SPECIAL TOPIC — Soft matter and biological physics

Protein–membrane interactions investigated with surface-induced fluorescence attenuation*

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Research on protein–membrane interactions has been undeveloped due to the lack of proper techniques to detect the position of proteins at membranes because membranes are usually only about 4-nm thick. We have recently developed a new method named surface-induced fluorescence attenuation (SIFA) to track both vertical and lateral kinetics of a single labelling dye in supported lipid bilayers. It takes advantage of strong interaction between a light-emitting dye and a partially reflecting surface. By applying the technique to membrane proteins being fluorescently labelled at different residues, here we show that SIFA can measure not only the insertion depth of a dye inside a lipid bilayer, but also the position of a dye in solution near the surface. SIFA can therefore be used to study membrane proteins of various types.

Keywords: single molecule surfaceinduced fluorescence attenuation, membrane–protein interactions, molecular mechanism

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1. Introduction

Biological membranes, which function as barriers between living cells and the environment, are fundamental elements of cells. Proteins are a primary membrane constituent, responsible for a variety of essential processes. Cooperation between the lipids and the membrane proteins are vital in many important biological processes. Knowledge about the orientation and depth of insertion of these proteins in biological membranes is crucial to understanding their functions.[1] Single-molecule techniques have become robust tools to study this fundamental process. Ensemble averaging inherent in bulk measurements can be avoided and unique properties of individual molecules can be revealed with single molecule methods. Unlike in bulk experiments, kinetic parameters can be recovered by simply observing the changes of a single molecule, even while the system as a whole is in dynamic equilibrium.[2] Single molecule fluorescence method has proven to be a highly versatile technique. By analyzing the fluorescent parameters including fluorescence intensity, life time, polarization, spectral diffusion and Förster resonance energy transfer (FRET), the underlying molecular mechanisms can be extracted.[3,4] The most commonly used single molecule technique is single molecule Förster resonance energy transfer (sm-FRET), by which the internal conformational dynamics of the membrane protein attached to the bilayer and the interaction between the proteins can be examined with a spatial resolution of better than 1 nm.[5] FRET is based on the dipole–dipole coupling of two fluorophores, i.e., a donor and an acceptor. The electric near-field of the emitting donor falls off as r^3, so the excitation efficiency of the acceptor follows an r^6 relationship.[6] A major limitation of sm-FRET is that the movement of the dyes along the direction perpendicular to the membrane surface is hard to be distinguished with that parallel to the surface. Tracking the accurate three dimensional movement of the biological molecule within the bilayer is hence difficult.

To overcome the shortage of sm-FRET, one may let the emitting donor transfers its energy to a layer of acceptors deposited on a surface. The idea is supported by the observation that graphene oxide (GO) quenches adsorbed fluorophores with a quenching efficiency of almost 100%.[7–9] It is also shown that a single-layered GO quenches the emission of dyes in a distance-dependent manner,[10] with a characteristic quenching distance estimated to be 4 nm$\sim$ 5 nm.[11,12] Based on these observations, we have recently developed a technique called surface-induced fluorescence attenuation (SIFA),[10] to track the movement of a target molecule along the direction perpendicular to the surface (Fig. 1). In this work, we show that the SIFA method can measure not only the insertion depth of a dye inside the lipid bilayer, but also the motion of a dye in solution near the surface of the bilayer. It is noteworthy that a method using environment-sensitive dyes had been used to detect whether the dyes are inside a lipid bilayer or in the solution near the bilayer,[13,14] but it does not yield more about the

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accurate position of the dyes. Also, fluorescence quenching by brominated lipids has nanometer sensitivity,[15] but suffers from the drawback that it does not distinguish the states in the two leaflets of bilayers. To the best of our knowledge, SIFA is currently the only method that can effectively detect the position of a dye above a bilayer because it is independent of the environment in which the dyes stay.

