Formation of Three-Dimensional Structures in Supported Lipid Bilayers

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ABSTRACT The creation of three-dimensional structures in supported lipid bilayers has been examined. In bilayers, shape transformations can be triggered by adjusting a variety of parameters. Here, it is shown that bilayers composed of phosphatidylcholine and phosphatidic acid can be induced to reversibly form cap structures when exposed to an asymmetry in ionic strength. The structures that form depend on the asymmetry in the ionic strength and the amount of anionic lipid. Other factors that may be of importance in the creation of the structures, expansion forces, osmotic forces, and the bilayer-support interaction are discussed. The cap structures have the potential to be of considerable utility in examining the effect that curvature has on membrane processes.

INTRODUCTION

Cells contain many membranes, two-dimensional fluids capable of occupying three-dimensional (3D) space. The shapes of cell membranes vary extensively and can be altered rapidly. There has been extensive theoretical work detailing the parameters that can trigger shape transformations (1). Theory suggests that charged membranes will bend when exposed to asymmetric screening environments (2,3) (Fig. 1). The goal of this work is to test if this process can be observed experimentally.

Supported lipid bilayers have been used extensively to examine membrane properties and are of interest for a number of technological applications (4). Due to the bilayer-support interaction supported lipid bilayers are flat, limiting their application. If bilayers can be induced to bend away from the solid support, then supported bilayers will be a highly convenient platform with which to study the effect of membrane curvature on protein binding/function. This new platform would allow direct comparisons to be made between protein properties in flat and curved regions on a single bilayer sample.

An area of the cell where significant membrane curvature changes are continually occurring is the trans-Golgi network. It has been shown that the creation of phosphatidic acid (PA) by phospholipase D mediates secretory vesicle budding (5,6). The details of the role that PA plays—recruitment of protein and/or membrane curvature changes—is unknown. Spontaneous curvature measurements, however, have shown that PA has a large negative spontaneous curvature that can be modulated significantly by ionic strength (7). As a consequence, we elected to examine the effect that asymmetric screening environments have on supported bilayers containing PA. That, in fact, the bilayers can be induced to bend away from the support will be shown. The necessary conditions will be discussed along with the mechanism.

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EXPERIMENTAL PROCEDURES

Materials

Chloroform stock solutions of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA), and 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) were purchased from Avanti Polar Lipids and used without further purification. 2-(*N*-Morpholino)ethanesulfonic acid hydrate (MES hydrate) and EDTA were purchased from Sigma Chemical (St. Louis, MO). KCl and KOH were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). The buffer used for lipid rehydration and fusion was composed of 50 mM MES hydrate, 0.1 mM EDTA, and varying amounts of KCl and adjusted to pH 5.0 with concentrated KOH. Exchange buffers were composed of 50 mM MES hydrate, 0.1 mM EDTA, and varying amounts of KCl (250–50 mM).

Vesicle and supported lipid bilayer preparation

Large unilamellar vesicles (LUVs) were prepared by the extrusion method. Briefly, the chloroform solvated lipids were mixed at the appropriate molar ratios, dried under nitrogen, and held under vacuum for 1 h; the dried lipids were rehydrated in the 50 mM MES hydrate, 0.1 mM EDTA, and varying amounts of KCl buffer, pH 5.0. The lipid suspension was then extruded 21 times through a polycarbonate membrane with 50-nm pores. After extrusion, the LUV solution was centrifuged for 5 min at 14,000 rpm (Eppendorf Minispin Plus). The extruded vesicles were stored at 20°C, shielded from light, and used within 1 day. Supported lipid bilayers were formed, by vesicle fusion, inside a 60-μL perfusion chamber (Molecular Probes, Eugene, OR) on appropriately treated glass slides. The slides were washed in dilute ICN 7X detergent (VWR International, Chicago, IL), rinsed extensively in distilled water, and baked at 450°C for 4 h; slides were used within a day of preparation. After 5 min of incubation, excess vesicles were removed from the perfusion chamber using the same buffer used in vesicle preparation. The perfusion chamber was used to exchange the outside buffer solution; at least 1 ml of buffer was passed through the perfusion chamber to ensure complete exchange.

