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Model Lipid Bilayer with Facile Diffusion of Lipids and Integral Membrane Proteins

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Abstract

A model membrane system is formed by the rupture of giant unilamellar vesicles (GUVs) onto a passivating layer comprising a PEG polymer cushion anchored in a lipid bilayer supported on glass. The novel use of pH-dependent electrostatic interactions between NeutrAvidin in the passivating layer and anionic lipids in the GUV drives vesicle rupture. The resulting “GUV pancakes” are single, planar lipid bilayer patches whose diameters vary from ~20 to 50 μm . The pancakes have several potential advantages for the in vitro study of protein–lipid interactions and integral membrane protein function. All components are commercially available. The pancakes resist nonspecific binding of vesicles containing protein. Both lipids and integral membrane proteins exhibit good lateral mobility in the GUV pancakes, as evidenced by single-particle tracking (SPT) of the DiD double-tailed fluorescent probe and of the integral membrane protein syntaxin-1A, labeled with AlexaFluor 633 (AF633-Syx). At least 80% of both probes exhibit free, homogeneous diffusion with a diffusion coefficient of $\sim 5.5 \mu\text{m}^2 \text{s}^{-1}$, which is more than 10 times faster than diffusion in a GUV pancake supported on bare glass. Atomic force microscopy (AFM) suggests that the polymer cushion has a height of ~4 nm. The mobility of a large fraction of the AF633-Syx probe suggests that even integral membrane proteins with large domains on both sides of the lipid bilayer should exhibit free diffusion within a GUV pancake.

I. Introduction

Biological membranes play an important role in all living systems by defining the boundary of cells and organelles. Integral membrane proteins enable communication and transportation across membranes. However, because of the complexity of real biological membranes, many different model systems have been developed in an effort to retain the physical properties of a lipid bilayer in a simplified system enabling the role of each component to be studied. The traditional model systems can be divided into three categories: vesicles,¹ lipid bilayers supported on a transparent solid substrate such as glass,² and “free-standing” lipid bilayers adhered to a small hole in a thin sheet of Teflon.³ Vesicles range in

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Supporting Information Available: Non-specific binding of fluorescently labeled v-SNARE vesicles on different surfaces. Fluorescent image of a large, heart-shaped GUV pancake with a smaller, adjacent, deflated GUV. Line scan of fluorescent image intensity and an AFM image. AFM images of a lipopolymer/lipid bilayer and a ruptured GUV. Theoretical estimate of the height of the PEG cushion. This material is available free of charge via the Internet at <http://pubs.acs.org>.

size from small unilamellar vesicles (SUVs, several tens of nanometers in diameter)⁴ to giant unilamellar vesicles (GUVs, tens of micrometers in diameter).⁵

Supported lipid bilayers on glass have been prepared by direct vesicle fusion,⁶ Langmuir–Blodgett (LB) transfer,⁷ or a combination of both.⁸ They enable spectroscopic studies of oriented samples using NMR,⁹ Fourier transform infrared spectroscopy,¹⁰ surface plasmon resonance,¹¹ and X-ray and neutron scattering.¹² They are ideal for total internal reflection fluorescence microscopy (TIRF),¹³ including single-particle tracking studies of binding and diffusion. There have been many successful studies of biomembrane structure,¹⁴ membrane dynamics,¹⁵ and protein–protein and protein–lipid interactions.^{6,13} However, the primary limitation has been the small, 1 to 2 nm distance between the lipid bilayer and the solid support.^{16,17} This is too small to accommodate large integral membrane proteins, leading to a loss of mobility and functionality.^{18,19} In addition, the thin space poorly mimics the extracellular or cytoplasmic space, which may affect the study of mass transfer across the bilayer, as in vesicle fusion.²⁰

A variety of methods have been devised to increase the space between the lipid bilayer and the supporting solid surface to increase the lateral mobility of lipids and membrane proteins. One strategy is to deposit a thin film of cellulose on glass surface by a Langmuir–Blodgett (LB) technique to serve as a polymer cushion.²¹ Supported planar bilayers were formed by the direct fusion of integrin-loaded vesicles onto slides coated with cellulose films. FRAP experiments reveal that 77% of lipids diffuse at $3.3 \pm 0.2 \mu\text{m}^2 \text{s}^{-1}$ in this model membrane, which is about 3 times faster than in a lipid bilayer on bare glass. Some 25% of integrins diffuse at $0.6 \pm 0.2 \mu\text{m}^2 \text{s}^{-1}$, but they are all immobile in a lipid bilayer on bare glass. Another method covalently tethers a lower lipopolymer/lipid leaflet onto the glass surface by the LB technique and then deposits the top lipid leaflet by either the LB technique or vesicle fusion.^{8,22,23} For these systems, the measured lipid diffusion coefficients vary widely. Both the Tanaka²³ and Tamm⁸ groups used silane-functionalized poly(ethylene oxide) tethers and obtained lipid diffusion coefficients of $\sim 1 \mu\text{m}^2 \text{s}^{-1}$, similar to that without polymer tethering. The Naumann group used grafted benzophenone derivatives and obtained lipid diffusion coefficients as high as $18 \mu\text{m}^2 \text{s}^{-1}$.²²

In recent work similar to that presented here, Groves and coworkers^{24,25} developed a double lipid bilayer system by rupturing GUVs onto a supported lipid bilayer through electrostatic interactions. This double bilayer system has been used to study the structure and dynamics of supported intermembrane junctions. Fluorescence resonance energy transfer (FRET) between the ruptured GUV patch and the glass-supported bilayer indicates an ~ 2 to 3 nm water layer in between. Lipid diffusion in the GUV patches was not measured directly. Boxer and co-workers²⁶ ruptured GUVs using binding between complementary single-stranded DNA molecules attached to the GUV lipids and to the underlying lipid bilayer.²⁶ The bilayer-bilayer separations were 10 ± 1 and 16 ± 1 nm using 24-mer and 48-mer DNA, respectively. The measured lipid diffusion coefficient in these GUV patches was $4.8 \mu\text{m}^2 \text{s}^{-1}$. This type of system combines the large surface area of GUVs with the advantages in surface techniques of a supported lipid bilayer.

