Membrane Fusion-Competent Virus-Like Proteoliposomes and Proteinaceous Supported Bilayers Made Directly from Cell Plasma Membranes

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ABSTRACT: Virus-like particles are useful materials for studying virus–host interactions in a safe manner. However, the standard production of pseudovirus based on the vesicular stomatitis virus (VSV) backbone is an intricate procedure that requires trained laboratory personnel. In this work, a new strategy for creating virus-like proteoliposomes (VLPLs) and virus-like supported bilayers (VLSBs) is presented. This strategy uses a cell blebbing technique to induce the formation of nanoscale vesicles from the plasma membrane of BHK cells expressing the hemagglutinin (HA) fusion protein of influenza X-31. These vesicles and supported bilayers contain HA and are used to carry out single particle membrane fusion events, monitored using total internal reflection fluorescence microscopy. The results of these studies show that the VLPLs and VLSBs contain HA proteins that are fully competent to carry out membrane fusion, including the formation of a fusion pore and the release of fluorophores loaded into vesicles. This new strategy for creating spherical and planar geometry virus-like membranes has many potential applications. VLPLs could be used to study fusion proteins of virulent viruses in a safe manner, or they could be used as therapeutic delivery particles to transport beneficial proteins coexpressed in the cells to a target cell. VLSBs could facilitate high throughput screening of antiviral drugs or pathogen–host cell interactions.

INTRODUCTION

Mammalian cell membranes are composed of a mixture of phospholipids, cholesterol, and proteins. Membranes are coated with a carbohydrate layer composed of glycosylated proteins and glycolipids that are collectively referred to as the glyocalyx. The properties of the glyocalyx dictate host–pathogen interactions, and these interactions play a key role in the pathogenesis of viruses such as influenza.

Influenza is a membrane-enveloped virus that causes seasonal outbreaks and occasionally pandemic illnesses in humans. Outbreaks occur because continuous viral evolution produces mutants capable of optimal interactions with the glyocalyx of target cells while also being able to successfully evade the immune system. Continuous viral evolution, therefore, requires scientists to be vigilant in determining both the host and viral factors that facilitate infection. Researchers must have tools available to quickly and safely assess virus-host interactions to (1) monitor virus evolution necessary for future vaccine development, (2) screen new antiviral drugs, and (3) study fundamental membrane fusion processes.

A relatively new approach for quantitatively studying virus–cell interactions and viral entry kinetics is single particle imaging using total internal reflection fluorescence microscopy (TIRFM). A key ingredient in this platform is a supported lipid bilayer (SLB) that coats the walls of microfluidic channels and acts as a host membrane mimic. This biomimetic material preserves lipid mobility in the bilayer plane and the planar geometry removes many experimental complications imposed by live cells. These features facilitate the study of virus–cell interactions and the membrane fusion processes required for viral infection, but in a convenient platform for imaging and quantitative data collection. These platforms have provided useful information about seasonal influenza, Sindbis, and other low pathogenic viruses. However, to study fusion processes of highly pathogenic membrane-enveloped viruses, like pandemic strains of influenza, Ebola virus, or severe acute respiratory syndrome (SARS) coronavirus, one must have laboratory biosafety level of 3 or more. Fewer facilities have this classification, which effectively limits the number of researchers that study these pathogens.

One strategy to safely study highly pathogenic viruses uses pseudovirus particles. One of the most common constructs for pseudovirus particles is the vesicular stomatitis virus (VSV). In the VSV pseudoparticle approach, a cell is transfected with a viral fusion protein of interest so that this protein is expressed in infected cells replicate the VSV following its usual infection pathway via endocytosis: endosomal escape, genome repli-
cation in the nucleus, viral protein production, and repackaging at the membrane surface. At the conclusion of this process, nascent viral particles bud from the cell plasma membrane surface. During the budding process, the emerging virion’s membrane acquires the fusion protein that was expressed in the plasma cell membrane of the infected cell. It is important to note that the pseudovirus does not contain the gene for the fusion protein of either VSV-G or the fusion protein coded in the plasmid that was used to transfect the cells prior to its infection with the VSVΔG*-G strain. Thus, the progeny pseudoviruses cannot propagate further infection, making it a safe strategy to study the activity of fusion proteins of virulent viruses.

Combining these virus-like particles with analytical techniques that can quantitatively assess virus–host interactions, such as single particle imaging, gives researchers powerful tools to study viral entry. However, the production of the pseudovirus particles is complex, requiring trained technicians and several days of culture, growth, and infection, which limits its wider use beyond research laboratories. Our goal was to create a simpler method to produce virus-like proteoliposomes that can be used for fundamental virus entry studies, as probes to assess virus–host interactions, or as packages for therapeutics where the viral fusion machinery ensures the delivery of cargo to the cytosol of a target cell.