Epifluorescence imaging of the supported lipid bilayer

A Nikon TE2000 fluorescence microscope (Tokyo, Japan) equipped with a Cascade 512B charge-coupled device camera (Roper Scientific, Tucson, AZ) was used to image the bilayers. An X-Cite 120 arc lamp (EXFO) was used as a light source, and the NBD fluorophores were imaged using a NBD filter set (Chroma Technology, Rockingham, VT). For Fig. 2 (and Figs. 4–7),

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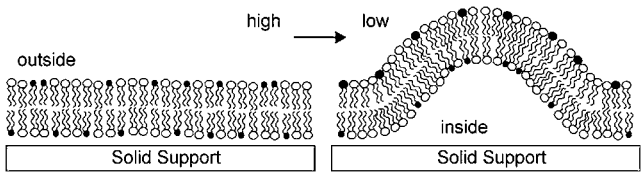

FIGURE 1 Schematic illustration of a shape transformation induced in a lipid bilayer when the ionic strength on the outside of the supported bilayer is lowered. DOPA lipids (*solid circles*), DOPC lipids (*open circles*).

a 100×, 1.3 numerical aperture (NA) oil immersion objective was used. For Fig. 3, a 100×, 1.45 NA oil immersion objective was used.

Confocal imaging of the supported lipid bilayer

Images were collected using an MRC-1024 ultraviolet confocal laser scanhead (Bio-Rad, Hemel, UK) on a Diaphot 300 (Nikon) inverted microscope using a 60× 1.4 NA objective. A 488-nm line of the krypton-argon laser (Ion Laser Technology, Salt Lake City, UT) was used to excite the NBD-conjugated lipid; the green fluorescence was collected with a 522/35 band-pass filter. A z-series was collected at 0.2 μm per plane, and a 3D projection of the lipid surface was made using Metamorph (Universal Imaging, West Chester, PA).

RESULTS

In Fig. 2, a DOPA/DOPC bilayer is exposed to an asymmetric screening environment. Vesicles containing 69 mol % DOPC/30 mol % DOPA/1 mol % NBD-PC in 250 mM KCl, 50 mM MES, 0.1 mM EDTA, pH 5, were fused to glass slides; after several minutes the vesicles were flushed out with the fusion buffer. This procedure results in uniform (intensity variation of $<$1% across the image), fluid, supported lipid bilayers (Fig. 2 *a*). The overall intensity of this bilayer is similar to that of a 99 mol % DOPC/1 mol % NBD-PC bilayer, indicating that a single bilayer has formed and not a multilamellar structure (8). When the solution above the bilayer is exchanged for a 50 mM KCl, 50 mM MES, 0.1 mM EDTA, pH 5 buffer, rings appear (Fig. 2 *b*). Upon increasing the ionic strength back to 250 mM, the rings disappear (Fig. 2 *c*). The bilayer does not fully return to its original uniform state; however, this residual fluorescence does not appear to affect the larger scale process of ring formation as rings can be reformed and dispersed repeatedly.

To determine if the rings appear because the bilayer has bulged away from the surface, as in Fig. 1, we acquired both epifluorescence images, using a higher NA objective, and confocal images. The bilayers in Fig. 3 were of the same composition as those in Fig. 2, and they were formed in the same way. Shown in Fig. 3, *a–e*, are epifluorescence images acquired with a 100×, 1.45 NA objective (a 100×, 1.3 NA objective is used for Figs. 2 and 4–7). In Fig. 3 *a*, the focus is on the flat part of the supported lipid bilayer; two dark spots are indicated with arrows. The spot indicated with the right arrow stays dark until the flat part of the bilayer is completely out of focus. It can therefore be assigned as a defect site. Such defects are rarely seen; here they are shown, as they are useful for noting the location of the bilayer. The left arrow indicates one of the two very large dark spots in the image. As the focus is adjusted up, rings appear and then finally a dot. These changes correspond to what would be expected if one scanned through, in the vertical direction, a structure like that indicated in Fig. 1. From these images we conclude that the rings observed in Fig. 2 are due to the bilayer bending away from the surface, creating a cap-shaped 3D structure. Barely visible dark spots are also observed in Fig. 3 *a*; these spots become bright (Fig. 3 *b*) until the focus is moved very high (Fig. 3 *e*). The behavior of these spots indicates that they are very small 3D structures. Due to the lack of a motorized z-stage, these images do not allow for precise measurements of the cap height; only an estimate can be made. The calculated depth of field of the objective used is $\sim$0.5 μm. The top of the large caps cannot be observed in Fig. 3 *a*; therefore, we conclude that the large caps are at least 0.5 μm in height.

