvironment of tryptophan present in FP. The tryptophan was excited at

295 nm, and the emission spectrum was monitored from 310 to 500 nm during sequential addition of IFP1 to the FP-bound membranes. The FP concentration was kept constant at 2 μM while the IFP1 concentration was varied from 0 to 16 μM. The spectrum of the 200 μM lipid-containing buffer solution was subtracted from the sample spectrum to eliminate the solvent Raman peak and other scattering effects. The fluorescence measurements were carried out at 37 °C, using a Hitachi model F-7000 (Japan) spectrofluorometer. For all measurements, a 1 cm pathlength quartz cuvette was used, and the excitation and emission slit widths were set at 5 nm.

Lipid Mixing Assay

The kinetics of lipid transfer (mixing) were measured by measuring FRET dilution as a function of time, as reported earlier.[7] 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, 8] In short, we prepared a set of vesicles that contain FRET pair probes in equal concentration (0.8 mol%), and hence this condition shows maximum FRET. These probe-containing vesicles were mixed with probe-free vesicles at a ratio of 1:9.[9] A small amount of PEG (either 4 or 5% (w/v)) was added to induce lipid mixing, and was measured by monitoring the reduction in FRET through the change in donor fluorescence intensity. To evaluate the effect of the peptide or the peptide complex, a requisite amount of peptide (or complex) was added in the presence of a small amount of PEG (either 4 or 5% (w/v)). To evaluate the effect of the peptide (or peptide complex), the kinetic data of LM in the absence of peptide were subtracted from the kinetic data in the presence of the peptide. The emission intensities of the donor were monitored in a Hitachi F-7000 (Japan) spectrofluorometer. The excitation and the emission wavelength for the donor (NBD-PE) are 460 nm and 530 nm, respectively. A minimum slit of 5 nm was used on both the excitation and emission sides throughout the experiment. Each experiment was repeated at least three times. The percentage of lipid mixing was calculated using the following equation,[8]

$$\% \text{ Lipid Mixing} = \left(\frac{F_t - F_0}{F_\infty - F_0}\right) \times 100 \quad (1)$$

where 'F_0', 'F_t', and 'F_∞' are the fluorescence intensities at the zeroth time, time = t, and time = ∞, respectively. F_∞ has been measured in the presence of TX-100, which is considered the complete mixing of lipids. The calculated %lipid mixing was plotted as a function of time and fitted to a double exponential equation, and the maximum %lipid mixing was calculated at time infinity.

Calculation of Change in % Lipid Mixing

The change in % Lipid Mixing was calculated using the following equation:

Change in %LM =

(% of LM in the presence of either FP, IFP1, or preformed FP-IFP1 complex) − (% of LM in the absence of either FP, IFP1, or preformed FP-IFP1 complex) (2)

The value of change in %LM provides the effect of either FP, IFP1, or preformed FP-IFP1 complex on the extent of lipid mixing. It reflects the impact of the peptide or peptide complex alone, as the effect of PEG on the lipid mixing is subtracted during the calculation of the change in %LM.

Calculation of % Change in Lipid Mixing by Preformed FP-IFP1 Complex

The effect of preformed FP-IFP1 complex on the lipid mixing was evaluated from the calculation of the % change in lipid mixing in the presence of preformed FP-IFP1 complex using the following equation:

%Change in LM =

(change in %LM in the presence of preformed FP-IFP1 complex) − (Numerical addition of change in %LM in the presence of FP and IFP1 individually) ***(3)***

Steady State Fluorescence Anisotropy Measurements

Steady state fluorescence measurements were carried out at 37 °C with a Hitachi F-7000 (Japan) spectrofluorometer using 1 cm path length quartz cuvettes. Fluorescence anisotropy measurements of DPH and TMA-DPH were performed, keeping the excitation wavelength at 360 nm, and emission was monitored at 428 nm. Excitation and emission slits with a nominal bandpass of 5 nm were used for fluorescence anisotropy measurements of DPH and TMA-DPH. Anisotropy values were calculated using the following equation:[10]