Here we report a method of GUV rupture that creates an ~4 nm space beneath the “GUV pancake” bilayer patch but does not require the synthesis of DNA-lipid conjugates. All materials are commercially available. Rupture is driven by pH-adjustable electrostatic interactions between NeutrAvidin molecules bound to the first passivating biotin-PEG-DSPE/POPC bilayer and anionic 1,2-dioleoyl phosphatidylserine (DOPS) lipids within the GUV. The diffusion coefficient of both the DiD lipid probe and the syntaxin single-helix integral membrane protein (Syx, labeled with Alexafluor 633) is $\sim 5 \mu\text{m}^2 \text{s}^{-1}$, as measured by single-particle tracking. At least 80% of both the DiD and AF633-Syx molecules are mobile. AFM measurements show that the distance between the upper surface of the GUV pancake and the upper surface of the underlying lipid bilayer is $\sim 7.9 \pm 0.6$ nm, indicating that the thickness of the PEG cushion beneath the GUV pancake is ~4 nm. The cytoplasmic domain of the AF633-Syx protein is presumably oriented both above and below the GUV pancake, indicating that the polymer cushion causes little friction. This suggests that the GUV pancakes would enable good mobility of a membrane protein with large protrusions on both sides of the bilayer.

II. Materials and Methods

The following materials were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification: 1-palmitoyl,2-oleoyl phosphatidylcholine (POPC), 1,2-dioleoyl phosphatidylserine (DOPS), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[biotinyl(poly(ethylene glycol))-2000] (biotin-PEG-DSPE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy-(poly(ethylene glycol))-2000] (PEG-DSPE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol))-2000-*N'*-carboxyfluorescein] (CF-PEG-DSPE), 1,2-di-(9*Z*-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine-*N*-(biotinyl) (biotin-PE). *N*-(Tetramethylrhodamine-6-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (TRITC-DHPE), 1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD), and AlexaFluor 633 maleimide were purchased from Invitrogen (Carlsbad, CA). β -Octyl-glucopyranoside (β -OG) and Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Sigma Chemical (St. Louis, MO). Other components include Accudenz (Accurate Chemical, Westbury, NY), CONTRAD 70 (Decon Laboratories, King of Prussia, PA), Nanostrip (Cyantek Corporation, Fremont, CA), ITO glass slides (Delta Technologies, Stillwater, MN), disposable PD-10 desalting columns (GE healthcare, Piscataway, NJ), and Zebra spin desalting columns (Thermo Scientific, Rockford, IL).

Protein Purification and Fluorescent Labeling

Synaptobrevin-2 (Syb, the v-SNARE) and full-length rat syntaxin 1A (Syx) were expressed, purified, and reconstituted into small unilamellar vesicles (SUVs) as described previously.⁶ Purified Syx was labeled with AlexaFluor 633 maleimide by shaking together in a ratio of 1:10 (protein/dye) for 1 h at room temperature in labeling buffer (25 mM HEPES, 400 mM KCl, 1 mM β -OG, and 0.1 mM TCEP). Free AlexaFluor 633 was removed from labeled Syx (AF633-Syx) by passage through Zebra spin desalting columns twice. AF633-Syx eluted out of the column, but free AlexaFluor 633 was retained. Labeled Syx was quantified by SDS-PAGE. The amount of AlexaFluor 633 was quantified by absorbance at 622 nm using the

value $\epsilon = 1.43 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ provided by Invitrogen. The labeling efficiency was calculated to be 110%. There are three cysteines in Syx, two in the transmembrane region, and one (145C) in the cytoplasmic domain. Presumably, 145C is the most frequently labeled.

Small Unilamellar Vesicle (SUV) Preparation

The AF633-Syx vesicles, v-SNARE vesicles, and lipopolymer vesicles were reconstituted by rapid dilution and dialysis and subsequently purified by flotation in an Accudenz step gradient as described previously.⁶ For AF633-Syx vesicles, the lipid to AF633-Syx ratio was $\sim 8 \times 10^6:1$, assuming the same reconstitution efficiency for lipids and proteins. The lipid composition of AF633-Syx vesicles was 85% POPC and 15% DOPS. For v-SNARE vesicles, the lipid to v-SNARE ratio was $\sim 300:1$ and the lipid composition was 84% POPC, 15% DOPS, and 1% TRITC-DHPE. A second type of v-SNARE vesicle containing 1% DiD and 0.2% biotin-PEG-DSPE was used to test the mobility of the passivating lipid bilayer. The lipid composition of lipopolymer vesicles was 95% POPC and 5% biotin-PEG-DSPE except as otherwise specified. The final buffer was 25 mM HEPES-KOH (pH 7.4), 100 mM KCl, 10% glycerol, and $\sim 10\%$ Accudenz for all SUV types. The final total lipid concentration was approximately 2.5 mM, assuming 67% recovery for all lipids.⁶

Formation of GUVs

Protein-free GUVs were made by the electroformation method in sucrose solution as described earlier²⁷ but with some modifications. First 85% POPC, 15% DOPS, and the desired amount of DiD were mixed at 10 mg/mL in chloroform. For GUVs with 5% biotin-PE, POPC was reduced to 80%. A 20 μL volume of the lipid mixture was spin-coated onto the ITO-coated side of a glass slide. The slide was left in vacuum for more than 1 h to remove chloroform. The completely dried slide and another ITO-coated glass slide were used to assemble an electroformation chamber with a Teflon spacer sandwiched in between.²⁷ The gap between glass slides is 3 mm. The Teflon spacer has two holes on the side for adding or removing solution. The entire chamber was held together with binder clips. Two flat aluminum bars were tightly attached to the coated sides of two ITO coating glass slides by binder clips to serve as electrodes. The chamber was filled with a 100 mg/mL sucrose solution. A function generator (B&K Precision Corporation, Yorba Linda, CA) was used to apply a 2 V, 10 Hz sinusoidal potential across the chamber for 2 h.