Due to instrument resolution limitations, we were not able to acquire confocal images as clear as those obtained with epifluorescence microscopy; but since the height could be controlled, it was possible to reconstruct a 3D image of the rings (Fig. 3 *f*). These protrusions look like the caplets that have been previously predicted (9); here they will be referred to simply as caps. From the confocal images, the heights of the large caps were calculated to vary between $\sim$1 and 2 μm based on the diameter of the ring; the basic 3D shape of the ring is that of a hemisphere. An *x-y* confocal slice taken midway up the cap shows that the caps are hollow inside (Fig. 3 *g*).

Fluorescence recovery after photobleaching (FRAP) experiments were performed to determine if the lipids in the caps and the flat areas freely exchange. At all ionic strengths the lipids continue to be mobile across the entire surface. Fig. 4

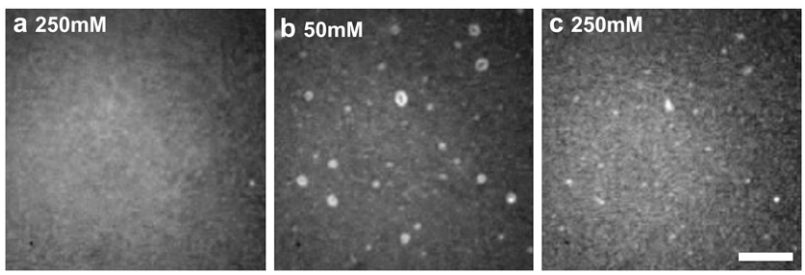

FIGURE 2 Epifluorescence images of a 69 mol % DOPC/30 mol % DOPA/1 mol % NBD-PC bilayer. The bilayer was formed in a 250 mM KCl, 50 mM MES, 0.1 mM EDTA, pH 5 solution. After formation, the excess vesicles were rinsed away and the bilayer imaged (*a*); the ionic strength of the outside solution was then dropped to 50 mM KCl (*b*), and raised back up to 250 mM KCl (*c*). A 100×, 1.3 NA objective was used to acquire the images. The scale bar represents 10 μm.

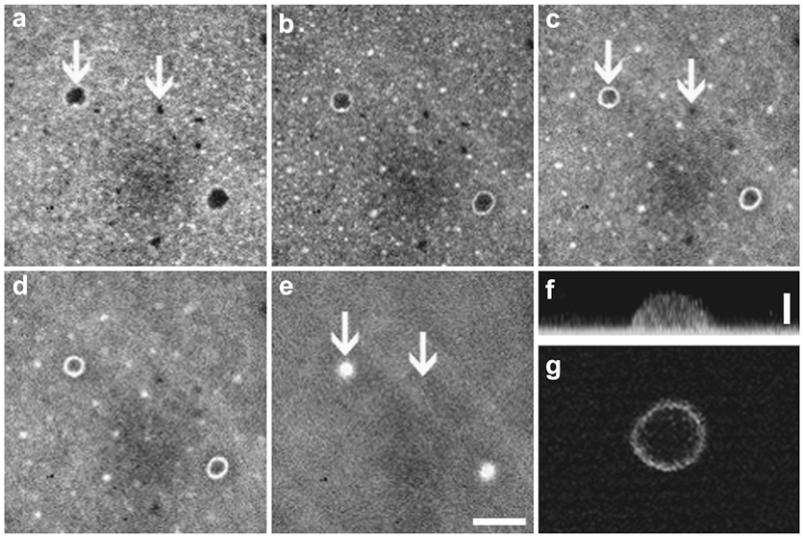

FIGURE 3 Epifluorescence (*a–e*) and confocal (*f* and *g*) images of two 69 mol % DOPC/30 mol % DOPA/1 mol % NBD-PC bilayers. The bilayers were formed in a 250 mM KCl, 50 mM MES, 0.1 mM EDTA, pH 5 solution. After the excess vesicles were removed, the outside bulk solution was exchanged to a 50 mM KCl, 50 mM MES, 0.1 mM EDTA, pH 5 solution and the images acquired. In the epifluorescence images (*a–e*), a 100×, 1.45 NA objective was used and the focus was adjusted to image through the caps. In the confocal images (*f* and *g*), a 3D reconstruction of a single cap is shown along with an *x-y* slice taken midway up the cap. The scale bars in *e* and *f* represent 10 μm and 2 μm, respectively.

shows an example FRAP recovery of a 69 mol % DOPC/30 mol % DOPA/1 mol % NBD-PC bilayer after the outside bulk solution was dropped to 50 mM KCl, 50 mM MES, 0.1 mM EDTA, pH 5. The lipids, including those in the caps, are clearly mobile over long distances.