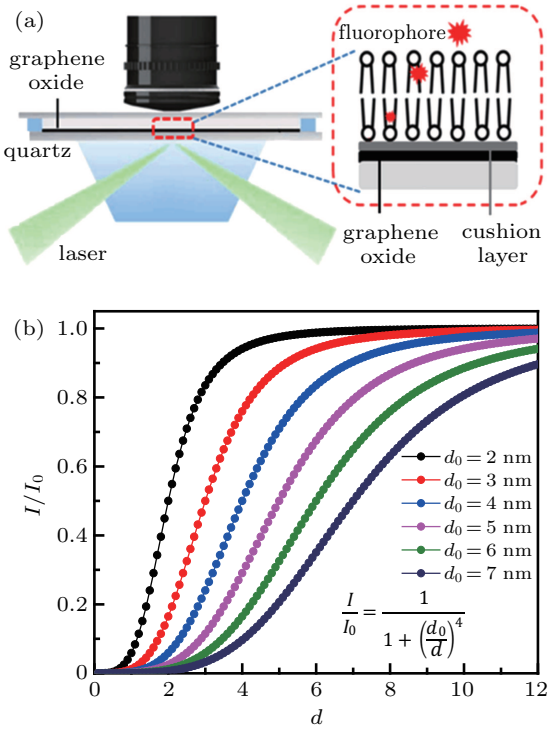


Fig. 1. (color online) (a) The experimental setup of SIFA. (b) The fluorescence attenuation by a GO layer follows approximately a d^{-4} relationship and is strongly dependent on the characteristic distance d_0.

2. Experiments

2.1. Single molecule surface-induced fluorescence attenuation experiments

The single layered graphene oxide was prepared by the modified Hummers method,[16] and deposited on the plasma-treated quartz by using the Langmuir–Blodgett method. Lipid bilayer or GO-supported lipid bilayer was made following a reported procedure.[13] Single-molecule SIFA measurements were carried out with a home-built prism-based TIRF microscopy (Fig. 1). A 532-nm solid state laser (Changchun New Industries Optoelectronics Tech. Co., Ltd.) through optical fiber coupler (PAF-X-5-A, Thorlabs) was used for excitation. Actual excitation intensity was set to about 3 mW–4 mW. The main optical setup was based on an Olympus IX71 microscope. A prism was placed on the top of a quartz slide of the sample chamber with a thin layer of immersion oil (Olympus, $n = 1.5$) in between. The laser beam was aligned through the objective (Olympus, NA = 1.45, $\times$100). The fluorescent emission of the fluorophores passes the dichromatic mirror (Semrock 570/20) and the emission filter (Olympus) which was set in the Olympus fluorescence filter cubes. The camera used was an Andor Technology iXon 897 EMCCD.

2.2. X-ray diffraction measurements

It is necessary to measure the thickness of lipid bilayers in order to calibrate the characteristic quenching distance of GO (c.f. Eq. (1) below). A lipid bilayer was prepared on a hydrophilic quartz substrate by using the Langmuir–Blodgett method. The x-ray reflectivity (XRR) was measured on a Bruker D8-Advance diffractometer equipped with a Goebel mirror to get parallel x-ray beams.[17] The incident beam was confined by a 0.1-mm slit 300 mm in front of the sample, and the scattering beam was confined by a 0.2-mm slit in front of the detector. The diffraction measurement was performed in a θ–2θ mode by keeping the incidence angle equal to the exiting angle.

2.3. Materials

Antimicrobial peptide LL-37 was labelled with rhodamine at the N-terminus (hereafter called N-Rh-LL-37 in this work) or C-terminus (hereafter called C-Rh-LL-37). All the labelled and unlabelled LL-37 was synthesized by China Peptides Co. Ltd. (Shanghai China). The Bid protein was labelled with tetramethylrhodamine-5-maleimide at residues 181 and 80, respectively. Bid was cloned and purified by our collaborator in the Institute of Biophysics, Chinese Academy of Sciences. All the lipids were purchased from Avanti, other organic and inorganic reagents were analytical-regent grade (from Beijing Chemical Reagents Company) and used as received without any purification. Distilled water (> 18.2 MΩ·cm) was obtained.