GUVs with AF633-Syx were prepared in a similar way to that described by Schwille and co-workers.²⁸ First, 400 μL of an AF633-Syx SUV solution (~ 2.5 mM lipid) was thawed. Accudenz and glycerol in the SUV solution were removed by buffer exchange using PD10 columns, leaving the purified vesicle solution in a membrane-making buffer (25 mM HEPES-KOH, 100 mM KCl, pH 7.4). The solution was pelleted by ultracentrifugation (430 000g, 90 min) with a Beckman Coulter Optima ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA). The supernatant was removed, and the pellet was resuspended in 60 μL of Milli-Q water. This resuspended SUV solution was deposited as 1 μL droplets onto a clean ITO glass slide. Before deposition, the ITO glass slide was cleaned with 2-propanol, rinsed extensively with Milli-Q water, and dried with Kimwipes. After the deposition of the SUV suspension, the ITO slide was lyophilized overnight to remove residual water. The dried ITO glass slide including the lipid and the AF633-Syx film was assembled with another ITO

glass slide to form an electroformation chamber as described above. Finally, the chamber was filled with a 100 mg/mL sucrose, and the 2 V, 10 Hz potential was applied for at least 6 h.

Most protein-free GUVs and AF633-Syx GUVs formed are evidently unilamellar, as indicated by the uniform fluorescence intensity observed by wide-field fluorescence microscopy. The GUV diameters range from several micrometers to $>100\ \mu\text{m}$. The yield of protein-free GUVs is higher than that of AF633-Syx GUVs. SUVs smaller than the diffraction limit can be observed in AF633-Syx GUV solution and occasionally in protein-free GUV solution. Trapping of these SUVs beneath the GUV pancakes may be the primary source of immobile, fluorescently labeled species.

Formation of the Passivating Layer on Glass

For the GUV tethering experiments, a glass surface was passivated with a lipid bilayer that anchors the lipopolymer cushioning layer. A schematic of the passivating layer and its fluorescence image is shown in Figure 1. This passivating layer was formed by vesicle fusion on a clean, hydrophilic glass coverslip as described earlier.⁶ Coverslips were cleaned by sonication in CONTRAD detergent for 1 h and thoroughly rinsed in Milli-Q water. They were soaked for 1 h at 90 °C in Nanostrip and rinsed again immediately before use. The cleaned slides were assembled into a homebuilt Teflon cell serving as an observation window. Lipopolymer/lipid SUVs were diluted 100-fold with membrane-making buffer to a final lipid concentration of $\sim 25\ \mu\text{M}$. Then 500 μL (~ 80 cell volumes) of the diluted lipopolymer/lipid SUV solution was added to the Teflon cell. After incubation at 4 °C for 2.5 h, the passivating layer was warmed up to 37 °C for 2 h and gently washed with buffer (40 cell volumes total). Such temperature cycling improves passivation of the glass by the bilayer. All subsequent experiments were performed on a 37 °C hot plate.

Rupture of GUVs on the Passivating Layer

For observations of the rupturing of GUVs, they were labeled with 0.1% DiD. We observed no difference in rupturing efficiency between protein-free GUVs and AF633-Syx GUVs. The buffers used during this step were warmed to 37 °C. A passivating layer was prepared at 37 °C and then incubated with 100 μL of 0.1 mg/mL NeutrAvidin solution for 1 min to bind NeutrAvidin to biotins at the end of the PEG molecules. Extra NeutrAvidin was gently rinsed away with 2 mL of the membrane-making buffer. A 2 mg/mL solution of NeutrAvidin was prepared in membrane-making buffer, stored at room temperature, and used within 2 weeks. In our experience, NeutrAvidin is deactivated after vortex mixing or freeze-thaw cycling. The stock NeutrAvidin solution was diluted to 0.1 mg/mL with membrane-making buffer and gently shaken by hand just before use.

Next the GUVs were tethered to the passivating layer through biotin–NeutrAvidin–biotin linkages. Five microliters of GUV solution was deposited into the prepared Teflon cell. The GUVs settle on the passivating layer because of the higher density of sucrose solution inside (Figure 1). Biotin molecules in the GUV bilayer bind to NeutrAvidin molecules bound to the PEG molecules. However, no spontaneous rupture of GUVs is observed at this stage.

Unbound vesicles were removed by gentle rinsing with membrane-making buffer. Tethering is confirmed by the persistence of bound vesicles after rinsing.

Finally, to rupture the tethered GUVs containing biotin-PE, the pH 7.4 membrane-making buffer was replaced with 200 μL of pH 4.2 membrane-making buffer. Because NeutrAvidin has an isoelectric point of 6.3, most NeutrAvidin molecules acquire one positive charge at pH 4.2. The electrostatic interaction between positively charged NeutrAvidin molecules and negatively charged DOPS within the GUV causes the GUV to rupture and flatten onto the passivating layer to form a second lipid bilayer (Figure 1), the GUV pancake. The largest GUVs ($>20\text{ }\mu\text{m}$ diameter) make pancakes immediately after buffer exchange. Smaller ones may rupture or simply bind tightly to the passivating layer, as explained in the Results section. The pH 4.2 membrane-making buffer is then replaced with a pH 7.4 membrane-making buffer after the GUVs have ruptured. The resulting GUV pancakes are stable on the surface during buffer rinsing. At least 10% DOPS in the GUVs was necessary for efficient rupture, confirming the idea that the dominant driving force toward rupture is electrostatic. GUVs with 5% biotin-PE but lacking anionic lipid heads did not rupture. However, tethering is not essential for rupture but enables untethered GUVs and SUVs to be washed prior to GUV rupture, which results in “cleaner” pancakes.