$$r = \frac{I_{VV} - G \times I_{VH}}{I_{VV} + 2G \times I_{VH}} \tag{4}$$

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.[6, 11] A pulsed light-emitting diode (LED) was used as the excitation source. This LED generates an optical pulse at 340 nm for exciting DPH and TMA-DPH with a pulse duration of 1.2 ns and is run at a 1 MHz repetition rate. The Instrument Response Function (IRF) was measured at the respective excitation wavelength using Ludox (colloidal silica) as a scatterer. To optimize the signal-to-noise ratio, 10,000 photon counts were collected in the peak channel. DPH and TMA-DPH lifetime of individual peptides and complex were performed using emission slits with band passes of 8 nm and 16 nm, respectively. 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:

$$F(t) = \sum_i^n \alpha_i exp(-t/\tau_i) \tag{5}$$

A considerable plot was obtained with a random deviation of 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:[10]

$$\tau_{avg} = \frac{(\alpha_1 \tau_1^2 + \alpha_2 \tau_2^2 + \alpha_3 \tau_3^2)}{(\alpha_1 \tau_1 + \alpha_2 \tau_2 + \alpha_3 \tau_3)} \quad (6)$$

where α_i is the fraction that shows τ_i lifetime.

Calculation of Apparent Rotational Correlation Time

Apparent rotational correlation time gives us information about the indulgence of rotational motion of fluorophores during their lifetime, which is mostly affected by the nearby microenvironmental rigidity. Thus, we know about the viscosity of the membrane bilayer in both interfacial and acyl chain regions by measuring the apparent rotational correlation time (θ_c) of TMA-DPH and DPH from steady-state fluorescence anisotropy and the average lifetime values using Perrin's equation[10] as follows:

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

Where "τ" is the lifetime of the probe and r is the anisotropy. The constant r_0 value was considered as 0.4 for both DPH and TMA-DPH.

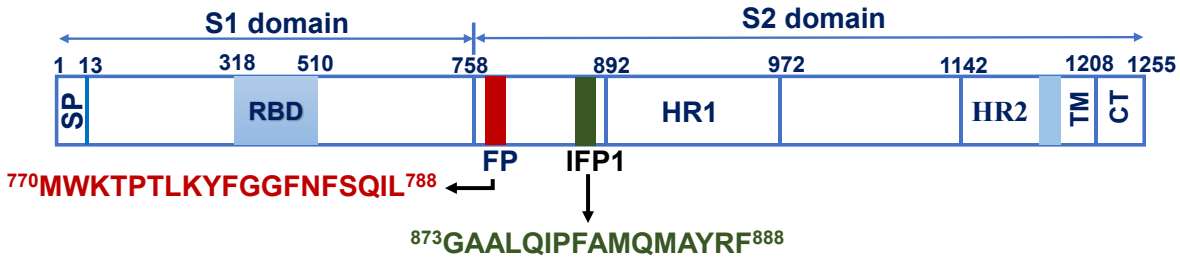
The Circular Dichroic (CD) spectroscopy

CD spectra of FP, IFP1, and preformed FP-IFP1 complex were measured in a Jasco 1500 (Japan) Spectrophotometer. CD spectra were recorded in the wavelength range of 180-250 nm in a far-UV range using a cylindrical quartz cuvette of 2mm pathlength. Spectra were scanned in 1 nm wavelength increments with 2 nm bandwidth and at a scan rate of 50 nm/min. All the circular dichroic spectra are the averages of at least 5 consecutive scans. The background spectrum (without peptide) was measured with the same parameters, and the background spectrum was subtracted from the sample spectrum. Instrument obtained ellipticity values were converted into molar ellipticity using the following equation:

$$[\theta] = \frac{< \theta >_{degree}}{10lnc}$$

where, '$< \theta >_{degree}$' is the ellipticity as measured by CD spectroscopy, 'l' is the pathlength in cm, 'n' is the number of amino acid residues present in the peptides, and 'c' is the concentration of protein solution in moles litre^{-1}.

RESULTS



Scheme ES1. The SARS-CoV spike glycoprotein (S) is made up of the S1 and S2 domains, which are separated by a protease cleavage site. The fusion peptide (FP) and the internal fusion peptide (IFP1) are located at the S2 domain.