3. Results and discussion

3.1. Calibration of the characteristic quenching distance d_0

A fluorophore near a GO layer undergoes energy transfer to the surface with a rate that depends on the fluorophore-to-GO distance.[18–20] The quenching equation reads as

$$\frac{I}{I_0} = \frac{1}{1+\left(\frac{d_0}{d}\right)^4} \quad (1)$$

where I_0 is the fluorescence intensity of a free fluorophore, I is the intensity near the GO surface, d is the fluorophore-to-GO distance, and d_0 is the characteristic quenching distance at which 50% of the intensity is quenched. We first prepared a lipid bilayer on the surface of a glass and measured its thickness with XRR. Simulation to the XRR curve in Fig. 2(a) yielded a thickness of $d = 3.98$ ($\pm$0.05) nm for the lipid bilayer. We then measured the brightness of the C-Rh-LL-37 on the GO-supported lipid bilayer surface by using the setup

in Fig. 1. The fluorescent intensity of the C-Rh-LL-37 on the GO-supported lipid bilayer surface is 54(±6)% of that on the quartz-supported bilayer (shown in Fig. 2(b)). The characteristic quenching distance for the GO used in this work is therefore 4.3(±0.5) nm according to Eq. (1).

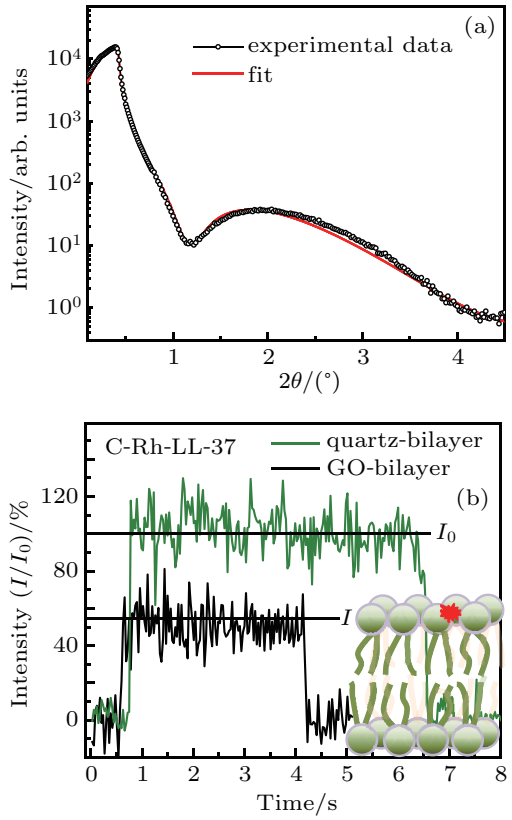


Fig. 2. (color online) (a) X-ray reflectivity profile of the single lipid bilayer on solid surface. (b) Comparison of the fluorescence intensity of the C-Rh-LL-37 on the GO-supported bilayer (black) with that on the quartz-supported bilayer (green).

In many cases, it is not necessary to know the absolute distance from the fluorophore to the GO surface. Information on the relative change in position of the fluorophore in the bilayer is enough to study the kinetics of membrane proteins. From a practical perspective, one can use a parameter d^*, which is the distance between the top of the supported bilayer under study and the GO layer, to replace d_0 in Eq. (1). Starting from Eq. (1), one can obtain the following equation for the relative position,

$$\frac{\mathrm{d}}{\mathrm{d}^*} = \left(\frac{I}{I_0 - I}\right)^{1/4} \left(\frac{I_0 - I^*}{I^*}\right)^{1/4}. \tag{2}$$

The parameters of I_0, I, and I^* in Eq. (2) can be obtained from single-molecule fluorescence measurements in Fig. 1.

3.2. The function of cushion layer

Figure 3 shows calculated quenching efficiency of a dye as a function of distance between the dye and the GO layer. The red segment on the curve represents the most sensitive range of SIFA. When a lipid bilayer is deposited directly on the graphene oxide surface, fluorophores in the lower leaflet of the bilayer which is less than 2 nm above the GO layer would be strongly attenuated so that the signals would be imbedded in the background noises (see the area with black oblique line in Fig. 3). This 'blind' region of SIFA limits our capability to study the kinetics of biomolecules in lipid bilayer. One can deposit a PEG cushion layer (∼ 1 nm thick) on the GO layer to lift the lipid bilayer out of the 'blind' region. After applying a 1-nm thick PEG cushion layer, the most sensitive range now covers the region from the surface to the middle of the lower leaflet (see the area with green oblique line in Fig. 3). If one is more interested in the protein dynamics in the lower leaflet of the lipid bilayer, one can deposit a layer of BSA protein which is about 3.3-nm thick.[21,22] By changing the cushion layer's thickness, SIFA can therefore be extended to different membrane system.

