Supporting information

Pathways for creation and annihilation of nanoscale biomembrane domains reveal alpha and beta-toxin nanopore formation process

Nirod Kumar Sarangi and Jaydeep Kumar Basu

Department of Physics, Indian Institute of Science, Bangalore--560 012, India

Materials and Methods:

Phospholipids such as 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, DO (> ~99% purity), N-octadecanoyl-D-*erythro*-sphingosylphosphorylcholine, SM (> ~99% purity) and Cholesterol, Chl (> ~99% purity) was obtained from Avanti polar lipids. Fluorescent probe 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine labeled with Atto 488 was obtained from ATTO-TEC GmbH, Germany. The lipid stock solutions (1 mM each) were prepared in chloroform (HPLC grade, Sigma Aldrich). The lipids were mixed in a ratio of 2:2:1:0.0005 (DO:SM:Chl:Atto488-PE) thoroughly and sprayed on the water subphase for the formation of interfacial monolayers. Ultrapure water with a resistivity of 18.2 Ωcm was used as the subphase for all monolayer studies produced by a two-stage Elix-3 and Milli-Q (Millipore Academic) system. Prior to the experiment, the mini trough was cleaned with ethanol (extra pure AR grade, Fine Chemicals, India) several times and finally rinsed with ultra-pure water. The supported lipid bilayers were prepared by using the Langmuir-Blodgett (LB) technique at a constant holding surface pressure of 33 mN/m as reported earlier by us.¹⁻⁵ In brief, during the preparation of supported lipid bilayers using the LB method, multiple compression-expansion cycles were followed at a constant trough temperature of 15 ± 1 °C before the collapse surface pressure, and subsequently, the bilayers were transferred at a highly condensed surface pressure to the hydrophilized glass slides by using the layer-by-layer transfer. Prior to transfer, glass substrates (20mm×20mm, Germany) were cleaned using piranha solution (a 30:70 mixture of 30% hydrogen peroxide and concentrated sulfuric acid at 80°C) for 30 min and washed multiple times with MilliQ water. The first monolayer was transferred at a holding surface pressure of 33 mN/m by the vertical withdrawal of the substrate at a speed of 5 mm/min with a transfer ratio of $\sim 1.0 \pm 0.1$. The second

monolayer transferred at the same surface pressure by a vertical downstroke at a speed of 3 mm/min yielded a centrosymmetric bilayer (Y-type).

Listeriolysin O was expressed in *E. coli* and purified by Ni-NTA affinity chromatography essentially as described earlier.⁶ The incubation of LLO (5µg/ml) was done at 37°C at a pH of 5.4 MES buffer. The plasmid expressing LLO was obtained from Dr. Gregor Anderluh. pGS1111 plasmid containing the ClyA gene as a fusion with glutathione S-transferase was obtained from Dr. J. Green, University of Sheffield, UK. The details of the purification processes were reported elsewhere.⁷ The incubation of ClyA (2µg/ml) was done at 37°C with phosphate buffer saline at a pH of 7.4.

Analyses of FCS:

The particle number fluctuations $N(t) = \langle N \rangle + \delta N(t)$ of fluorescent molecules entering and leaving the focus of a confocal microscope observation spot ($d=200$ nm) during the excitation can be calculated by measuring the emitted intensity $I(t)$ as $I(t) = \langle I \rangle + \delta I(t)$; where $\langle I \rangle$ and $\delta I(t)$ represents a time averaged intensity and fluctuations respectively. In FCS, the autocorrelation function $G(t_c)$ from the intensity signal $I(t)$ measured in the microscope is calculated using;

$$G(t_c) = \frac{\langle \delta I(t) \cdot \delta I(t+t_c) \rangle_t}{\langle I(t) \rangle_t^2} \quad (S1)$$

where $\langle \dots \rangle_t$, denotes a time average over the time variable t .

The correlation data are normalized and fitted to single component 2D equation using Eq.(1) (see main text). For all of our FCS data in a confocal mode in the absence of toxin, the anomaly α was found to be ~ 1 suggesting the diffusion is Brownian in both L_o and L_d regions. A similar observation with $\alpha=1$ was observed in the presence of ClyA. In contrast, in the presence of LLO, α was found to be 1 ± 0.02 and 0.87 ± 0.1 in L_d and L_o regions respectively, indicating the diffusion is not purely Brownian in the later region. The representative FCS autocorrelation data of LLO reconstituted DO:SM:Chl bilayers are shown in Fig.S1a. The black and red open symbols are the experimental data collected from L_d and L_o regions (left panel) respectively. The solid lines are the fit using Eq.1. The right panel shows residual plots showing the accuracy of fit.

Figure S1. (a) Normalized autocorrelation data of DO:SM:Chl bilayers after LLO reconstitution. Open symbols are the experimental data. Solid lines (black and blue) are the fit to Eq. 1. Right panel illustrates the residuals of the anomalous diffusion model fit, showing the accuracy of the fit.