Herein we describe a simple method to create virus-like proteoliposomes from cell plasma membranes that express a viral membrane protein of interest using a cell blebbing technique. Cell blebs are chemically induced membrane vesicles that bud from the cell plasma membrane. These chemically induced vesicles were first generated in the 1970s. Since then, blebs have been used to make giant plasma membrane vesicles (GPMVs) to study lipid raft and domain formation, and to elucidate membrane protein behavior, but not as a means to study lipid raft and domain formation, to test fusion protein, hemagglutinin (HA). Pure blebs have difficulties from cell blebs contain fusion proteins that maintain full fusion capability, compared to traditional methods to produce proteoliposomes, is that proteins can be incorporated directly into supported bilayers without the requirement of detergent membrane solubilization, protein purification steps, and reconstitution into liposomes. These steps can result in loss of native conformation of the membrane protein and disruption of the local lipid–protein interactions that may be necessary for regulating protein activity. Thus, a virus-like supported bilayer produced from cell blebs with fully functional proteins, combined with a microfluidic platform, could facilitate high throughput screening of antiviral drugs and host cell–pathogen interactions.

**MATERIALS AND METHODS**

**Cells and Plasmids.** Baby hamster kidney-21 (BHK-21) cells, a generous gift from Michael Whitt, were grown and maintained in Dulbecco’s modified Eagle medium (DMEM, CellGro) supplemented with 10% fetal bovine serum (Gibco), 100 μM penicillin, and 10 μg/mL streptomycin (CellGro), 1% HEPES buffer (CellGro) in a 37 °C, 5% CO₂ incubator.

The pCAGGS-H3/X31 and pcDNA3APN plasmids were used for transfections of BHK-21 cells. They encode the influenza hemagglutinin HA X-31 and feline aminopeptidase N (fAPN) and are generous donations from David Steinhauser of Emory University and Kathryn Holmes of the University of Colorado, respectively.

**Preparation of Cell Blebs.** For transfections, 1.5 × 10⁶ cells were seeded in 10 cm culture dishes, and incubated for 24 h. Transfections were performed using TurboFect transfection reagent (ThermoScientific) according to manufacturer’s instructions and using 12 μg of plasmid DNA for each plate. Buffer used in cell culture will be referred to as buffer A (2 mM CaCl₂, 10 mM HEPES, 150 mM NaCl at pH 7.4) and to induce cell blebbing, as buffer B (2 mM CaCl₂, 10 mM HEPES, 25 mM formaldehyde, 2 mM dithiothreitol (DTT), and 150 mM NaCl at pH 7.4). Cell blebs were dialyzed using cellulose dialysis tubing (Fischer Scientific) in two 100 mL volumes of buffer A for 24 h to remove the blebbing buffer. The size distribution of cell blebs was determined by dynamic light scattering using a Malvern Zetasizer Nano (Worcestershire, UK). A plot of the size distributions is provided in the Supporting Information.

**Preparation of Liposomes.** The following lipids were used in the experiments: 1-oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine (POPC), 1-oleoyl-2-palmitoyl-sn-glycero-3-phosphoethanolamine (POPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), cholesterol, sphingomyelin, and total ganglioside extract. These materials were all purchased from Avanti Polar Lipids (Alabaster, AL). Fluorescent lipids and lipophilic labels used to label membranes include Oregon green DHPE and Octadecyl Rhodamine (R18), all purchased from Molecular Probes, Eugene, OR. The water-soluble fluorophore, Sulforhodamine B (SRB), was purchased from Molecular Probes, and used to label interiors of liposomes and virus.

Two formulations of liposomes were used in these studies. The first preparation, referred to as BHK-liposome, contained POPC, POPE, sphingomyelin, and cholesterol in the ratio 37:3:4:2:0.5. This composition was formulated to match the native lipid content of BHK cells as closely as possible and primarily used in the formation of bleb supported bilayers. The second preparation, referred to as SA-liposome (SA = sialic acid), was composed of DOPC, POPE, cholesterol, and total ganglioside extract (which contain sialic acid) in the ratio 4:4:2:0.5. SA-liposomes were used both to form supported lipid bilayers containing SA and as liposomes to verify HA functionality in cell bleb-derived HA-supported bilayers. Standard procedures were used to form the liposome solutions. Our detailed procedure is described in the Supporting Information.

**Formation of Supported Lipid Bilayers from Pure Liposome Solutions.** Liposome solutions were added to either a polydimethylsiloxane (PDMS) well or drawn into a microfluidic device and incubated for at least 5 min before rinsing with buffer A. Fabrication procedures for PDMS wells and microfluidic devices are provided in the Supporting Information. Supported lipid bilayers self-assemble on clean glass during vesicle fusion of these liposome formulations. In some experiments, liposome membranes were fluorescently labeled with Oregon green DHPE or with R18 prior to bilayer formation. Fluorescent labels enabled visualization of the supported lipid bilayer.
were indicators of bilayer acidification in fusion experiments, and were probes for mobility measurements.