Effect of Peptide and Peptide Complex on the Lipid Mixing

Polyethylene glycol (PEG)-mediated fusion assay offers a convenient platform to evaluate the effect of the peptide on the hemifusion and pore formation.[5] This has been shown earlier that the PEG-mediated fusion data support the cellular fusion results.[9] We have evaluated the effect of FP, IFP1, and FP-IFP1 complex on membrane fusion utilizing a PEG-mediated fusion assay. In this assay, we use a small amount of PEG to induce fusion by inducing osmotic stress and removing water from the inter-bilayer space (control). The effect of the peptide was evaluated through the addition of PEG in membranes, which were pre-incubated with the peptide. Therefore, the effect of the peptide could be easily evaluated by subtracting the kinetic data of the control from the peptide (PEG + peptide). We have carried out the fusion experiments in the presence of 5% (w/v) PEG for the membranes with lipid composition DOPC/DOPE/DOPG (60/30/10 mol%) and DOPC/DOPE/DOPG/CH (50/30/10/10 mol%) for both control and in the presence of peptides and preformed FP-IFP1 complex. However, 4% (w/v) PEG was used for

the membranes containing a higher concentration of membrane cholesterol, i.e., DOPC/DOPE/DOPG/CH (40/30/10/20 mol%) and DOPC/DOPE/DOPG/CH (30/30/10/30 mol%), considering the higher fusogenic behavior of cholesterol. Nonetheless, the control kinetic data have always been subtracted from the peptide data; therefore, the effect of the peptide remains independent of PEG concentration. The kinetic data of lipid mixing for the four different lipid compositions in the absence (control) and presence of FP, IFP1, and preformed FP-IFP1 complex are shown in **Fig. ES2**. The subtracted kinetic data (demonstrating the effect of the peptide) for the DOPC/DOPE/DOPG/CH (30/30/10/30 mol%) have been shown in **Fig. 2A.**

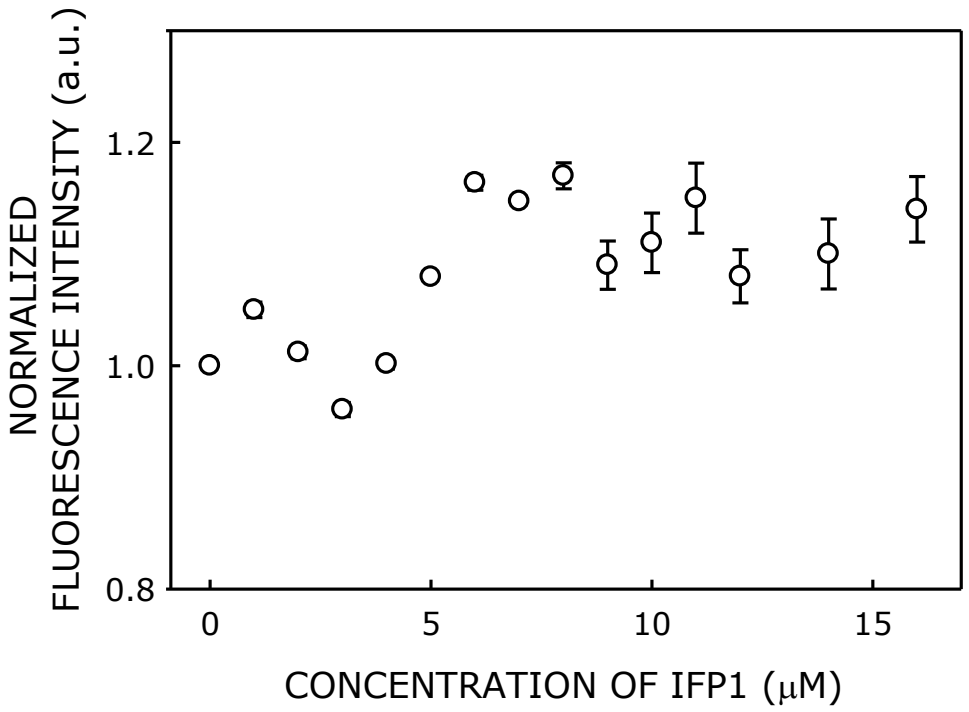


Fig. ES1. Plot of fluorescence intensities of tryptophan in FP as a function of added IFP1 in 10 mM TES, 100 mM NaCl, 1 mM $CaCl_2$, and 1 mM EDTA, pH 7.4, at 37 °C in the absence of any lipid membranes. The FP concentration was kept constant at 2 μM while the IFP1 concentration was varied from 0 to 16 μM. Data points shown are means ± SE of at least three independent measurements.

