Supporting Information

Insertion Mechanism of Cell-penetrating Peptides into Supported Phospholipid Membranes Revealed By X-ray and Neutron Reflection

D. Choi, J.H. Moon, H. Kim, B.J. Sung, M.W. Kim, G. Tae, S.K. Satija, B. Akgun, C.-J. Yu, H.W. Lee, D.R. Lee, J.M. Henderson, J.W. Kwong, K.L. Lam, K.Y.C. Lee, and K. Shin

Experimental details

A synthesized TDP, H_3N^+-YGRKKRRQRRR-COO^-, with purity over 97%, was purchased from Anygen (Gwangju, Korea). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was purchased from Avanti Polar Lipids and used without further purification. To prepare homogeneous supported planar DPPC bilayers, we developed a modified vesicle fusion technique (McKiernan *et al*., Biophysical Journal, 73, 1987-1998, 1997); Lipids were dissolved homogeneously in chloroform. The solvent was then evaporated under nitrogen gas, and was further dried under vacuum for 4 hrs. The dried lipids were then hydrated and suspended in 10 mM Trizma buffer at pH 8.0 with 100 mM NaCl by vortexing at a temperature above the gel-fluid transition temperature ($T > T_{lc} \sim 42\ ^oC$). While maintaining $T \sim 50\ ^oC$, the hydrated lipid solution was then extruded through a 50 nm filter by using a mini extruder, to produce small unilamellar vesicles (SUV). The lipid-deposited substrate was then immersed into a *Trizma* buffer solution, which was prepared with D_2O for NR and H_2O for XR. When the solution with SUV solution was spread onto the silicon slab, it spontaneously formed a single bilayer on the silicon slab within a few minutes.

Prior to lipid deposition, silicon substrates were subjected to the following cleaning procedures in order to ensure the formation of a native oxide layer on the silicon surface: the 76 mm and 100 mm diameter Si slabs for NR and the silicon wafers pre-cleaved into 5 mm x 10 mm squares for XR were sonicated in methanol and deionized (DI) water respectively for 3 min. The samples were dried in N2 gas before each immersion in the different solvents. To get a uniform oxide layer, removal of pre-existing oxide layer and re-oxidation are necessary. Therefore, the substrates were immersed in very dilute hydrofluoric acid solution ($HF:H_2O$, v:v=1:10) for 3 min. and followed by subsequent rinsing in DI water, for 2 min. Then, we applied the modified Shiraki technique which has been shown to create a thin oxide layer (~ 20 Å). A sulfuric acid solution ($H_2SO_4:H_2O_2:H_2O$, v:v:v=1:1:3) was mixed and the silicon substrates were immersed for 15 min while it was being heated to ~ 100 ^oC.

After washing the excess vesicles away from the supported bilayer by shaking it gently in deionized (DI) water, we directly assemble the bilayer covered silicon slab with

liquid cells in DI water without an exposure to air to avoid the rupturing of bilayer in air. When the assembly was finished, D_2O was exchanged through the liquid filling ports, making the sample ready for the neutron experiment. Design of the liquid cell for the reflectivity measurement is well described elsewhere (Lee et al., *JKPS*, 53, 1944-1950, 2008). The supported lipid membrane was then rinsed by shaking it gently at a temperature above the transition temperature in an aqueous environment. During the whole process of vesicle fusion described above, maintaining temperature well above the gel-liquid transition temperature was very important. Several test runs were conducted to optimize the process, and ca. 85 - 92 % lipid coverage was usually observed using by neutron reflectometry.

Only TDP Induces Increase in Defect Coverage at the Membrane

SLB was made by fusing small unilamellar vesicles (SUVs) of DPPC onto a Si substrate. The vesicle solution was exposed to the Si substrate over a period of two hours, after which the solution was flushed out using a desired medium. Analysis of NR data of a pure DPPC bilayer sample made in this fashion shows that the bilayer did not have complete coverage but rather a ~ 88% coverage. Hence the initial defect coverage was ~ 12% at the start of any experiment. Cycling the temperature of the SLB does not result in additional loss of lipid materials, and the % defect coverage remains at ~ 12%. In addition, no increase in pore coverage was observed when a non-cell penetrating peptide was injected into the system. Figure S1 shows the sequence of TDP with that of a non-cell penetrating peptide, TAT-control (TAT-C), used to check if a non-cell penetrating peptide could result in a similar increase in defect coverage as TDP. Figure S1 further shows the NR profiles when a supported DPPC bilayer was subjected to 0 (black), 2.4 (red), 4.8 (green), and 9.6 (yellow) µM of TAT-control. As the data show, subjecting the bilayer to TAT-control does not result in any increase in the % defect coverage. The fact that increase in % defect coverage is only observed when TDP is present indicates that the additional defect formation has to do with the TDP (presumably due to the solubilzation of lipids in the presence of TDP), a peptide capable of going through the membrane. While we cannot rule out that some