To obtain the rupture of untethered GUVs (lacking biotin-PE), it was necessary to introduce pH 4.2 buffer into the cell before adding the GUVs. At pH 4.2, the larger GUVs (10 to 100 μm diameter) would rupture immediately upon touching the passivating layer covered with positively charged NeutrAvidin.

Temperature variations in the cell can cause the decomposition of the passivating layer and thus the loss of the GUV pancakes. When the temperature was kept stable at 37 $^{\circ}\text{C}$ within 2 $^{\circ}\text{C}$, the GUV pancakes remained stable for at least 30 min and perhaps much longer.

GUVs can also be ruptured on the passivating layer at pH 7.4 without the pH shifting if Avidin is used in place of NeutrAvidin. We believe that this is again due to electrostatic interactions because Avidin ($\text{pI} = 10$) has one positive charge at neutral pH. However, the charged Avidin caused the severe nonspecific binding of lipid vesicles and proteins at pH 7.4, so it is not well suited for a model membrane system. Streptavidin was also able to rupture GUVs at pH 7.4, but not nearly as efficiently as NeutrAvidin at pH 4.2 or Avidin at pH 7.4. The streptavidin pI is between 6.8 and 7.5, consistent with our electrostatic arguments. However, streptavidin induced much more severe nonspecific binding of v-SNARE vesicles than did the preferred NeutrAvidin protocol.

Fluorescence Microscopy

Single-particle tracking experiments were carried out with a modified commercial widefield microscope (Eclipse TE2000-U, Nikon, Melville, NY) using total internal reflection (TIR) illumination. Through-the-objective TIR used a 100 $\times$, 1.45 numerical aperture oil-immersion objective (Olympus, Melville, NY). Each pixel corresponds to $160 \times 160\text{ nm}^2$ at the sample. A 633 nm laser (HeNe, Coherent) was used to excite either AlexaFluor 633 or DiD labels. The laser intensity was 0.04 kW/cm^2 to locate and characterize the GUVs and

0.32 kW/cm² for single-particle tracking. A 514 nm laser (Ar⁺, Melles Griot) was used to excite TRITC-DHPE in v-SNARE vesicles at a laser intensity of 0.002 kW/cm².

Fluorescence from DiD or AlexaFluor 633 passes a 650-700 nm band-pass filter (D675/50M, Chroma, Rockingham, VT). A 535-585 nm band-pass filter was used for TRITC-DHPE (D560/50M, Chroma, Rockingham, VT). An EMCCD camera (DV887ECS-UVB, Andor Technologies) was used to record all of the signals. The camera pixels are square, $16 \times 16 \mu\text{m}^2$. The frame duration was varied to match the events being recorded.

Single-Particle Trajectory Analysis

The lateral mobility of DiD and AF633-Syx within GUV pancakes was characterized by an analysis of the distribution of short-time experimental diffusion coefficients D_e obtained from segments of 2D single-particle trajectories. Trajectories of individual AF633-Syx and DiD were measured using Grier and Crocker's spatial filtering and particle tracking software,²⁹ adapted for MATLAB (Blair and Dufresne, <http://physics.georgetown.edu/matlab/>) with minor in-house modifications. The spatial filter in effect flattens and zeroes the background between particles and filters high-frequency noise within the image of particles while preserving information at the diffraction limit. This is especially useful for tracking rapidly diffusing particles, which produce images with irregular shapes even at 20 ms/frame. Particles in each frame are identified with an intensity threshold and are located at subpixel resolution with a centroid algorithm. The tracking algorithm finds many short trajectories and fewer long trajectories. Trajectories may terminate because of photobleaching, blinking, or a frame in which rapid movement blurs the intensity below the chosen threshold.

The statistics of single-particle diffusion are noisy, especially for the short trajectories observed here. Our treatment borrows heavily from that of Moerner and co-workers³⁰ and Saxton.³¹ First a minimum trajectory length of l_{traj} steps is chosen to include a majority of the observed particles, typically either 13 or 7 steps (Figure 2 and Table 1). Trajectories shorter than l_{traj} are discarded, and longer trajectories are truncated after the first l_{traj} steps (l_{traj} 1 image, $l_{\text{traj}}\delta t$ total duration, with δt equal to the frame duration). For a given lag time t_{lag} , each truncated trajectory was broken into N_l statistically independent (nonoverlapping) segments of duration t_{lag} , with extra frames at the end of the trajectory discarded. The squared displacement of each particle is averaged over the N_l segments to obtain $\langle r^2(t_{\text{lag}}) \rangle$, which is converted into the best estimate of the diffusion coefficient for each particle at the chosen values of l_{traj} and t_{lag} :

$$D_e = \frac{\langle r^2(t_{\text{lag}}) \rangle}{4t_{\text{lag}}} \quad (1)$$

The distribution of D_e is used to assess whether diffusion is free and homogeneous. For free, homogeneous diffusion, the theoretical distribution was first given by Qian³² as

$$p(D_e)dD_e = \frac{1}{(N_I - 1)!} \left(\frac{N_I}{D_0} \right)^{N_I} D_e^{N_I - 1} \exp \left(-\frac{N_I D_e}{D_0} \right) dD_e \quad (2)$$

Here D_0 is the free, homogeneous diffusion coefficient. Equation 2 has been verified by simulations³¹ and applied to experimental results.^{33,34}