**Fluorescent Labeling of Blebs, Liposomes, or Native Virus.** Influenza X-31 (H3N2) with a hemagglutinin (HA) concentration of 2 mg/mL (as determined by Charles River Laboratories) was used in virus membrane fusion experiments. To visualize membrane fusion using a fluorescence dequenching technique, membranes must be labeled with a semiquantitative amount of fluorophores following standard procedures. In these experiments, HA-bleb membranes, SA-liposome membranes, and viral membranes of influenza X-31 virus were labeled with R18 according to the general procedure outlined in the Supporting Information.

**Atomic Force Microscopy (AFM).** AFM was used to image the supported bilayer samples and measure bilayer thicknesses. Samples for AFM were prepared as described and the surfaces were gently scratched so as to allow a step height measurement of the lipid bilayer. Samples were imaged with a PicoPlus 2500 AFM (Molecular Imaging) using DNP-S10 contact mode tips (Bruker). Images were taken under aqueous buffer in contact mode at 10 μm/s, 256 lines per image, and 10 × 10 μm² scan size.

**Ellipsometry.** Ellipsometry was used to confirm thickness measurements made by AFM. An EP3 Imaging Ellipsometer (NanoFilm) with a liquid cell, filled with deionized water, was used for all ellipsometric experiments. Measurements of both bare silicon and bilayer films were taken to confirm oxide layer thickness. The area of the surface over which the measurements were made was 0.5 cm × 0.5 cm. Variable angle measurements of 57°−60° were taken of areas of about 0.25 mm² at a wavelength of 531.7 nm. The data from the ellipsometric measurements was modeled using a Marquardt−Levenberg simulation in Thin Film Companion software (Semi-consoft, Inc.). The thickness of the oxide layer on the bare silicon wafer used for these experiments was determined to be approximately 4.1 nm, and this value was incorporated into the calculation of total supported bilayer thickness. For the supported bilayers, we used frequency-dependent complex refractive index parameters, \( n = 1.38 \) and \( k = 0 \), based on previously published results.

**Antibody Binding Experiments.** To label HA proteins in cell bleb-derived support bilayers, the bilayer was incubated with Alexa Fluor 488-conjugated mouse monoclonal IgG, anti-hemagglutinin antibody (16B12) (Invitrogen) at a concentration of 1 μg/mL for 20 min. Following incubation, bilayers were rinsed extensively with buffer A to remove unbound antibodies and visualized using an inverted fluorescence microscope with appropriate filter set.

**Diffusion Measurements in Supported Bilayers Using Fluorescence Recovery after Photobleaching.** Integrity of bilayers and diffusion of the lipids within it was examined by fluorescence recovery after photobleaching (FRAP). Bilayers were formed following the above procedures inside PDMS wells. R18 was used to label the bilayer and carry out the photobleaching experiments. Once formed, bilayers were gently scratched with a dissection tool to remove a thin section of the supported bilayer. This line aided in the optical pathway of the microscope using a Laser TIRF 3 slider (Carl Zeiss, Inc.), which controlled the angles of incidence. Exceeding the critical angle for water−glass system (≈62°) ensured total internal reflection of the lasers and created evanescent waves about 100 nm thick. At this thickness, the evanescent waves excited fluorophores positioned within the supported bilayer, within fluorescently labeled liposomes bound to hemagglutinin proteins embedded in the supported bilayer, or within fluorescently labeled virions bound to SA-containing lipid bilayers. The excitation laser light was band-pass filtered through a Semrock 74 HE GFP/mRFP filter cube, and then combined with a dichroic mirror before being focused on the outer edge of the back aperture of the objective. The fluorescence emission signal was filtered through a 525/31 and 616/57 nm dual band-pass emission filter and then sent to an electron multiplying CCD camera (Hamamatsu ImageEM C9100−13, Bridgewater, NJ).

**pH-Triggered Membrane Fusion Assay.** Membrane fusion between bound virions (or liposomes) and supported bilayers was initiated by flow mixing (150 mM NaCl, 1.5 mM MOPS, 5 mM citric acid) precalibrated to the optimal acidic triggering pH for X-31, pH = 4.25. This acidic buffer was sent into the microfluidic device at a flow rate of 100 μL/min for 2 min. The time at which acidification of the flow cell occurred was marked by an obvious decrease in fluorescence of the pH-sensitive fluorophore, Oregon green DHPE, present in the supported bilayer for this purpose. TIRFM was used to monitor fusion and images were collected at an interval of 100 ms for 3 to 4 min.

