Calcium-dependent Hydrolysis of Supported Planar Lipids Were Triggered by honey bee venom Phospholipase A_2 with Right Orientation at Interface

Siqi Kai, Xu Li, Bolin Li, Xiaofeng Han, Xiaolin Lu*

1. Materials

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-sn-glycero-3–phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (18:1 Liss Rhod PE), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (16:0 Liss Rhod PE), 1-Oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-Glycero-3-Phosphocholine (18:1-12:0 NBD PC), 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino] dodecanoyl}-sn-glycero-3-phosphocholine (16:0-12:0 NBD PC) were purchased from Avanti Polar Lipids**.** The fluorescein-5-isothiocyanate (FITC) was purchased from Fanbo Biochemicals. Phosphalipase A_2 from honey bee venom (Apis mellifera) ($bvPLA_2$) was purchased from Sigma Aldrich. Chloroform, ethylenediaminetetraacetic acid tetrasodium salt (EDTA·4Na), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), calcium chloride dihydrate ($CaCl_2·2H_2O$), sodium chloride (NaCl) were purchased from Aladdin. All the reagents were used without any further purification. All solutions were prepared using deionized water (18.2 MΩ·cm) purified with a Milli-Q system (Millipore).

2. Preparation of supported lipid bilayers for SFG..

Supported lipid bilayers were prepared on CaF_2 prisms by the Langmuir-Blodgett (LB)/Langmuir-Schaeffer (LS) methods for SFG spectra collection. The mixture of DOPC: DPPC (1:1 mol %) was dissolved in chloroform at a total concentration of 1 mg/mL in a glass bottle and stored under -20 ˚C. CaF_2 prisms were pretreated by toluene and plasma to remove the surface contaminants. The proximal leaflet was prepared by KSV NIMA Langmuir-Blodgett instrument under the surface tension of 34 mM/m and then air-dried overnight. The distal leaflet was formed on the surface of water (in a Teflon dish) with a surface pressure of 34 mN/m. By contacting the proximal leaflet with the distal leaflet, a supported bilayer was formed.

3. Preparation of supported lipid bilayers for LSCM

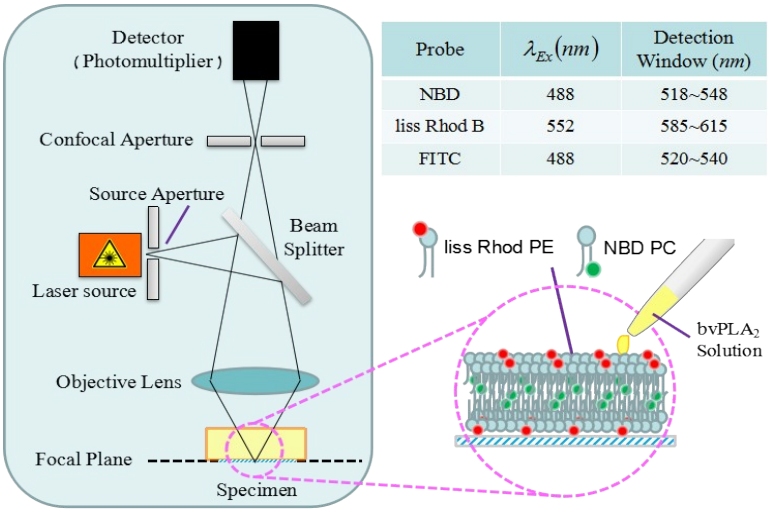
We prepared supported lipid bilayers for laser scanning confocal microscopy (LSCM) on the glass substrates. First, the mixture of DOPC: DPPC (1:1 mol%) and fluorescent lipid (2 wt% as compared to mixture of POPC: DPPC) was dissolved in chloroform at a total concentration of 2 mg/mL in a centrifuge tube. The solution was then dried under nitrogen and a film on the bottom of the centrifuge tube was obtained. The centrifuge tube was then put into vacuum oven overnight to eliminate chloroform completely. The buffer solution (20 mM NaCl, 20 mM HEPES, 0.1 mM EDTA·4Na, pH=7) was added into the tube and the lipid thin film was dispersed into the solution under vortex and sonication (Heating is needed when necessary) at a concentration of 2 mg/mL. After three freeze-thaw cycles, the lipid solution was ready for vesicle preparation. Vesicles with a diameter of ~50 nm were prepared via the extrusion method at 60 °C. The vesicle solution was diluted to reach a concentration of 0.25 mg/mL and then incubated on a glass substrate surface (which was pretreated by piranha solution/oxygen plasma) at 60 °C to form the supported lipid bilayer. To track the lyso-PCs and fatty acids in one experiment, a supported bilayer containing