Analysis of the secondary structure of peptides and their complex

The secondary structures of FP, IFP1, and the preformed FP-IFP1 complex were calculated using the DICHROWEB program.[12, 13] The program considers the mean residue ellipticity data in the wavelength region of 180-250 nm. The analyzed data of the CD spectra (**Fig. 1B**) of peptides and their complex in DOPC/DOPE/DOPG/CH (30/30/10/30 mol%) membranes are shown in **Table ES1**. Our results show that FP is a majorly unstructured peptide (80.30%) with a small amount of α-helix (0.30%) and some amount of β-sheet component (18.80%). On the other hand, IFP1 is a mixture of α-helix (32.50%) and unstructured component (51.20%) with some amount of β-sheet (16.30%) component. However, the preformed FP-IFP1 complex is majorly unstructured (75.50%) with 24% of β-sheet conformation (**Table ES1**). Taken together, the results clearly indicate that the FP and IFP1 are interacting with each other in the membrane milieu.

Table ES1. Secondary structure of FP, IFP1, and preformed FP-IFP1 complex calculated using the DICHROWEB program

Secondary Structure	**FP (%)**	**IFP1 (%)**	**Preformed FP-IFP1 Complex (%)**
α-helix	0.30	32.50	0.20
β-sheet	18.80	16.30	24.30
Unstructured	80.90	51.20	75.50