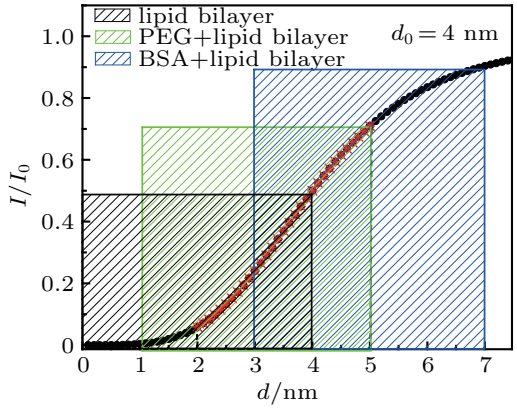


Fig. 3. (color online) Observed region in our experiments with different cushion layer. Black oblique line area stands for lipid bilayer system with no cushion; green one for PEG-supported lipid bilayer with PEG as the cushion layer; blue one for BSA-supported lipid bilayer with a BSA film as the cushion layer.

3.3. Insertion of a peptide into a lipid bilayer

We first studied the interaction of antimicrobial peptide LL-37 with a lipid bilayer. LL-37 is a 37-residues peptide of sequence LLGDF FRKSK EKIGK EFKRI VQRIK DFLRN LVPRT ES. It is involved in diverse biological processes such as immunomodulation, apoptosis, angiogenesis and wound healing.[23] We deposited a lipid bilayer directly on top of a GO layer which was in turn on top of a glass surface. When we flushed-in the LL-37 labelled at N-terminus with a dye, the LL-37 molecules landed on the surface of the GO-supported lipid bilayer. The intensities of most of the molecules have a mean intensity of 52(±6)% of the intrinsic intensity of the dyes. However, one sees from Fig. 4 that some LL-37 molecules (about 1%) insert into the lipid bilayer shortly after they landed. The intensity of such molecules is initially 52(±6)% of the intrinsic one but reduces to 18(±6)% before they are photo-bleached (Fig. 4). The calculated positions corresponding to the two intensities are 4.1(±0.4) nm and 2.7(±0.4)nm above the GO layer, respectively.

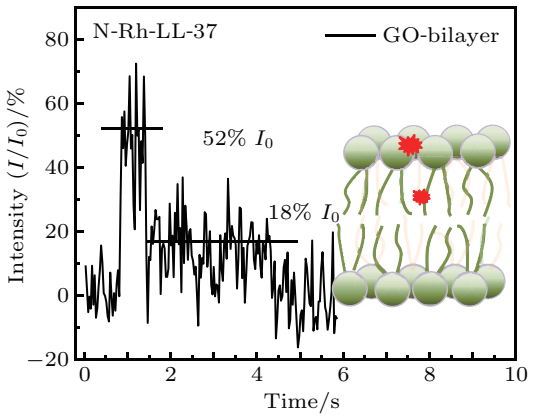


Fig. 4. (color online) Trace of the N-Rh-LL-37 on the GO-supported bilayer indicating the insertion of an LL-37 molecule into the lipid bilayer.

3.4. Configuration of tBid on a membrane surface

SIFA was originally designed to study the insertion depth of membrane proteins in the lipid bilayer.[10] Before the invention of SIFA, researchers have already been able to get information about whether a dye is in solution or inside the membrane by monitoring the fluorescent intensity of environment-sensitive dyes although the method does not yield high resolution data. Here we show that SIFA can do more than it was initially expected, namely, it can measure with high precision the positions of a dye near the surface of a lipid bilayer. We illustrate this feasibility by studying the configuration of a tBid protein on a lipid bilayer. Bid is a BH3-interacting domain death agonist protein, playing an essential role in the intrinsic and extrinsic apoptosis pathways in some cells.[24] During apoptosis, Bid is cleaved by caspase-8 in response to death stimuli.[25] The two fragments of the cleaved Bid (cBid) remain together through hydrophobic interactions until cBid encounter with the membranes. We took movies before and after adding labeled tBid with lower concentration to GO-PEG-bilayer and bilayer system respectively. When the fluorescence labeled cBid molecules encounter the membrane, the fate of them can be recorded till the fluorophores photobleach. Hydrophobic helix 7 of tBid plays a central role in the hydrophobic interaction between the tBid protein and the membranes.[24] To observe the behavior of helix 7 when individual tBid bound to supported lipid bilayers, residue 181 was fluorescence labeled. When interacting with membranes, BH3 region of a protein molecule can interact with other proteins' BH3 region. To study the process of tBid homo-oligomerization on the membrane, residue 80 was labeled. The labelled sites were shown in Fig. 5(a).