both Liss Rhod PE and NBD-PC was prepared. The formed bilayer was rinsed gently by ultrapure water for 5 times for LSCM experiment.

4. Supplementary LSCM image and Matlab Analysis

The bilayer was deposited on a dish with a glass bottom customized for LSCM experiment. The experiment was conducted via a Leica TCS SP8 microscope. The dish was placed on the objective table and aligned with a 63×/1.40 OIL objective lens. First, to position the bilayer, which was labeled by NBD-PC and/or liss Rhod PE, the height of the objective lens was adjusted until image of a smooth and flat film could be observed. For the NBD labeled bilayer (tracking fatty acids), the excitation laser was at 488 nm and the detection window was in the range from 518 nm to 548 nm. For the liss Rhod PE labeled bilayer (tracking lyso-PCs), the excitation laser was at 552 nm and the detection window was in the range from 585 nm to 615 nm. Two sequences can be settled simultaneously which allow for tracking signals from NBD and liss Rhod PE at the same time. Three images (channel 1, channel 2, overlap) can thus be generated for one snapshot. For FITC (tracking bvPLA$_2$), the excitation laser was at 488 nm and the detection window was in the range from 520 nm to 540 nm. Therefore, fluorescence signals of NBD and FITC can not be detected simultaneously.

Before adding bvPLA$_2$ into the solution, a small amount of water was pipetted from the dish and mixed with the bvPLA$_2$ solution. The mixing process should be gentle in order to reduce the mechanical disturbance and avoid the sudden increase of local momentary concentration. After adding bvPlA$_2$, the morphological evolution of the substrate was observed over the time and the images were captured.



Probe	$\lambda_{Ex}(nm)$	Detection Window (nm)
NBD	488	518~548
liss Rhod B	552	585~615
FITC	488	520~540

Figure S1. LSCM experimental scheme (left). The zoomed-in bilayer deposited on the dish (bottom right), and the excitation wavelengths and detection windows for the three dye molecules (upper right).

Figure S2 indicates, without adding Ca^{2+}, bvPLA$_2$ can only be adsorbed onto the lipid bilayer surface (first two images in the second row); but the hydrolysis was not initiated (first two images in the first row). Upon adding 2mM Ca^{2+}, the catalysis of the interfacial lipids can be activated (first row for tracking lyso-PCs and third row for overlay).

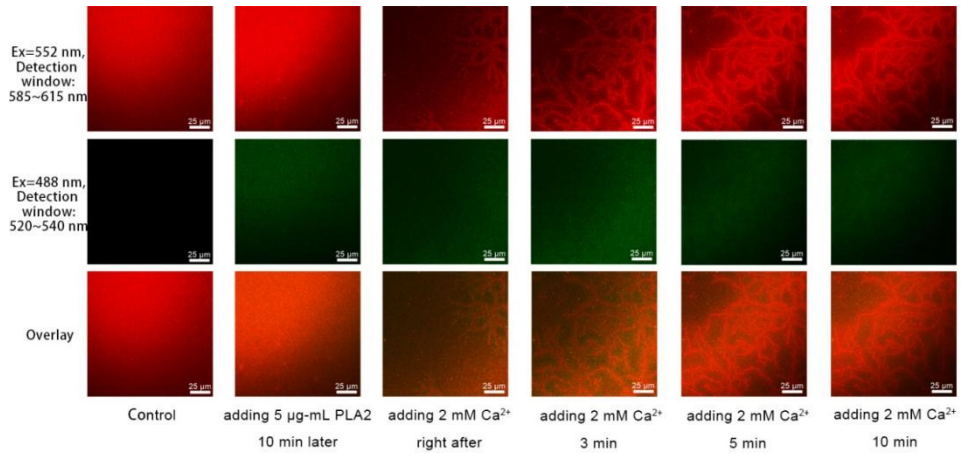


Figure S2. Interfacial adsorption and co-localization of bvPLA$_2$ and lyso-PC monitored by Liss Rhod PE and FITC-bvPLA$_2$ from no Ca^{2+} to adding 2 mM Ca^{2+}.