**Image Processing.** The images acquired during the membrane fusion assays were analyzed using both ImageJ (NIH) and MATLAB (Mathworks). Fusing particles were manually selected in Imagej and the fluorescence intensity in a 4 × 4 pixel region around each particle was collected as a function of time. In videos where background noise was high due to a large amount of fusion events, a rolling ball background subtraction algorithm was applied to all of the images to remove excess background noise as the fluorophores diffused into the membrane after hemifusion. The rolling ball algorithm determines the local background for every pixel by averaging over a large circular region around the pixel. This background value is then subtracted from the original image. The fluorescence trajectories for the particles were then imported to MATLAB (Mathworks) for further analysis. A code written in MATLAB determined the onset time of the dequenching ‘spike’ for each particle by finding the time of the maximum intensity in each particle trajectory.

### RESULTS AND DISCUSSION

**Formation of Cell Blebs and Size Characterization.** Cell blebs are produced from baby hamster kidney (BHK) cells, transfected with the transmembrane influenza fusion protein hemagglutinin (HA). Twenty-four hours after transfection of BHK cells with the desired plasmid, cells were washed twice with buffer A. Three milliliters of buffer B, which contains the chemical that induces the formation of bleb vesicles from the cell plasma membrane, was added to each culture dish. The dishes were then incubated for 1 h at 37 °C with gentle rocking. After incubation, the cell supernatant, containing detached cell blebs, was decanted into a 15 mL falcon tube and placed on ice for 20 min to allow any detached cells to settle out. The supernatant was then transferred to new test tube. Buffer B was removed from the supernatant by dialysis with buffer A for 24
h. An excellent recent review of protocols for cell bleb production is given in ref 15.

The size and distribution of cell blebs in the supernatant was determined by dynamic light scattering. The bleb supernatant showed three populations typically, with three main peaks at 40 nm, 100 nm, and 600 nm. A plot of the size distribution is provided in the Supporting Information (Figure S1). For reference, the size of BHK-liposomes and SA-liposomes were relatively monodisperse with diameters ranging between 90 and 100 nm. Aliquots of these solutions were used to carry out single particle membrane fusion experiments, as will be described later.

Formation of Proteinaceous Supported Bilayers from Cell Blebs. To visualize the formation of supported bilayers derived from BHK cell blebs, the membranes of HA-blebs were first labeled with a lipophilic fluorophore, R18. A small aliquot of the labeled bleb solution was added to a PDMS well and incubated for 10 min at room temperature. During this time, blebs contact and adsorb to the glass surface. Excess blebs that were not adsorbed to the glass surface were removed by gently rinsing the well with buffer A. Observation of samples under 40× and 100× magnification show that the blebs are not ruptured at this stage, but are adsorbed to the glass surface intact, as seen in Figure 1A as punctuate bright spots. Although the absolute concentration of the bleb solution is unknown, the solution was observed to be sufficiently concentrated to absorb blebs over the entire glass surface.

To induce the formation of a supported bilayer from the adsorbed blebs, we adapted a procedure Dodd et al.16 used to form supported bilayers from the inner membrane vesicles of E. coli. In our case there are two distinct procedural differences from Dodd et al. First, the liposomes used to rupture the BHK cell blebs were formulated to closely match the BHK endosomal composition17 (referred to as BHK-liposome) so that the resulting bilayer would most closely resemble the native cell membrane lipid content. Second, we adsorb cell blebs to the substrate first, rather than mix in solution with BHK-liposomes prior to adding to the well. This latter step...
ensures that the liposomes do not outcompete the cell blebs for glass adsorption.

In this experiment, the BHK-liposomes do not contain any fluorophore labels. The rupturing of the liposomes in the spaces between the adsorbed blebs rapidly induced the rupture of the blebs, as indicated by the dispersion of the bright, punctuate spots (Figure 1B,C). This observed dispersion in fluorescence is due to the spread of R18 that originated in the membranes of the adsorbed blebs, diffusing within the newly formed bilayer plane after rupture. Photobleaching a small section of this newly formed bilayer and monitoring the temporal recovery also confirms that the blebs have ruptured to form a planar film. Photobleaching experiments are used to determine diffusion coefficients in the supported bilayers and will be described in a later section.

**Supported Bilayer Quality, Planarity, and Thickness Characterization.** AFM and ellipsometry were used to confirm the formation of supported bilayers from cell blebs, visually assess their uniformity, and to characterize the film thicknesses. Three different samples were characterized by each method: (A) a supported lipid bilayer formed from protein-free, BHK-liposome solution only, (B) a supported bilayer formed from HA-blebs+BHK-liposomes (VLSB), and (C) nonruptured, adsorbed HA-blebs (VLPLs) on glass, prior to BHK-liposome addition.