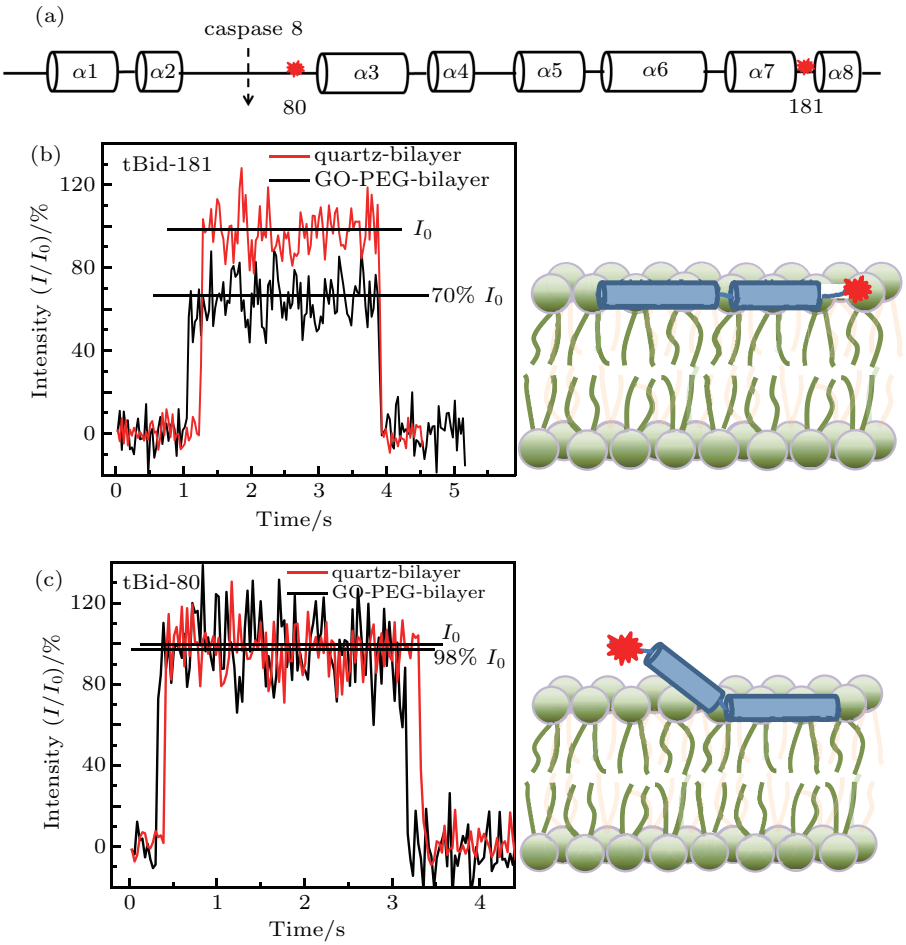


Fig. 5. (color online) (a) Schematic diagram of Bid and the fluorophore labeled sites. (b) The fluorescence intensity of the 181-labelled tBid on the GO-PEG-supported bilayer and the quartz-supported bilayer. (c) The fluorescence intensity of the 80-labelled tBid on the GO-PEG-supported bilayer and the quartz-supported bilayer.