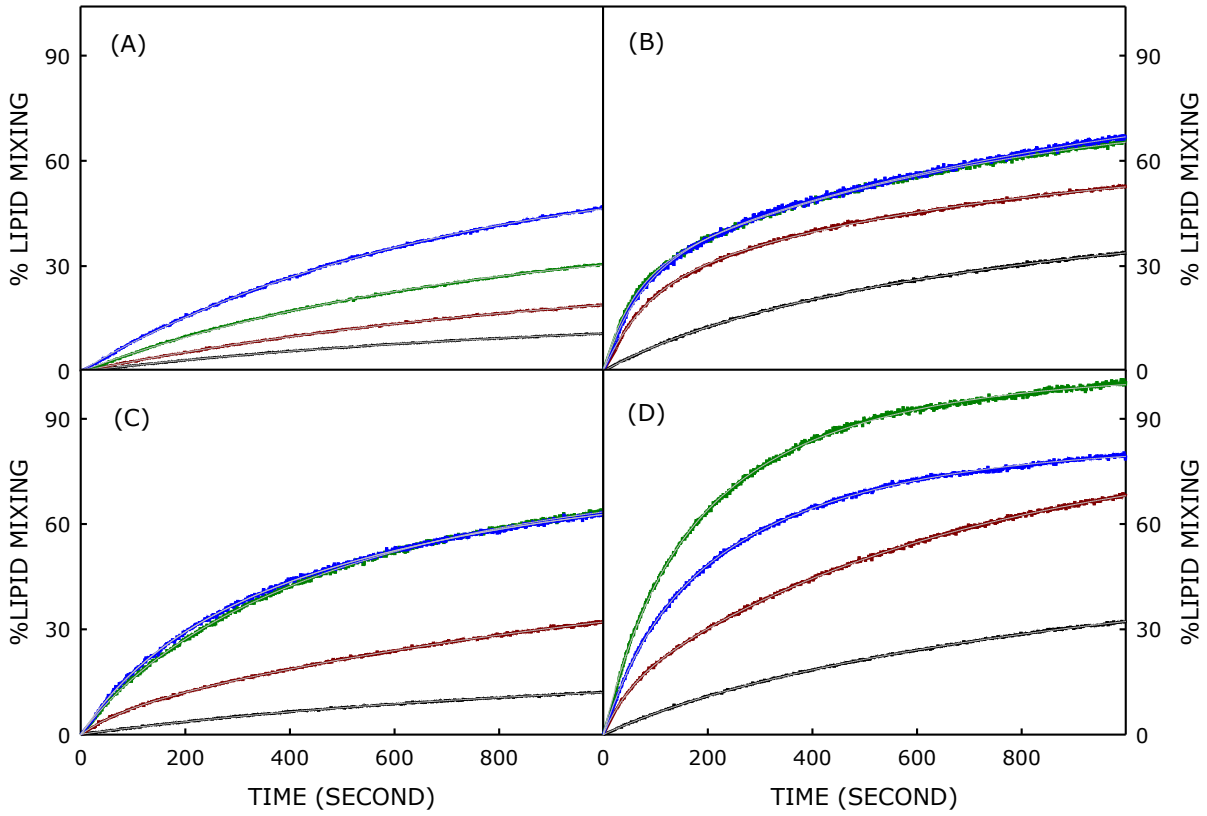


Fig. ES2. Plot of lipid mixing kinetics of DOPC/DOPE/DOPG (60/30/10 mol%), DOPC/DOPE/DOPG/CH (50/30/10/10 mol%), DOPC/DOPE/DOPG/CH (40/30/10/20 mol%), and DOPC/DOPE/DOPG/CH (30/30/10/30 mol%) membranes, in the presence of FP (Red), IFP1 (Green), numerical addition of FP and IFP1 (Yellow) and FP-IFP1 complex (Blue) in 10 mM TES, 100 mM NaCl, 1 mM $CaCl_2$ and 1 mM EDTA of pH 7.4. Fusion was induced by 5% (w/v) PEG for DOPC/DOPE/DOPG (60/30/10 mol%), DOPC/DOPE/DOPG/CH (50/30/10/10 mol%), and 4% (w/v) PEG for DOPC/DOPE/DOPG/CH (40/30/10/20 mol%), DOPC/DOPE/DOPG/CH (30/30/10/30 mol%) membranes at 37 °C. The FP and IFP1 concentrations are kept at 2 µM and 12 µM, respectively, and the total lipid concentration is 200 µM. The data shown are the average of at least three independent measurements. The percentage of LM was calculated using Eq. 1.

The binding affinity of FP in membranes of varied compositions

We have evaluated the binding affinity of FP to the membranes in the absence and presence of cholesterol by measuring the emission intensity of tryptophan by exciting at 295 nm. The fluorescence intensity was plotted as a function of lipid concentration, and the data were fitted to the classical Langmuir model of ligand binding (Fig. ES3). The binding affinities of FP in DOPC/DOPE/DOPG (60/30/10 mol%) and DOPC/DOPE/DOPG/CH (30/30/10/30 mol%) membranes are 1.25 and 1.40 μM, respectively. This indicates that the binding of FP to the membrane does not depend on membrane composition. However, the interaction of FP and IFP1 is dependent on the composition of the membrane (**Fig.1A**).