Matlab Release 2015a software was used for the brightness analysis of the image, see Figure S3. The image used in the analysis was a grayscale image transformed from the LSCM image. The image was saved in bmp format and imported into matlab workspace. Then the 3D brightness distribution image (Figure 5b) and the contour map (Figure 5c) of the brightness distribution were obtained by executing the corresponding commands. Besides, a horizontally cross section line was selected to get the 2D brightness image (Figures 5d, 5e, and 5f).

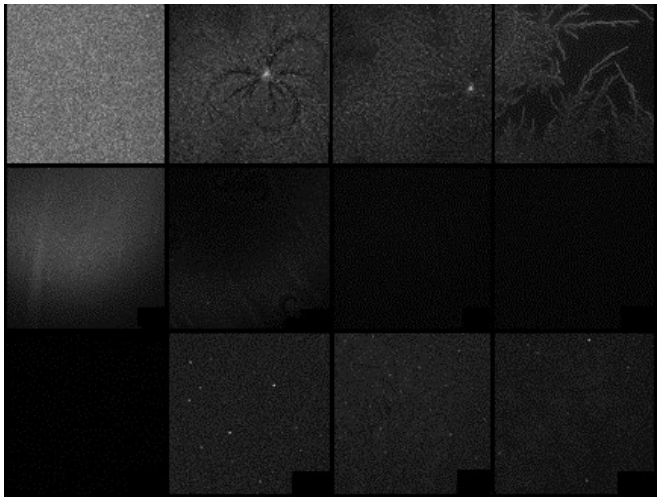


Figure S3. Image used for Matlab analysis.

5. SFG spectra collection and Fitting

As a second-order nonlinear optical process, SFG is surface or interface sensitive; and for materials with inversion symmetric, SFG is forbidden under the electric dipole approximation.[1] Because on a surface or at an interface, the symmetry is generally broken, SFG is an ideal analytical technique to study the events happening on a surface or at an interface. It involves two incident beams and one output beam. With one of the incident beams being frequency tunable in the mid-infrared range and the other being visible, vibrational signals of the surface or interfacial molecules can be detected. In this communication, we used a commercial SFG spectroscopic system from EKSPLA, Lithuania. All SFG spectra were collected with ssp (s-polarized SFG signal, s-polarized visible, and p-polarized IR beam) polarization combination.Figure S4 shows the schemtic for the SFG experiment. The bilayer was prepared at the bottom of the right-angle

prism in a total internal reflection geometry. The SFG signal change can be monitored when Ca^{2+} or $bvPLA_2$ was added.

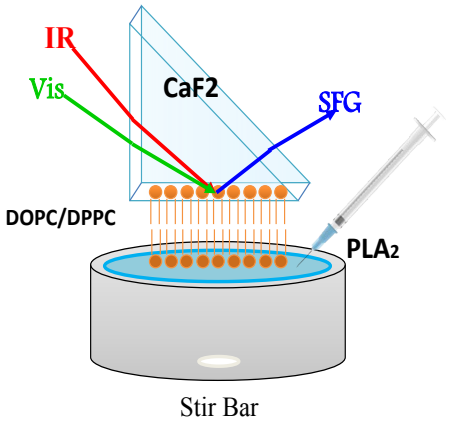


Figure S4. The schematic SFG experiment in a total internal reflection geometry.