AFM on the Passivating Layer with GUV Pancakes on Top

To accommodate the AFM tip, a special open cell was made by gluing an O-ring onto a clean glass coverslip with nail polish. The procedure for making the supported passivating layer and rupturing GUVs is the same as described above except that the measurements were made at room temperature to prevent temperature fluctuations. When the sample was ready, the O-ring was removed and the glass coverslip with the sample on top was transferred without drying to a commercially available liquid cell (Veeco Instruments Inc., Santa Barbara, CA). Images of the passivating layer were acquired with a Nanoscope IIIa Multimode scanning probe microscope (Veeco Instruments Inc., Santa Barbara, CA). Silicon nitride cantilevers with a nominal spring constant of 0.32 N/m were used. Images were taken at 256 points per line and a scan rate of 1 Hz. Images of GUV pancakes atop the passivating layer were acquired with a BioScope Catalyst scanning probe microscope (Veeco Instruments Inc., Santa Barbara, CA), which combines the functions of a fluorescence microscope and an AFM. A GUV pancake was first identified by the fluorescence microscope. Then an AFM image was taken on the same GUV pancake. The AFM parameters are the same as those for the Nanoscope IIIa.

The AFM tip severely damages the GUV pancake surface as it scans (Figure S4). In addition, the GUV pancakes as observed with the fluorescence microscope in a Teflon cell are cleaner than those from AFM images because they are more thoroughly washed free of small vesicles and fragments. The open-top cell used to take AFM images does not allow complete rinsing as in a closed flow cell.

III. Results

Formation and Characterization of the Passivating Layer

Small unilamellar vesicles (SUVs) containing 5% biotin-PEG-DSPE were deposited onto glass substrates as described in Materials and Methods. The integrity of the passivating layer was tested by incubation in TRITC-DHPE-labeled v-SNARE vesicle solution (1 nM vesicles) for 5 min, followed by rinsing with 2 mL of 37 °C membrane-making buffer. If the number of bound vesicles is 20 or fewer for the entire image (6700 μm^2), then we judge the passivating layer to have good integrity (Figure S1) and proceed to the next step. Most passivating layers have very good resistance to the nonspecific binding of v-SNARE vesicles. We also tried to make the passivating layer with biotin-PE but without the polymer part. It was significant that more nonspecific binding of v-SNARE vesicles was observed (Figure 2C), consistent with previous work.^{35,36} Fluorescent images of the passivating layer were obtained by using SUVs reconstituted with 0.1% CF-PEG-DSPE and 4.9% biotin-

PEG-DSPE. A smooth distribution of fluorescence was observed (Figure 1a), indicating the homogeneous distribution of PEG molecules over the entire surface on the 250 nm scale of the microscope resolution.

Tapping-mode AFM was used to study the surface roughness of the passivating layer (Figure S4). An rms roughness of 0.28 nm was found on a $7 \times 7 \mu\text{m}^2$ area. To measure the height of the surface being imaged above the glass, the AFM tip was used to create a hole in the bilayer, after which the hole was imaged. The depth of the hole was measured to be ~3 to 4 nm (data not shown). This is the height typically observed for a supported lipid bilayer alone. The result is consistent with the polymer layer lying on top of the lipid bilayer (as illustrated in Figure 1). Evidently the AFM tip does not image the polymer layer but instead pushes through the polymer to image the top of the glass-supported lipid bilayer.

Characterization of GUVs and GUV Pancakes

GUVs without proteins were formed efficiently in 2 h of electroformation. Most GUVs are unilamellar (Figure 1). Diameters ranged from several micrometers to more than $100 \mu\text{m}$. The formation of GUVs containing proteins required more time, as described in Materials and Methods. Many small vesicles coexist with GUVs. The diameter of protein-containing GUVs ranged from several micrometers to $\sim 50 \mu\text{m}$, and again most GUVs were unilamellar. GUVs with diameters from 10 to $50 \mu\text{m}$ were selected for diffusion studies.

It seemed possible that the GUVs might merely “deflate” on the PEG cushion, forming double bilayers. However, for the larger GUVs used for the diffusion studies, fluorescence imaging demonstrates that the pancakes comprise a single bilayer pancake atop the PEG cushion. First, the fluorescence intensity is uniform within a pancake and the average intensity is the same from pancake to pancake. The pancakes are either entirely single bilayer or entirely double bilayer. Second, in seven cases we imaged the same GUV before and after flattening (Figure 1). The diameter of the intact GUV was always half the diameter of the corresponding flattened GUV pancake to an accuracy of 5%, proving that the larger GUVs rupture and open to form a single lipid bilayer patch.

The results from tapping mode AFM are consistent with this picture under the assumption that the AFM tip images the upper surface of both lipid bilayers but is oblivious to the PEG layer in between (Figure 1c). The GUV pancakes were fragile under our AFM conditions; a single scan with the AFM tip badly damaged GUV pancakes (Figure S4), making it impossible to measure the surface roughness of the pancakes. For most GUV pancakes with diameter $>20 \mu\text{m}$, the measured difference in heights between the upper surface of the pancake and the imaged surface of the passivating layer was $7.9 \pm 0.6 \text{ nm}$. Assuming that the AFM tip senses the top of the passivating lipid bilayer but not the PEG layer and that the single bilayer of the GUV pancake is $\sim 4 \text{ nm}$ thick, this would leave a thickness of $\sim 4 \text{ nm}$ for the (unimaged) PEG cushion that lies between the two lipid bilayers. This thickness agrees with the expected extension of a PEG 2000 layer in brush form, estimated by the theory of de Gennes (Supporting Information).⁸ The NeutrAvidin molecules in our system may contribute little to the measured height difference, perhaps because they are largely buried within the PEG layer.