The size and the number of caps change as a function of time. In Fig. 5, a bilayer composed of 69 mol % DOPC/30 mol % DOPA/1 mol % NBD-PC was monitored as a function of time after the ionic strength of the buffer was dropped to 50 mM KCl (Fig. 5, *a–i*). At short timescales, $<$1 min, it can be seen that some of the caps grow at the expense of others. Examining several samples we observe that, after 60 s, the number of caps reduces by $\sim$52%, whereas the average size grows by $\sim$20%. At longer timescales, many minutes, the caps gradually disappear with the smallest disappearing first. After 20 min the ionic strength of the buffer was raised back to 250 mM KCl, (Fig. 5 *j*), no further change was observed. Many bilayer samples have been continuously observed and evidence of cap detachment from the bilayer has not been seen. Thus, we do not think that the caps detach from the bilayer but rather that they collapse. If the large caps were to leave the surface, we would expect to see the creation of defect sites and these have not been observed. There is also no evidence of fluorescently labeled lipids floating in the bulk solution, further indicating the collapse and not detachment of the caps.

To examine the extent of the asymmetry needed to produce caps, bilayers of 69 mol % DOPC/30 mol % DOPA/1 mol % NBD-PC were formed in varying KCl solutions with 50 mM MES, 0.1 mM EDTA, pH 5. After the excess vesicles were rinsed away, the ionic strength of the outside solution was dropped directly to the amount indicated and the bilayers quickly imaged (Fig. 6). Below 50 mM the lipids no longer uniformly mix (L. R. Cambrea, F. Haque, J. L. Schieler, J. C. Rochet, and J. S. Hovis, unpublished); as a

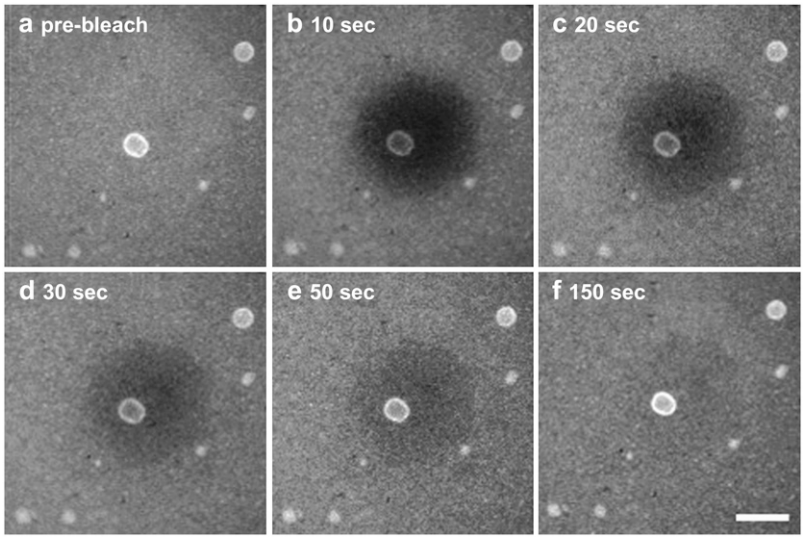

FIGURE 4 FRAP experiment showing that the lipids freely exchange between the flat and curved regions. The bilayer was composed of 69 mol % DOPC/30 mol % DOPA/1 mol % NBD-PC. The fusion buffer was 250 mM KCl, 50 mM MES, 0.1 mM EDTA, pH 5 solution. To form the caps, the outside bulk solution was exchanged to a 50 mM KCl, 50 mM MES, 0.1 mM EDTA, pH 5 solution. A 100×, 1.3 NA objective was used to acquire the images. The scale bar represents 10 μm.