Figure 5(a) compares the fluorescence intensity I_0 of a tBid labelled at residue 181 on a quartz-supported lipid bilayer with the fluorescence intensity I of a tBid labelled at the same position but on a GO-supported bilayer. A PEG cushion layer is used to lift the bilayer by ~ 1 nm. The intensity of the latter is 70(±4)% of the former, indicating that the residue 181 is on the membrane surface, consistent with previous report.[26] In contrast, as indicated in Fig. 5(b), the ratio of I to I_0 for residue 80 was 98(±4)%. The residue 80 is therefore located in the solution. Previous studies show that it is the tBid BH3 domain that directly binds and activates BAX, which is involved in mitochondrial targeting, homo-oligomerization and pore formation.[27,28] Considering this, we suggest that this conformation with the BH3 domain fully exposed may be the key point of tBid binding with BAX, which could further induce mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome c.[25]

3.5. Dynamics of antimicrobial peptides in pores inside lipid bilayers

It is known that LL-37 induces pores in lipid bilayers when the surface density of LL-37 is high enough.[29–32] We mixed labelled LL-37 with unlabelled LL-37 and flushed the mixture into the chamber. The final concentration of LL-37 is about 1 μM, while the labelled LL-37 is about 4 nM. A GO-BSA-lipid system was used in the experiment. Although we could not see pores with the SIFA technique, we inclined to believe that pores could form in our systems because the experimental conditions are very similar to those under which many antimicrobial peptides induce toroidal pores in bilayers.[30–32] About 15% of the N-Rh-LL-37 molecules were less mobile and flickered repeatedly and four fluorescence levels were recorded. As it is shown in Fig. 6, four transmembrane positions were (i) 7.5±0.9 nm (top surface of the lipid bilayer), (ii) 5.2±0.4 nm (center of the lipid bilayer), (iii) 4.2±0.4 nm (lower leaflet of the lipid bilayer), and (iv) 3.4±0.4 nm (bottom surface of the lipid bilayer) above the GO layer, respectively. The phenomenon was not observed for N-Rh-LL-37 on quartz-supported bilayers. The flickering of the fluorescence means that the N-Rh-LL-37 molecule moves up and down in the bilayer repetitively.

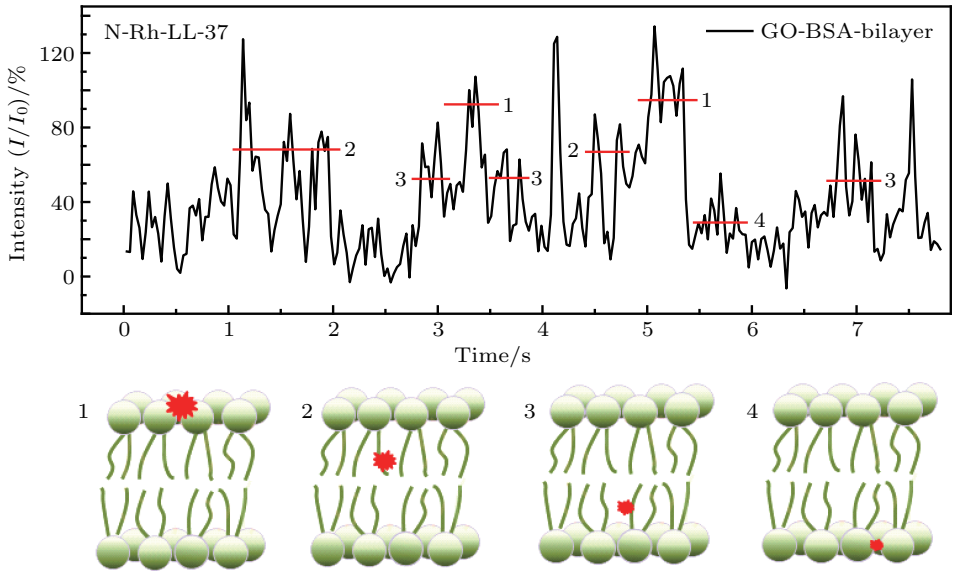


Fig. 6. (color online) Trace of N-Rh-LL-37 on the GO-BSA-supported bilayer (upper panel). The LL-37 monomer transfers among 4 transmembrane positions as illustrated in the lower panel.

4. Conclusion

The single-molecule SIFA is a powerful method to study protein–membrane interactions with high resolution. Using large single-layered GO as the two-dimensional quenchers, the distance between a fluorophore and GO can be derived from the intensity of the fluorescent molecules. With SIFA, the motion of a fluorescent molecule along the normal of the membrane can be readily distinguished from its motion in the direction parallel to the membrane. Moreover, by choosing dyes that are not environment-sensitive, SIFA can be used to study the kinetics of a protein in the solution layer near a supported lipid bilayer.

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