Lipid Monolayer Assay and in-situ confocal microscopy imaging

Pore-forming toxin (PFT) - lipid interactions were studied at 25°C with the Langmuir film balance technique coupled with inverted confocal microscopy, Leica SP5 Germany, a homemade technique as described earlier.⁸ The interaction of PFTs monomers and their association to the membrane is an interfacial phenomenon which can be studied by surface pressure (π) measurements of lipid monolayers at the air/water interface as well as in-situ confocal microscopy imaging techniques. The underlying idea is that the insertion of the toxin underneath the lipid monolayer can be detected, at the constant area and/or constant time, by an increase in the surface pressure. This increase in the surface pressure is caused by the initial binding of the toxin to the cholesterol present between the polar heads groups of phospholipid interfacial monolayer, which is not counterbalanced by an increase of the area of the monolayer. Monomolecular films of the DO:SM:Chl (2:2:1) lipid mixtures with 0.001 mol% Atto647-PE were spread on air/water interface. Figure S2 shows the Π -t curve showing the monolayer behavior of indicated lipid mixtures at the air/water interface before and after toxin addition. The addition of toxin increases the surface pressure suggesting lipid-toxin interaction at the interface.

The adsorption of LLO is more prominent when compared with ClyA indicating interaction is stronger for the case of LLO than ClyA. Inset figure indicates the real-time Π -t curve during the recording of confocal images (cf. Fig. S3) at different constant surface-pressure as indicated by a horizontal plateau (arrow mark) with respect to time.

Figure S2. Surface-pressure vs time plot for DO:SM:Chl (0.001 mol% Atto647-PE) monolayer at air/water interface before (black) and after injection of ClyA (red) and LLO (blue) addition in the subphase. The inset shows some of the holding surface pressures as shown by arrow marks where the confocal imaging was carried out.

STED-FCS calibration: The observation spot at various STED power can be obtained by measuring FCS autocorrelation data of Atto488-PE (0.0005 mol%) probes stained in pristine DOPC supported lipid bilayers (SLBs prepared by the LB method) and estimating the transit time (τ_D) at each STED power. Figure S3a represents the fluorescence signal bursts from Atto488-PE at zero (black) and 230 mW (blue) STED power. The respective auto correlation raw data along with an intermediate STED power (150 mW) are shown in Figure S3b. Upon increasing the STED power, the amplitude increases (see arrow in Fig. S3b) as a result of decrease in number of molecules (N) and this leads to the effective reduction in the illumination

area. Figure S3c represents the normalized autocorrelation at the respective STED power showing the shift (see arrow) in correlation data towards low lag time (t_c).

Figure S3. (a) Intensity-time plot of Atto488-PE diffusing in DOPC SLBs in the absence (black) and in the presence of STED depletion laser power (230 mW). (b) Represents the respective autocorrelation raw data curve which measures the self-similarity of the fluctuating fluorescent burst signal using Eq. (S1) along with an intermediated STED power data (red). (c) Normalized correlation data showing the shift in correlation data upon increasing STED power as shown in arrow. (d) Transit time (τ_D) versus the square of observation spot diameter, d^2 plot.

The transit time, τ_D values at different STED power was obtained from fitting using Eq.2 (main text). At STED power zero, d ($P_{STED} = 0$) the observation spot d was found to be 200 nm and this was obtained by scanning the fluorescence chromeo bead of size 40 nm. The other observation spot at different STED power d ($P_{STED} \neq 0$) can be obtained using equation S2;

$$\frac{d(P_{STED}=0)}{d(P_{STED})} = \sqrt{\frac{\tau_D(P_{STED}=0)}{\tau_D(P_{STED})}} \quad (S2)$$

where τ_D is the transit times correspond to each values of the STED power used. In the above equation, we assume that the lipid diffusion in pristine DOPC SLBs undergoes a two dimensional free Brownian diffusion where the diffusion time scales linearly with the observation spot diameter, d^2 . Figure S3d shows the τ_D vs. d^2 plot obtained by using Eq. 1 and Eq. S2 respectively.

Figure S4. Confocal microscopy images of DOPC bilayer in the absence (a), and in the presence of ClyA (b) and LLO (c) toxin. The bilayer is stained with ATTO488-PE and the scale bar is 2 μm . The toxin reconstituted images (b,c) were acquired after 120 min. (c) Represents the autocorrelation data in the absence of toxin (filled square), and in the presence of ClyA (after 30 min: filled circle, 120 min: filled triangle) and in the presence of LLO (after 30 min: inverted filled triangle, 120 min: filled diamond).

Figure S5. Confocal microscopy image of DOPC:Chl (3:1) bilayers (a) in the absence and (b) in the presence of ClyA toxin. The bilayer was stained with ATTO488-PE (0.0005 mol%). (c) Represents the FCS diffusion law plot with (open symbol) and without (closed symbol) ClyA. The solid lines are fit to Eq.(3) (see main text) and the dotted lines are the extrapolation to y-axis to show the intercept values.

Figure S6. Time-lapse confocal microscopy image of PO:SM:Chl (2:2:1) bilayer in the presence of (a) ClyA and (b) LLO toxin. The bilayer is stained with Atto488-PE (0.0005 mol%) and the

scale bar is 5 μ m. (c) Represents the change in diffusion coefficient with respect to time in presence of ClyA (red) and LLO (black).

Figure S7. Confocal microscopy image of DOPC:DPPC:Chl (2:2:1) bilayers in the (a) absence, and in the presence of (b) ClyA and (c) LLO toxin. The bilayers were prepared using the LB technique and stained with ATTO488-PE (0.0005 mol%). The bottom panel in the respective images is the fluorescence intensity line profile obtained from a region of interest as shown by solid green lines in the image.

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