**AFM.** Samples for AFM were prepared in PDMS wells as described in the Methods section. Details of the AFM setup and images (Figure 2) are provided in the Supporting Information. Contact mode under water was used to image and measure the thickness/height of the bilayer. For a point of comparison, we first characterized a standard bilayer made from BHK-liposome solution only (Sample A). Typical supported bilayers formed by vesicle fusion to glass are ~4 nm thick and have about a 1 nm water layer between the bottom leaflet and glass support. An AFM image in Figure 2A shows that the BHK-liposome bilayer is a relatively uniform film of thickness ~4–6 nm, as determined by scanning across the depth of a thin line intentionally scratched on the bilayer. This thickness is in good agreement with literature values for similar supported lipid bilayers.28–30

In the next experiment, we imaged the VLSB (Sample B). From the AFM image (Figure 2B, Supporting Information) we see in general that the bilayer is uniform, but there are some defects present in the film. The thickness of the VLSB was determined by measuring the depth of a defect. The measured depths of several defects were consistently between 3 and 4 nm, which are consistent with the thickness measured for the standard BHK-liposome bilayer. However, to be sure we were not measuring thicknesses based on areas primarily consisting of BHK-liposome bilayer; we also measured film thicknesses of these samples over and extended area (0.5 cm × 0.5 cm) using ellipsometry.

We also attempted to image adsorbed cell blebs prior to supported bilayer formation (Sample C), but found that images were very irregular and difficult to interpret as is consistent with irregularly adsorbed material and incomplete film formation.

**Ellipsometry.** To corroborate that the addition of lipid vesicles to adsorbed cell blebs induced the formation of a supported bilayer film, ellipsometry was also performed on the same three kinds of samples used in AFM imaging. For these measurements, it was necessary to prepare samples on silicon wafers instead of glass coverslips, but the procedure was otherwise the same as described in the Methods section.

<table>
<thead>
<tr>
<th>sample</th>
<th>thickness (nm)</th>
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<tbody>
<tr>
<td>(A) BHK-liposome SLB</td>
<td>3.2</td>
</tr>
<tr>
<td>(B) HA-bleb+BHK-liposome SLB</td>
<td>3.9</td>
</tr>
<tr>
<td>(C) Adsorbed HA-blebs</td>
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**Table 1. Average Thickness of Supported Bilayer Films Measured by Ellipsometry**

Additional experimental details are provided in the Supporting Information. The thickness of the oxide layer of the silicon wafer used for these experiments was determined to be approximately 4.1 nm. This value was taken into account when modeling the supported lipid bilayer data to determine the film thickness, as described in the Supporting Information. A water layer of ~1 nm exists between the oxide support and a supported lipid bilayer film typically. We did not determine the thickness of the protein layer protruding from the supported cell-bleb bilayer. The total amount of expressed HA protein is not dense enough to create a contiguous film, as inferred by results of antibody binding experiments presented in the next section.

The film thicknesses are summarized in Table 1 and match reasonably well to the thicknesses measured using AFM. No film thickness could be determined from the sample of adsorbed cell blebs prior to BHK-liposome addition, presumably because of the irregularity of the adsorbed bleb surface density.

**Antibody Binding to Confirm Presence of Proteins Derived from Cell Blebs in the Supported Bilayer.** To confirm that the hemagglutinin proteins expressed in the cells used to produce blebs were both incorporated into the supported bilayer and oriented facing the bulk (i.e., not inverted toward the glass support), the bilayer was incubated with anti-HA antibodies specific for X-31 HA protein (Figure 3). Anti-HA labeled with Alexa Fluor 488 conjugate was incubated with HA-bleb supported bilayers for 20 min. The bilayer was then rinsed extensively with buffer A to remove unbound antibodies. Control experiments were performed where the anti-HA antibodies were incubated for 20 min on a HA-free bilayers (derived from empty vector blebs and blebs expressing aminopeptidase N (fAPN) instead of HA). Binding was assessed by imaging samples with an inverted fluorescence microscope. The images for the antibody binding experiments (Figure 3) show that a significant amount of specific anti-HA binding occurs on the VLSB, while minimal nonspecific binding occurs for the HA-free bilayers. Also included in this series of experiments is a control using another antibody directed to aminopeptidase N (fAPN), as well as bilayers formed from blebs expressing fAPN. These data show that both anti-HA and anti-fAPN are both specific to their respective targets (i.e., working properly) and have minimal nonspecific binding to other bilayers. The specific binding not only indicates that HA was present in the bilayer, but also that a significant amount of protein was in the correct orientation facing the bulk solution, and not toward the glass support.