The Boxer group directly observed the expanding hole at the top of a deflating GUV as the total area of contact expanded, demonstrating the orientation of the ruptured GUVs.²⁶ We observed transient intermediate GUV shapes (deflated GUVs bound tightly on the surface) similar to those reported by the Boxer group.²⁶ Ruptured GUVs typically formed round or heart-shaped pancakes (Figures 1, S3, and S4), but other shapes occurred as well, similar to the observations of Musser and coworkers.³⁷ On the basis of previous work, we suspect that the outer leaflet of the GUV becomes the lower leaflet of the GUV pancake after rupture.

Smaller GUVs with a diameter of $<10\ \mu\text{m}$ tether efficiently to the PEG cushion but evidently deflate like a balloon to form a double-bilayer structure, as also observed by Boxer and coworkers.²⁶ These deflated GUVs are about twice as bright as the GUV pancakes in the fluorescent images (Figure S3). In confocal imaging,²⁶ they have a dome shape with flattened bases. In our tapping-mode AFM experiments, the top of a deflated GUV is flat, presumably because of the force of the AFM tip. The measured vertical distance from the top of these deflated GUVs to the top of the underlying bilayer varied from 14 to 18 nm (Figure S4). Allowing $\sim 4\ \text{nm}$ for the height of the polymer cushion (inferred from the GUV pancakes), this indicates a total top-to-bottom height of the deflated GUVs of $\sim 10\text{--}14\ \text{nm}$. Subtracting two bilayer heights leaves $\sim 4\ \text{nm}$ of the aqueous medium within the flattened GUV, which is comparable to the $\sim 2\text{--}3\ \text{nm}$ layer of water inferred for double bilayers by Groves and co-workers.^{24,25}

Lateral Mobility Studies

We carried out several tests of lateral mobility within the GUV pancakes and also within the passivating layer. The first test shows that the PEG monomers within the passivating layer are mobile. Here the 5% biotin-PEG-DSPE component of the SUVs was replaced with 4.9% PEG-DSPE (lacking biotin) and 0.1% biotin-PEG-DSPE to lower the density of binding sites in the passivating layer. The passivating layer was formed as usual, treated with NeutrAvidin, and washed. Then v-SNARE vesicles containing 1% DiD and 0.2% biotin-PEG-DSPE were tethered to the layer. The diffusion of single, tethered vesicles was tracked using a 633 nm laser beam at $0.04\ \text{kW}/\text{cm}^2$ in TIR mode. Movies were taken at 100 ms/frame for 100 frames. Using the SPT program described above, we found that all of the vesicles diffuse in the passivating layer with $D_{\text{lipopolymer}} \approx 0.3\ \mu\text{m}^2\ \text{s}^{-1}$. These experiments track v-SNARE vesicles bound to single PEG polymer molecules that are in turn bound to biotin-PEG-DSPE embedded in the layer. The friction in this system is probably dominated by PEG-PEG interactions, which explains the relatively small diffusion coefficient. For comparison, the diffusion coefficient of a vesicle in solution is 10 times larger, $D_{\text{vesicle}} \approx 3.3\ \mu\text{m}^2\ \text{s}^{-1}$.⁶

To characterize the mobility of lipids within GUV pancakes, GUVs were reconstituted with $10^{-4}\ \text{mol}\ \%$ DiD and ruptured onto the polymer cushion through the pH-sensitive electrostatic interactions described above. A 633 nm laser beam at $0.04\ \text{kW}/\text{cm}^2$ was used to locate ruptured GUVs containing DiD. Single DiD molecules were observed by TIRF using $0.32\ \text{kW}/\text{cm}^2$ (Figure S2). Movies were taken at 21.7 ms/frame for 500 frames. In each case, the best-estimate diffusion coefficient D_e (eq 1) of 497 individual particles was obtained by cutting trajectories after the first segment of $l_{\text{traj}} = 13$ steps and measuring the mean-square

displacement using time lags of 1, 2, and 4 steps ($t_{\text{lag}} = 22, 43, \text{ and } 87 \text{ ms}$). The histogram of D_e (Figure 2a) reveals two fairly distinct populations. About 80% of trajectories have $D_e \sim 1 \mu\text{m}^2 \text{ s}^{-1}$. These “fast” molecules apparently obey the theoretical equation for free, homogeneous diffusion reasonably well (eq 2). The other 20% of trajectories, the “slow” molecules, have $D_e \sim 1 \mu\text{m}^2 \text{ s}^{-1}$. Some of these values are clearly nonzero, and others are essentially zero within the estimated measurement error. The slow DiD molecules cannot belong to the same population as the fast molecules. As argued in the Discussion section, we believe that most of the slowly moving DiD molecules reside within small unilamellar vesicles (SUVs) that enter the sample cell with the GUV mixture and are trapped beneath the GUV pancake as it forms.

In practice, we discarded the slow molecules from the analysis and fit only the fast-molecule distribution to eq 2 in a least-squares sense. The fit is qualitatively good (Figure 2a), indicating that the fast-moving population diffuses freely and homogeneously. The best-fit value of the diffusion coefficient is $D_0 = 5.5 \mu\text{m}^2 \text{ s}^{-1}$, which agrees well with the mean of the fast-moving values of D_e , $\langle D_e \rangle = 5.4 \mu\text{m}^2 \text{ s}^{-1}$. Importantly, D_0 values derived from the three different time lags are in good agreement (Table 1), further demonstrating free diffusion of the fast-moving population.