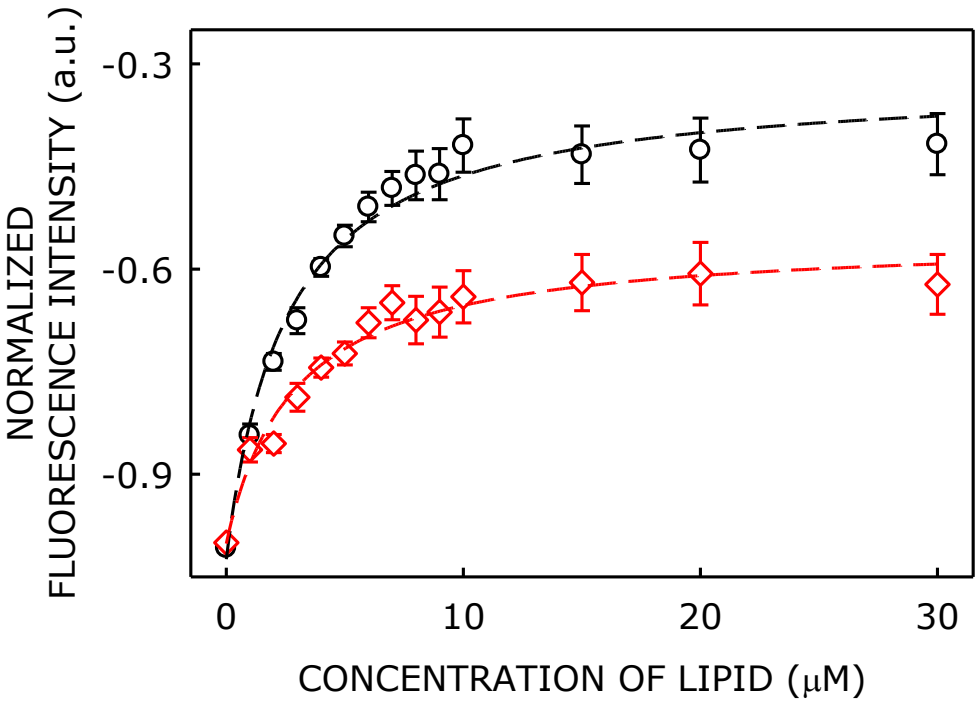
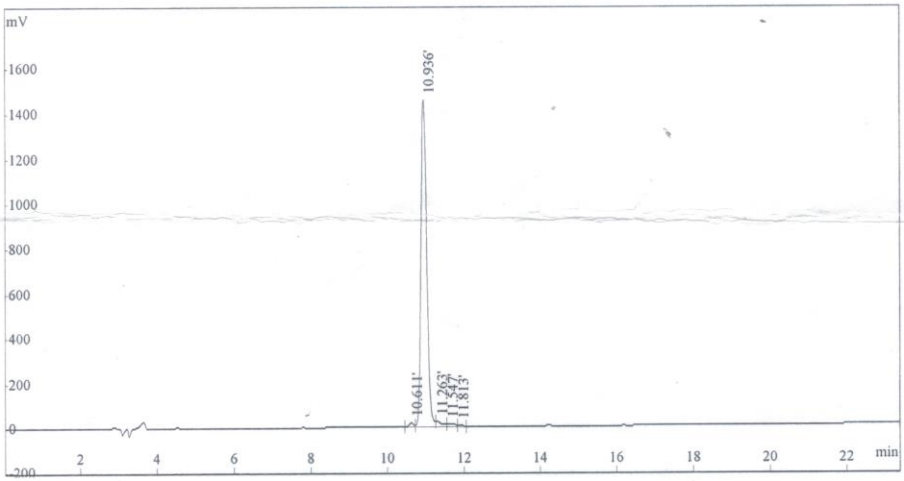


Fig. ES3. Determination of the binding affinity of FP in DOPC/DOPE/DOPG (60/30/10 mol%, Black) and DOPC/DOPE/DOPG/CH (30/30/10/30 mol%, Red) membranes. The fluorescence intensity of tryptophan in FP has been plotted as a function of the concentration of lipid. The concentration of FP was kept constant at 2 μM. Measurements were carried out at 10 mM TES, 100 mM NaCl, 1 mM $CaCl_2$, and 1 mM EDTA, pH 7.4, at 37 °C. Data points shown are means ± SE of at least three independent measurements.

HPLC REPORT

Product Name	:ML-19
Lot No	:P220214-HS284152
Column	:Gemini-NX 5μ C18 110A, 4.6*250mm
Solvent A	:0.1%Trifluoroacetic in 100% Acetonitrile
Solvent B	:0.1%Trifluoroacetic in 100% Water
Gradient	: A B
	0.01min 20% 80%
	25min 80% 20%
	25.01min 100% 0%
	30min Stop
Flow rate	:1.0ml/min
Wavelength	:220nm
Volume	:20ul