**Mobility in Supported Bilayers. Lipid Mobility.** Fluorescence recovery after photobleaching (FRAP) was employed to determine the fluidity of the cell bleb bilayers. Samples were prepared on glass surfaces as described in the Methods section. Figure 4A–C shows the typical bleaching and recovery of R18 probes in various supported bilayers after bleaching with a 561 nm circular laser beam. Sample A is the BHK-liposome

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supported lipid bilayer, sample B is a glass surface coated with adsorbed VLPLs (HA-blebs) prior to BHK-liposome solution addition, and sample C is a VLSB derived from HA-blebs after BHK-liposome addition. The diffusion coefficients of R18 in samples A and C were found to be around 0.25 μm/s² with mobile fractions near 1. For the samples containing only absorbed cell blebs (sample B), photobleached spots did not recover, as expected if lipids and R18 fluorophores were confined to the adsorbed proteoliposomes and unable to exchange with surrounding unbleached adsorbed blebs.

To determine if the binding of an anti-HA antibody to HA in the VLSB reduces the mobility of R18 in the supported bilayer, the mobility of R18 was tested before and after antibody binding. It was found that antibody binding did not hinder the diffusion of R18 or cause the bilayer to lose mobility (Figure 4D). This result indicates that the VLSB is not near a phase transition and should remain in the fluid state after receptor binding. This is a key point as binding-induced phase transition could impact the ability of membrane fusion to occur, or at minimum, slow down the membrane fusion kinetics.

**Protein Mobility.** The mobility of HA protein in the supported bilayers was assessed by two methods. First, the position of bound, fluorescently labeled antibodies to HA were tracked at 100× magnification for an extended period of time. Second, fluorescence recovery after photobleaching was monitored as described for the lipid diffusion measurements. In both methods, limited protein mobility was observed.

There are two possibilities to explain immobilization of the protein. First, the thin water layer beneath the supported bilayer may not be thick enough to keep the extended portions of the HA from coming into contact with the underlying support and nonspecifically adsorb to it. Second, because the fluorescent antibody used to label the HA can bind multiple proteins, it is possible that cross-linking due to antibody binding can cause a reduction in mobility as well.

There are a number of cushioning strategies that can be used to improve protein mobility, from tethered polymer layers to dextran cushions. None of these cushioning strategies were integrated here because, as we will describe next, protein
immobility did not seem to block hemagglutinin’s ability to bind sialic acid or carry out its function of inducing the fusion of membranes. We elected not to use cushions to keep the system simple for this study.

Membrane Fusogenicity of HA Protein in Cell Blebs and in Bleb-Derived Supported Bilayers. The function of hemagglutinin in influenza entry virus is to mediate the fusion between viral and host endosomal membranes. HA protein undergoes a conformational change at pH values less than 5.5 to trigger the fusion of the viral and endosomal membrane during virus infection. Antibody binding experiments verified that HA was present in the supported bilayer in the correct orientation. However, the blebbing buffer, which contains a small amount of DTT and formaldehyde, can induce the cleavage of disulfide bonds and the cross-linking of HA proteins before dialysis takes place to remove these chemicals from the blebs. Thus, the fusogenicity of the proteins must be tested to ensure the blebbing buffer did not destroy the function of the protein. To test the functionality of HA proteins expressed in cell blebs and in bleb-derived bilayers, we performed the following series of experiments.

Membrane Hemifusion. Membrane hemifusion is the merging of the two outermost leaflets of the viral and host membranes. At this stage, the complete fusion pore has not opened, but a stalk-like structure connects the viral and host membrane. When this stalk forms, fluorescent labels can mix with lipids from the host cell and diffuse radially away from the hemifusion site. By labeling one membrane with a quenched amount of fluorophore, and the other membrane with nothing, hemifusion can be marked by a “dequenching” event when the stalk is formed. These dequenching events are easy to observe using TIRFM at the individual virion level and are distinct from a simple dissociation event by their intensity signal. The readout for dequenching is a sudden spike in fluorescence intensity, followed by a finite decay in fluorescence, as will be described next. On the other hand, simple dissociation is indicated by a sudden disappearance of the entire punctate spot where the virus was originally bound.

In the first experiment, we tested the ability of HA expressed in cell blebs (VLPLs) to hemifuse to sialic acid-containing supported lipid bilayer. SA-liposomes were drawn into a microfluidic device at a flow rate of 100 μL/min for 1 min and incubated in the channel for 20 min. The channel was rinsed with buffer A at 100 μL/min for 2 min to form a supported lipid bilayer containing sialic acid receptor. HA-blebs labeled with R18 were drawn into the channel at a flow rate of 30 μL/min for 5 min and bound to SA in the supported bilayer during 20 min incubation. Unbound HA-blebs were removed from the channel by rinsing with buffer A at 100 μL/min for 2 min. HA-mediated fusion of the blebs to the supported lipid bilayer was initiated by flowing pH 4.5 buffer into the channel at a flow rate of 30 μL/min for 2 min. Hemifusion was marked by dequenching of the R18 that originated in the HA-bleb, and its subsequent radial diffusion among the lipids in the supported SA-bilayer (Figure SA).