Figure S5 shows the SFG spectra in the CH range for the DOPC/DPPC bilayer, upon adding 5 μg/mL $bvPLA_2$, and upon adding 2 mM Ca^{2+}, sequentially. Initially, owing to the high symmetry of the lipid bilayer, only weak CH vibrational signals were observed. Upon adding $bvPLA_2$, again, weak CH vibrational siganls were observed since the hydrolysis was not initiated. Upon hydrolysis by adding Ca^{2+}, the CH signals increased substantially, indicating the inversion symmetry of the original lipid bilayer was broken. Since fatty acids were desorbed from the interface, such CH signals should mainly come from the retained lysolipids.

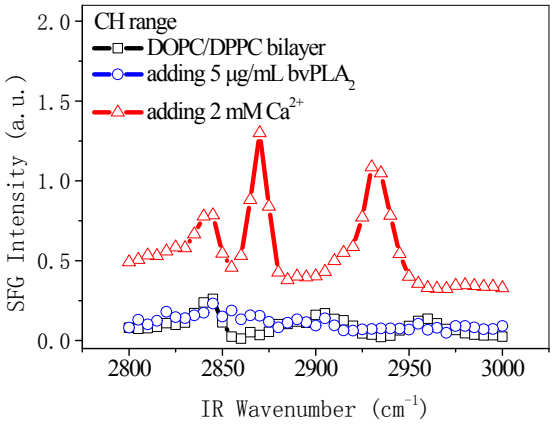


Figure S5. SFG spectra in the CH range (from 2800 cm^{-1} to 3000 cm^{-1}) for the DOPC/DPPC bilayer, upon adding 5 μg/mL $bvPLA_2$, and upon adding 2 mM Ca^{2+}, sequentially..

The static spectra in Figures 5a and 5b for the $bvPLA_2$ amide I band were fitted using the Lorentzian equation. Before hydrolysis, there was no resonant signal in the amide I range. Only SFG spectra after hydrolysis were fitted.

$$\chi = \chi_{nr} + \sum_q \frac{A_q}{\omega - \omega_q + i\Gamma_q}$$

Curves for the time dependent intensity change at 1660 cm^{-1} in Figures 2c and 2d were fitted using an equation for simulating the adsorption process, where k_a is an adsorption constant. The fitting parameters were listed in Table S1.

$$\sqrt{I(t)} = \sqrt{I_\infty}(1 - e^{-k_a(t-t_0)})$$

The desorption process for the fatty acids shown in Figure 5h was fitted using the following equation

$$I(t) - I_\infty = (1 - I_\infty)e^{-k_d(t-t_0)}$$

Where k_d is a desorption constant. It should be noted, for the above fitting equations, in comparison to the infrared dynamic curve, due to the coherent nature of SFG, square root for the SFG intensity is needed.[2] The fitting parameters were listed in Table S1.

Table S1. Fitting Parameters for SFG spectra, time-dependent curves for SFG at 1660 cm^{-1} (Figure 2) and ATR-FTIR experiment (Figure 5).

Equation		$\chi = \chi_{nr} + \sum_q \frac{A_q}{\omega - \omega_q + i\Gamma_q}$	
Fitting Parameters	ω_q (cm^{-1})	Γ_q (cm^{-1})	A_q
Fig. 2a / adding 2 mM Ca^{2+}	1660	21.7±2.2	22.7±2.7
Fig. 2b / adding 5 μg/mL PLA_2 after 2 mM Ca^{2+}	1660	21.0±1.4	21.5±1.2
Equation		$\sqrt{I(t)} = \sqrt{I_\infty}(1 - e^{-k_a(t-t_0)})$	
Graph / Curve	I_∞	k_a (s^{-1})	t_0 (s)
Fig. 2c	0.73±0.04	0.00147±0.00008	980
Fig. 2d	1.05±0.07	0.00140±0.00014	500
Equation		$I(t) - I_\infty = (1 - I_\infty)e^{-k_d(t-t_0)}$	
Fitting Parameters	I_∞	k_d (min^{-1})	t_0 (min)
Fig. 5h / peak 1	0.478±0.004	1.35±0.07	0
Fig. 5h / peak 2	0.455±0.007	1.30±0.14	0

References:

[1]. Y. R. Shen, *Nature,* 1989, **337**, 519−525.

[2]. M. S. Yeganeh, S. M. Dougal, B. G. Silbernagel, *Langmuir,* 2006, **22**, 637−641.