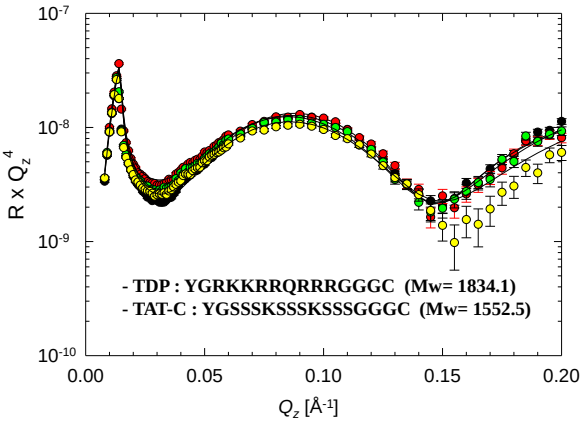

Fig. S1. NR profile of DPPC bilayer with 0 (black), 2.4 (red), 4.8 (green) and 9.6 (yellow) µM TAT-control (TAT-C). Primary sequences of TDP and TAT-C are shown in the inset.

peptides can reach spots of bare silicon from the solution due to the initial non-100% bilayer coverage, that alone cannot account for the observed increase in SLD in the headgroup region of the proximal leaflet only when TDP is present. The only logical conclusion therefore is that TDP is translocated to the proximal leaflet in the process, giving rise to the increased SLD observed.

Temperature Cycling Does Not Result in Loss of Lipid Materials

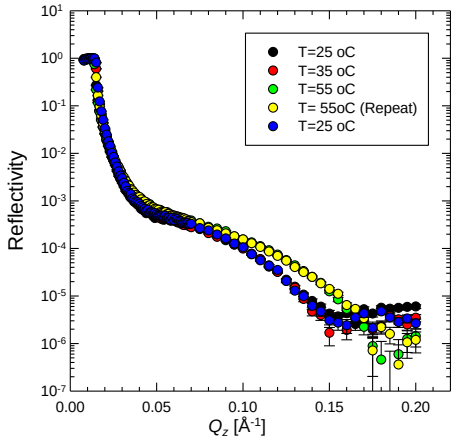

Fig. S2 XR profiles from temperature cycling experiment for pure DPPC bilayers.

In order to assure that the temperature cycling protocol employed in both XR and NR experiments do not result in the loss of lipid materials, a process which will complicate interpretation of the experimental results, we have carried out measurements using pure DPPC bilayers but subjecting it to a similar temperature cycling as we did in the presence of the peptide. Fig. S2 shows the XR curves taken for the same sample starting at 25°C (black), then heating the sample up to 35°C (red) and then to 55°C (green). The system was held at 55°C for 2 hours (yellow) before being cooled back down to 25°C (blue). As can be seen from the curves, they all overlap in the entire Q_z range, indicating that no D_2O penetrates into the bilayer even after the temperature cycling. The small differences at higher Q_z are due to the more disordered state of the bilayer (with slightly thinner membrane) at the higher temperature (green and yellow). The overlap of the green and the yellow curves taken at 55°C but 2 hours apart shows that the bilayer at the high temperature state is stable. Finally, this type of cycling experiment in the absence of any peptide was performed before each peptide injection experiment to ensure that the bilayer was stable. This and other similar sets of experiments performed on pure DPPC bilayers clearly demonstrate that the temperature cycling protocol does not lead to the loss of lipid materials.

Schematic of the SLD profiles and simulated NR data

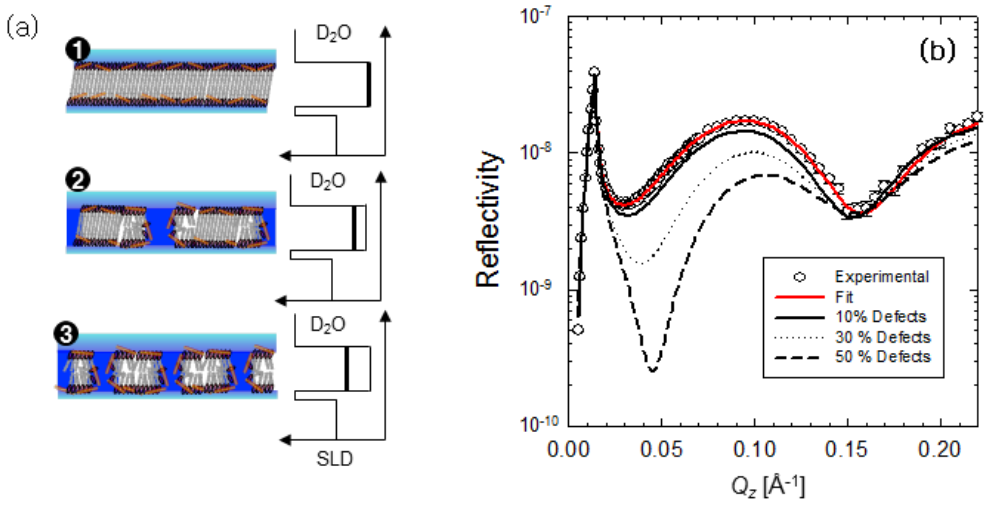

Fig. S3. (a) Schematic of the SLD profiles for different lipids absent volumes, (1) the lowest SLD of the lipid bilayer without defects, (2,3) the increases in the SLD profiles when D_2O solution occupies the defects. (b) NR data (open circles) for the SLB before the TDP injection, together with the best fit (red line). The simulated NR curves are obtained from models with 10 % (black line), 30 % (dots), and 50 % (dashed line) defect volume.