In a second set of experiments, we tested the functionality of HA in supported bilayers derived from HA-blebs. HA-VLSB were formed at neutral pH in microfluidic channels, following the general procedure outlined above. First, HA-bleb solution was drawn into the microchannel at a flow rate of 30 μL/min...
for 1 min and incubated for 20 min to allow the blebs to adsorb to the channel walls. The channel was subsequently rinsed with buffer A at 100 μL/min for 2 min to remove excess blebs. Second, BHK-liposome solution was drawn into the microchannel at 100 μL/min for 2 min and incubated to induce the rupture of adsorbed blebs. After 10 min elapsed, more BHK-liposome solution was drawn into the channel at 10 μL/min for 5 min to heal defects in the membrane. Third, the channel was rinsed with buffer A at 100 μL/min for 2 min to rinse out the excess liposomes. Following the formation of the VLPL, SA-liposomes labeled with R18 were then added to the channel and incubated for 20 min. During this time, the SA-liposomes bound to the HA proteins in the supported bilayer. Excess vesicles were rinsed from the channel. To activate the HA protein to induce hemifusion, buffer A at pH 4.5 was drawn into the channel. Hemifusion of the SA-liposome to the VLPS was indicated by R18 dequenching and radial diffusion through the bleb membrane away from the point of fusion (Figure 5B).

The third experiment was a control case of an R18-labeled X-31 influenza virion fusing to SA-containing supported lipid bilayer. The SA-bilayer was formed as described above in the first experiment of this series, and fusion initiated in the same manner. The hemifusion results for this control case are presented in Figure 5C. A qualitative comparison of the HA-blebs and HA-VLSB results to the native virion shows that hemifusion proceeds without much variation among these geometries.

For a more quantitative comparison among the hemifusion events, the fluorescence intensity curves plotted to the right of Figure 5 can be fit with a two-dimensional diffusion equation to determine the diffusion coefficient of the R18 fluorophores as they move radially away from the fusion site. The equation used to fit the decay in the fluorescence intensity data is

\[ I(t) = I(0)[1 - \exp\left(-\frac{r^2}{4Dt}\right)] \]

(2)

where \( I(t) \) is the intensity at time \( t \); \( I(0) \) is the intensity value at maximum dequenching (the peak intensity in each plot); \( r \) is the initial size of the radial spot containing the fluorophores (here taken to be 0.85 μm to match the area monitored for intensity); and \( D \) is the diffusion coefficient. In fitting these data, we chose only the portion from the highest part of the dequenching peak to the decay to the background level. At the highest intensity of the peak, the curve reflects a transition from fluorescence dequenching to the relaxation of fluorescence (decay of fluorescence signal) due to only the radial diffusion of the fluorophores away from the fusion site.

The intensity data was fit with eq 2. \( D \) was determined as the value that minimized the sum of the square of the error between the data and the fit. The results for each kind of sample are summarized in Table 2. These values are averages of the diffusion coefficients obtained from several (at least 4) single particle fusion events for each sample type and fall within the range of values obtained from the FRAP experiments. This good match confirms that the lipids remain mobile during the fusion process and that the VLPL and VLSB exhibit similar characteristics during hemifusion as the native virion.

The percentage of the number of observed fusion events (fusion extent), relative to the amount of bound native virus under these experimental conditions, was ∼27%. For the VLPLs, the fusion extent was ∼12%. For the VLSBs, the extent dropped to ∼5%. There may be two explanations for this drop, which we have not yet attempted to optimize. First, there may be a higher number of vesicles that appear to be bound to the bilayer, but may in fact be nonspecifically interacting with the glass through a defect site and therefore incapable of fusion. Second, the density of HA in the bilayer is probably significantly lower than that in the VLPL or native virus, which may reduce the fusion competency.

**Pore Formation.** Membrane hemifusion is directly followed by the formation of a fusion pore, where in the native virus, the viral RNA is released into the cytosol of the host cell. To show that HA maintained full functionality to induce pore formation after membrane hemifusion, the single vesicle fusion experiments carried out for hemifusion were repeated here. In this case, the internal compartment was fluorescently labeled instead of the membrane. Release of the interior contents and subsequent loss of fluorescence at the initial viral binding site indicates pore formation. In this case, we note that distinguishing pore formation from simple dissociation is less obvious than the dequenching events that result from hemifusion. However, there is a distinct and finite decay in the drop of the signal, which is notably slower than the intensity drop that occurs from a virus unbinding and leaving the evanescent field.