For comparison, GUVs with tracer amounts of DiD were ruptured directly onto a clean glass surface to create GUV pancakes without the passivating layer. Movies of these more slowly moving DiD molecules were taken at 41.0 ms/frame for 500 frames. Analogous histograms of D_e were prepared (Figure 2b). These D_e distributions do not separate into a majority of the population that obeys eq 2 and a minority population. The vast majority of molecules have $D_e \sim 1 \mu\text{m}^2 \text{ s}^{-1}$. The diffusive behavior of DiD in the GUV pancakes deposited directly on glass is evidently poorly inhomogeneous and much slower than that of the majority of the fast population in the pancakes ruptured on the polymer cushion. We characterize the distribution of Figure 2b only by the mean of all measurements, $\langle D_e \rangle = 0.4 \mu\text{m}^2 \text{ s}^{-1}$. This is more than 10 times smaller than the fitted value of D_0 for the freely diffusing molecules in Figure 2a. The much slower diffusion is presumably caused by interactions between lipids in the lower leaflet and the glass surface, but it is clear that these interactions are somehow transmitted to both leaflets of the GUV pancake.

To characterize the diffusion of integral membrane proteins in the GUV pancakes, syntaxin molecules were labeled with Alexa-Fluor 633 (AF633-Syx) and reconstituted into GUVs at the nominal lipid to protein ratio of $8 \times 10^6:1$. Such GUVs were ruptured on the passivating layer or directly on bare glass. Single AF633-Syx movies used the same experimental parameters as before, except that the frame duration was 41.7 ms/frame in both cases. For GUVs ruptured on polymer cushions, the resulting histograms of D_e (Figure 2c) again exhibit two distinct populations. About 80% of trajectories diffuse rapidly and homogeneously with $D_e \sim 1 \mu\text{m}^2 \text{ s}^{-1}$, and the other 20% of trajectories have reduced or nearly zero D_e . The rapidly diffusing population is reasonably well fit by the theoretical model for homogeneous diffusion (eq 2). For the fast population using a lag time of two frames, the best-fit value of D_0 is $5.5 \mu\text{m}^2 \text{ s}^{-1}$, in good agreement with $\langle D_e \rangle$ (Table 1). The value of D_0 is again sensibly independent of the chosen time lag, consistent with free diffusion. In contrast, the AF633-Syx molecules in GUV pancakes on bare glass diffuse

some 10 times more slowly ($\langle D_e \rangle = 0.4 \mu\text{m}^2 \text{s}^{-1}$) and do not exhibit a majority of the population that undergoes homogeneous, free diffusion (Figure 2d and Table 1).

IV. Discussion

At least 80% of DiD lipid probes and at least 80% of integral membrane protein AF633-Syx in the GUV pancakes ruptured on the passivating layer, display free, homogeneous diffusion. In both cases, the diffusion coefficient, $D_0 \approx 5 \mu\text{m}^2 \text{s}^{-1}$, is more than 10 times faster than that observed in GUV pancakes ruptured directly on glass. These estimated percentages are lower limits because the tracking algorithm discriminates against mobile molecules. Essentially all immobile or slowly moving molecules are found by the tracking algorithm because they are bright and concentrated in intensity. Rapidly moving molecules often make short trajectories that are not included in the analysis because they may often fall below the threshold intensity. In addition, we believe that most of the slow-moving DiD and AF633-Syx molecules are not stuck to exposed patches of bare glass but rather reside within small unilamellar vesicles (SUVs) that enter the sample cell with the GUV mixture and are trapped beneath the GUV pancake as it forms. Several observations support this inference. First, these vesicles appear in unrinsed samples. Although we try to rinse them away after GUV tethering but before the GUV rupture step, the rinsing must be done very gently to keep the tethered GUVs intact. Some SUVs remain on the surface near the GUVs before rupture. Second, some of the molecules with $D_e < 1 \mu\text{m}^2 \text{s}^{-1}$ do in fact move, albeit slowly. Finally, the fraction of molecules that are slow-moving varies greatly from sample to sample and from pancake to pancake within a given sample. The immobile population is less apparent for GUVs ruptured directly on glass than on the passivating layer (Figure 2b,d vs Figure 2a,c). This is likely because the entire population now has limited mobility, so very slow vesicles are obscured under the broad overall distribution.

This model membrane system minimizes the influence of the glass surface on lipid and protein mobility, as evidenced by the large diffusion coefficient of both the lipid and integral membrane protein markers on the cushioned GUV pancakes ($\sim 5 \mu\text{m}^2 \text{s}^{-1}$ at 37 °C). For comparison, the DNA-tethered membrane patches formed by GUV rupture in the Boxer group²⁶ exhibit lipid mobility very similar to that of our GUV pancakes. For an egg PC membrane patch, they found $D_{\text{lipid}} = 4.8 \mu\text{m}^2 \text{s}^{-1}$ compared with our value of $5.5 \mu\text{m}^2 \text{s}^{-1}$ for DiD. Membrane protein diffusion was not studied. It could be argued that the true benchmark for free lipid or protein diffusion is the free-standing GUV. Two studies using free-standing GUVs found similar diffusion coefficients in the range of $3\text{--}5 \mu\text{m}^2 \text{s}^{-1}$ for single-membrane helix proteins analogous to Syx.^{40,41} The measured values of D_{lipid} varied from 6.3 to $11.4 \mu\text{m}^2 \text{s}^{-1}$,^{40–42} which is significantly faster than our result for DiD.

Our method is similar in spirit to the supported double-membrane system of Kiessling and co-workers.³⁸ However, in that system only 10% of the Syx molecules were mobile, with the faster population diffusing at $0.25 \mu\text{m}^2 \text{s}^{-1}$. In another variation, the Tamm group³⁹ made a bilayer on a polymer cushion on glass by first transferring the lower leaflet with 3% triethoxysilane-PEG-PE onto glass and subsequently forming a t-SNARE-containing upper leaflet by vesicle fusion. In this system, 75% of the t-SNARE molecules (Syx plus SNAP-25) were mobile, but the diffusion coefficient was only $0.75 \mu\text{m}^2 \text{s}^{-1}$.