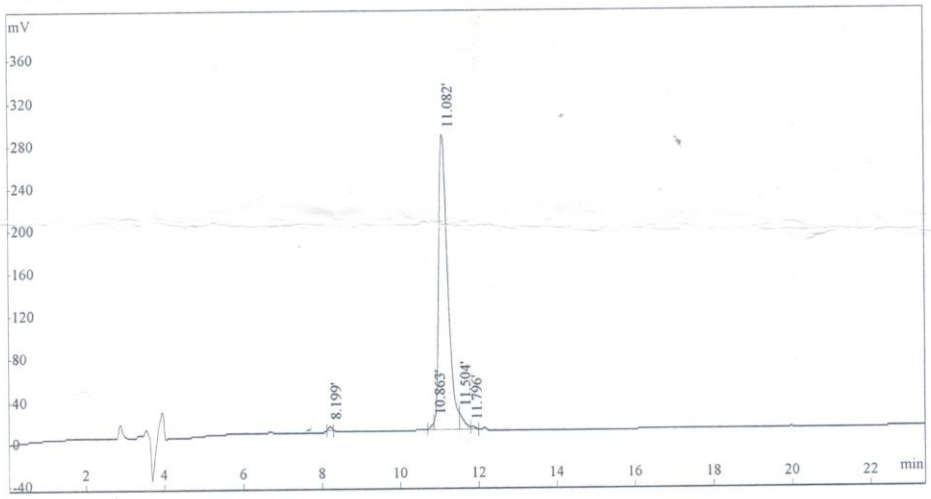


Rank	Time	Conc.	Area	Height
1	10.611	0.9164	136651	17123
2	10.936	95.8718	14295416	1453045
3	11.263	1.8161	270805	24401
4	11.547	1.1050	164763	12291
5	11.813	0.2907	43350	4931
Total		100	14910985	1511791

Fig. ES4. HPLC report of fusion peptide (FP).

HPLC REPORT

Product Name	:GF-16
Lot No	:P220214-HS301756
Column	:Gemini-NX 5μ C18 110A, 4.6*250mm
Solvent A	:0.1%Trifluoroacetic in 100% Acetonitrile
Solvent B	:0.1%Trifluoroacetic in 100% Water
Gradient	: A B
	0.01min 30% 70%
	25min 90% 10%
	25.01min 100% 0%
	30min Stop
Flow rate	:1.0ml/min
Wavelength	:220nm
Volume	:20ul



Rank	Time	Conc.	Area	Height
1	8.199	0.4333	19229	3350
2	10.863	0.7140	31685	7495
3	11.082	95.6362	4244124	276495
4	11.504	2.7741	123108	15239
5	11.796	0.4424	19631	2654
Total		100	4437777	305233

Fig. ES5. HPLC report of internal fusion peptide 1 (IFP1).