HA-blebs, sialic acid liposomes, and native virus were labeled with SRB, a water-soluble dye which intercalates through lipid membranes and partitions into the aqueous interiors of liposomes and virions. When the fusion pore opens the SRB is released and diffuses away from the fusion site, so a drop in fluorescence indicates when the pore formation event occurred. Both the HA-blebs and SA-liposomes dropped in fluorescence, indicating that pore formation occurred in both these cases (Figure 6A,B). For comparison, we also ran the control case of the native virion forming a pore with a SA-containing supported lipid bilayer, shown in Figure 6C.

The hemifusion and pore formation experiments taken together show that HA membrane proteins, either in cell bleb proteoliposomes or embedded in supported bilayers, remain fully fusion-competent. There are two important outcomes of these results.

First, these results illustrate that virus-like particles can be created using a straightforward cell blebbing technique. This simple approach to creating virus-like particles has several advantages over a prominent strategy that employs VSV virus to create pseudovirus particles. Both approaches require that a cell express the viral fusion protein of interest in its plasma membrane. In the blebbing approach, the bleb-inducing buffer is added to the culture and induces proteoliposomes to “bud” from the cell resulting in virus-like proteoliposomes containing the fusion protein of the virus. In the pseudovirus approach, several more intricate infection steps with VSV and pseudovirus are required; these steps take about 3–4 additional days. Depending on the application, the cell bleb approach may be advantageous for producing virus-like particles because of its much simpler procedure. Additionally, it should be possible to coexpress additional viral coat proteins into the cell bleb to

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**Table 2. Diffusion Coefficients Determined from the Radial Dispersion of R18 Fluorophores during Membrane Hemifusion**

<table>
<thead>
<tr>
<th>Type</th>
<th>Diffusion Coefficient</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>R18 HA-bleb to SA-SLB</td>
<td>0.14 ± 0.08</td>
<td>μm²/s</td>
</tr>
<tr>
<td>R18 SA vesicle to HA bilayer</td>
<td>0.27 ± 0.11</td>
<td>μm²/s</td>
</tr>
<tr>
<td>R18 virus to SA-SLB</td>
<td>0.22 ± 0.03</td>
<td>μm²/s</td>
</tr>
</tbody>
</table>
incorporate components of the virus as desired (in the case of influenza this might be neuraminidase or the M2 ion channel). By doing so, a useful research tool emerges that can decouple or isolate specific proteins or protein pairs for fundamental fusion studies. An exciting and related application for this technique is incorporating other desired proteins in cell blebs, in addition to the virus fusion machinery, as an avenue to reconstitute virus-like particles with designer functions such as therapeutic protein delivery. These applications are currently being undertaken in our laboratory.

The second important outcome these data illustrate is that functional, virus-like planar surfaces can be created from the cell blebs. This geometry may be useful for biosensing applications or for high throughput assays designed to quantify pathogen—host or pathogen—antiviral drug interactions. Additionally, if one can express a membrane protein of interest (not necessarily a viral protein) in a cell bleb, this technique can be used to incorporate that protein into supported bilayers without having to detergent-solubilize the membrane, purify, and reconstitute extracted proteins into liposomes. This is a significant advantage for reducing the possibility of (1) denaturing proteins and suffering a consequent loss of function, and (2) disrupting critical protein—lipid interactions necessary to maintain proper protein activity. This method also affords the possibility to create support bilayers that capture much more of the complexity of the cell membrane, i.e., the glycocalyx, into a planar format. A planar version of the complex plasma membrane architecture can offer new features for many in vitro biological assays and biosensing applications that already use standard supported lipid bilayers and biophysical—biochemical analytical techniques compatible with these materials.

**SUMMARY AND CONCLUSIONS**

In this work, membrane fusion-competent virus-like proteoliposomes and proteinaceous supported bilayers were made directly from cell plasma membranes using a cell blebbing technique. It was demonstrated in both geometries that hemagglutinin from X-31 influenza virus maintained the ability to both bind and fuse to sialic acid containing membranes. Analysis of the decay dynamics of the fluorescent probe during membrane hemifusion showed that fusion dynamics in the HA-proteoliposome and HA-supported bilayer are in line with the native virus fusing to chemically similar host membrane mimics. Both the virus-like proteoliposomes and proteinaceous supported bilayers could also proceed to full pore formation, illustrating that fusion function of the HA is not disrupted by the blebbing technique. The spherical or planar geometry of the virus-like membrane has many possible applications from fundamental fusion studies and therapeutic delivery to high throughput screening of host—pathogen interactions and antiviral drugs.

**ASSOCIATED CONTENT**

* Supporting Information
Preparation of surfaces for supported bilayers, fabrication of PDMS wells and microfluidic devices, liposome preparation, labeling procedures, and dynamic light scattering data. This material is available free of charge via the Internet at http://pubs.acs.org.
The authors declare no competing financial interest.

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