An advantage of our GUV pancakes is that all of the materials are commercially available; no special polymer synthesis is required. The pancakes exhibit excellent resistance to nonspecific interactions with anionic vesicles containing proteins. The polymer layer provides a space of ~4 nm thickness between the passivating lipid bilayer and the lower leaflet of the GUV pancake, which is substantially larger than the 1 to 2 nm thickness of the water layer between a supported lipid bilayer and the glass substrate. The cytoplasmic domain of AF633-Syx presumably occurs equally often on both surfaces of the GUV pancake. Homogeneous diffusion of AF633-Syx then indicates that friction between the cytoplasmic domain and the PEG cushion is minimal. This in turn suggests that even membrane proteins with large water-soluble domains on both sides of the bilayer should diffuse freely within the GUV pancakes. Ruptured GUVs thus hold great promise for studies of integral membrane proteins in vitro. The most difficult remaining question is the degree to which such proteins can be reconstituted into GUVs while retaining full functionality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Fluorescence microscopy images (left) and schematic diagrams (right) of the passivating layer on glass, a tethered GUV, and a GUV pancake (from top to bottom). In the schematics, lipid bilayers are black, PEG polymers are blue, and NeutrAvidin molecules are green. (a) A TIRF image of the passivating layer with 0.1% PEG-DSPE labeled with carboxyfluorescein. The area is $40 \times 40 \mu\text{m}^2$. (b) An epi image of a GUV tethered to the passivating layer by biotin–NeutrAvidin–biotin interactions at pH 7.4. Fluorescent labeling is via 0.1% DiD in the GUV. The focal plane is set at the GUV midplane. The GUV diameter is $17 \mu\text{m}$. (c) An epi image of the same GUV after rupture to form a pancake was induced by lowering the pH to 4.0. The pancake diameter is 34 nm, proving that the pancake is a single bilayer.

Figure 2.

Distributions of best-estimate diffusion coefficients D_e for individual DiD and AF633-Syx molecules within GUV pancakes. (a) 330 DiD molecules in GUV pancakes ruptured on the passivating polymer cushion. The analysis uses $l_{\text{traj}} = 13$ steps (282 ms) and $t_{\text{lag}} = 2$ steps (42 ms). The solid curve is the least-squares fit to eq 2 including only data with $D_e \geq 1 \mu\text{m}^2 \text{s}^{-1}$, yielding $D_0 = 5.5 \mu\text{m}^2 \text{s}^{-1}$. The mean is $\langle D_e \rangle = 5.4 \mu\text{m}^2 \text{s}^{-1}$. See the text for details. (b) DiD molecules (497) in GUV pancakes ruptured directly on bare glass. The analysis uses $l_{\text{traj}} = 13$ steps (542 ms) and $t_{\text{lag}} = 2$ steps (84 ms). The solid curve is the least-squares fit of all of the data to eq 2. The best-fit value is $D_0 = 0.21 \mu\text{m}^2 \text{s}^{-1}$, but the fit is poor. The mean is $\langle D_e \rangle = 0.36 \mu\text{m}^2 \text{s}^{-1}$. (c) AF633-Syx molecules (107) in GUV pancakes ruptured on the passivating polymer cushion. The analysis uses $l_{\text{traj}} = 7$ steps (291 ms) and $t_{\text{lag}} = 2$ steps (83 ms). The solid curve is the least-squares fit to eq 2 including only data with $D_e \geq 1 \mu\text{m}^2 \text{s}^{-1}$, yielding $D_0 = 5.5 \mu\text{m}^2 \text{s}^{-1}$. See the text for details. (d) AF633-Syx molecules (1079) in GUV pancakes ruptured directly on bare glass. The analysis uses $l_{\text{traj}} = 7$ steps (291 ms) and $t_{\text{lag}} = 2$ steps (83 ms). The solid curve is the least-squares fit of all of the data to eq 2. The best-fit value is $D_0 = 0.25 \mu\text{m}^2 \text{s}^{-1}$, but the fit is poor. The mean is $\langle D_e \rangle = 0.36 \mu\text{m}^2 \text{s}^{-1}$. For the conditions in parts a and c, at least 80% of the molecules had $D_e \geq 1 \mu\text{m}^2 \text{s}^{-1}$ but these fast molecules are underrepresented in the histograms because their trajectories tend to be short; see the text for details.

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Table 1
Diffusion Characteristics of GUV Pancakes on a Polymer Cushion and on Bare Glass

system	l_{traj} (steps) ^a	n^a	t_{lag} (ms) ^a	$\langle D_e \rangle$ ($\mu\text{m}^2 \text{s}^{-1}$) ^b	D_0 ($\mu\text{m}^2 \text{s}^{-1}$) ^b	“fast” fraction ^c
DiD in GUV pancake on passivating layer	13	330	21.7 (1 step)	5.1	5.4	>0.8
			43.5 (2 steps)	5.4	5.5	>0.8
			87.0 (4 steps)	5.6	5.5	>0.8
DiD in GUV pancake on glass	13	497	83.5 (2 steps)	0.36		
AF633-Syx in GUV pancake on passivating layer	7	107	83.5 (2 steps)	5.8	5.5	>0.8
			125.2 (3 steps)	5.8	5.7	>0.8
AF633-Syx in GUV pancake on glass	7	1079	83.5 (2 steps)	0.40		

^aTrajectories are broken into independent segments of l_{traj} steps each; there were n such segments. The lag time t_{lag} for the mean-square displacement analysis varied as shown.

^b $\langle D_e \rangle$ is the mean of D_e values for all segments analyzed. D_0 is the best-fit value of the diffusion coefficient for the fast fraction of trajectory segments (i.e., those segments for which $D_e \geq 1 \mu\text{m}^2 \text{s}^{-1}$). See the text for details.

^cFraction of molecules with $D_e \geq 1 \mu\text{m}^2 \text{s}^{-1}$. See the text for details.