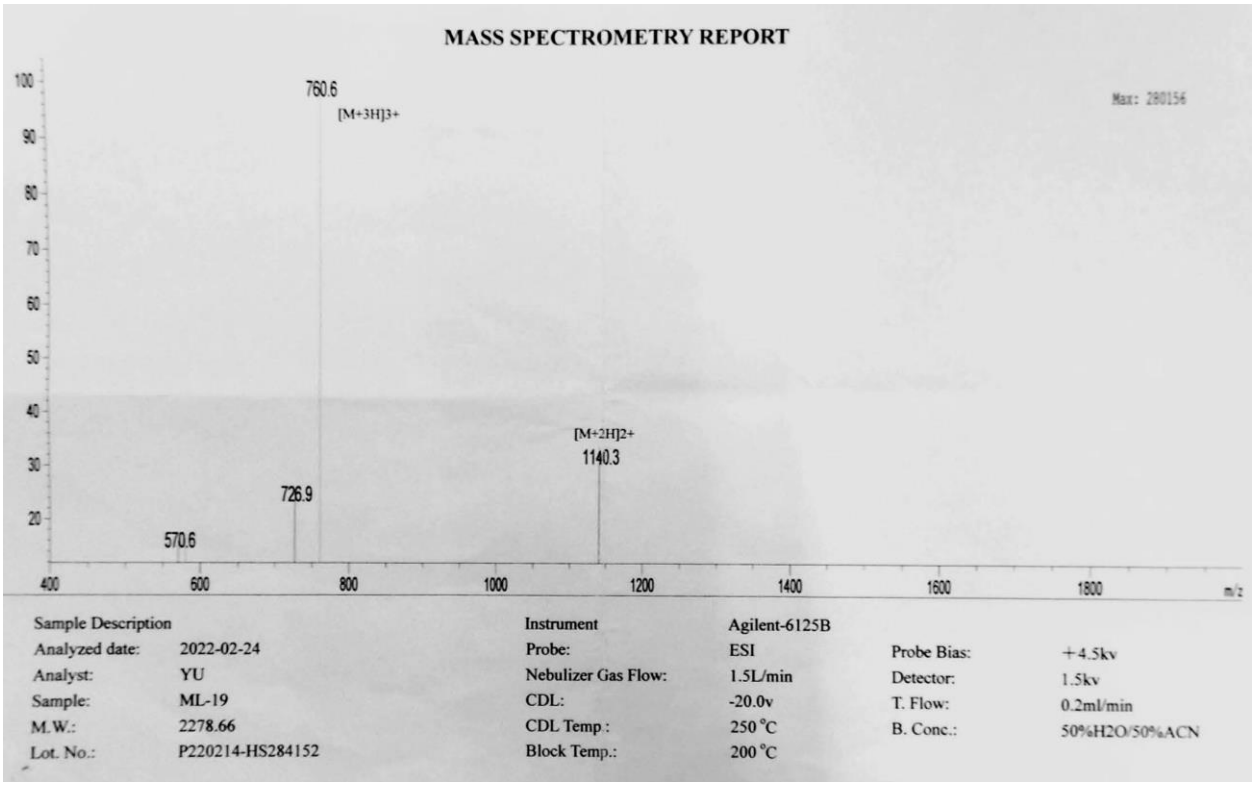


Fig. ES6. Mass Spectrometry report of fusion peptide (FP).

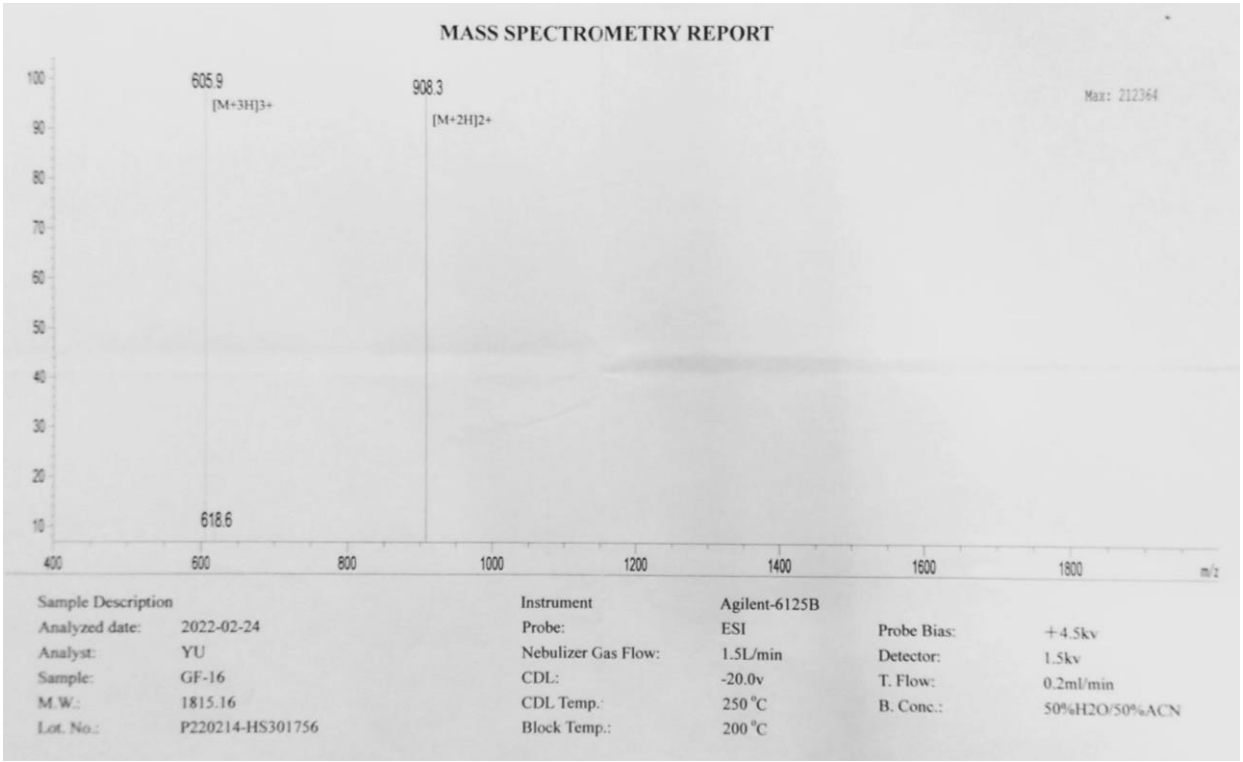


Fig. ES7. Mass Spectrometry report of internal fusion peptide 1 (IFP1).

References

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