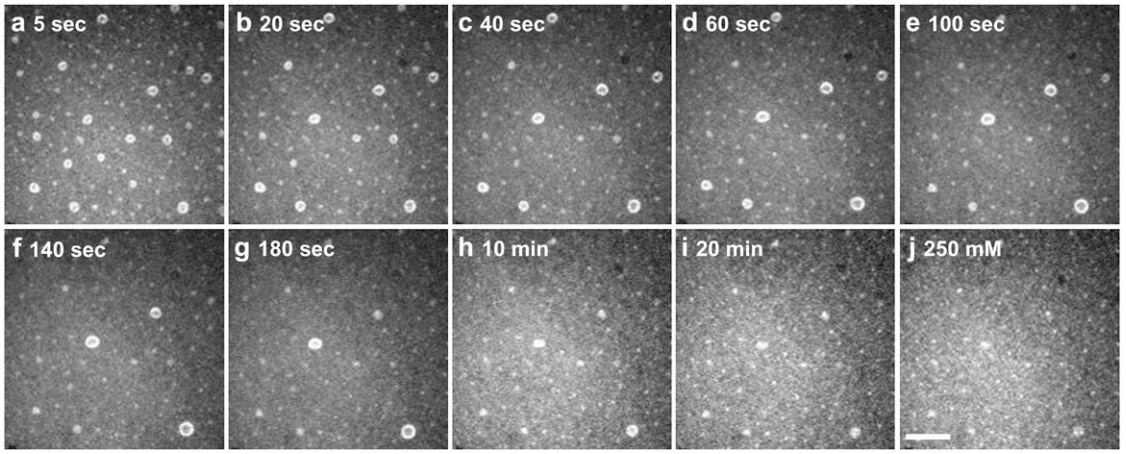

FIGURE 5 After the formation of caps, their size and number were followed as a function of time with epifluorescence microscopy (*a–i*). The bilayer was composed of 69 mol % DOPC/30 mol % DOPA/1 mol % NBD-PC. The fusion buffer was 250 mM KCl, 50 mM MES, 0.1 mM EDTA, pH 5 solution. To form the caps, the outside bulk solution was exchanged to a 50 mM KCl, 50 mM MES, 0.1 mM EDTA, pH 5 solution. After 20 min, the outside solution was returned to 250 mM KCl (*j*). A 100×, 1.3 NA objective was used to acquire the images. The scale bar represents 10 μm.

consequence, lower ionic strengths were not examined. It appears that an initial asymmetry of between 51–100 mM KCl is needed for the caps to form. As the asymmetry is increased, the number of caps increases.

The role of PA was investigated by examining bilayers containing 0−45 mol % DOPA in DOPC with 1 mol % NBD-PC. Attempts to form bilayers containing more than 45 mol % DOPA were unsuccessful. Again the vesicles were formed and fused in a 250 mM KCl, 50 mM MES, 0.1 mM EDTA, pH 5 solution; after fusion the excess vesicles were rinsed away and the ionic strength dropped to 50 mM (Fig. 7). From this figure it is clear that a certain amount of DOPA, somewhere between 11–20 mol %, is required for caps to form. The effect of the fluorophore has also been examined. The NBD-PC was varied between 0.5–2 mol %, and it was also replaced with 1 mol % Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine with no effect on cap formation observed.

DISCUSSION

The key observations from the Results section can be summarized as follows:

1. Cap formation requires both PA and a certain asymmetry in KCl concentration.
2. Caps disappear either with time or when the KCl concentration is returned to starting levels.

To account for these observations the following model is proposed: First, the asymmetry in ionic strength provides the trigger for cap formation. As indicated in Fig. 1, the curvatures of the monolayers are no longer equal in magnitude, opposite in sign. The increase in headgroup area of the PA in the upper leaflet decreases the curvature of that leaflet, and the bilayer bends away from the support. Second, osmotic pressure differences between the water in the nascent cap and the external environment further drive cap formation. The large volume expansion means that the asymmetry in ionic strength, which triggered the cap formation, is eliminated. LUVs are typically formed by nonequilibrium methods, and over time they equilibrate back to multilamellar, liquid-crystalline structures. Similarly, we propose that the caps form by a nonequilibrium process and their disappearance over time is reequilibration to the original state. Lastly, due to osmotic effects, the caps disappear when the external solution is returned to starting conditions. The salt concentration inside the cap is $\ll$250 mM; consequently, when exposed to a 250 mM KCl external solution more water will flow out of, than into, the caps, causing them to shrink and collapse. This model and the factors that have been left out of the model will be discussed in further detail below.

For lipids to leave or bulge away from the surface, the bilayer-support interaction needs to be overcome. Lipids are held close to the support through a combination of hydration, electrostatic, steric, and van der Waals forces (11–13). The interaction is significant; for example, it dampens out the undulations that free bilayers undergo. Lipids in supported bilayers have previously been observed to move into three dimensions (14). In these experiments, gel phase bilayers were heated above the transition temperature, and tubules were observed to leave the surface; unlike our experiments, these were irreversible. We hypothesize the bilayer-support interaction keeps bilayers with $<$20 mol % PA (Fig. 7) or those exposed to a small asymmetry in ionic strength (Fig. 6) from forming caps. In our work with anionic membranes, we have exposed several other compositions to asymmetric screening environments; these include 0, 20, 30, 40, and 50 mol % egg phosphatidylglycerol in egg PC and 0, 30, and

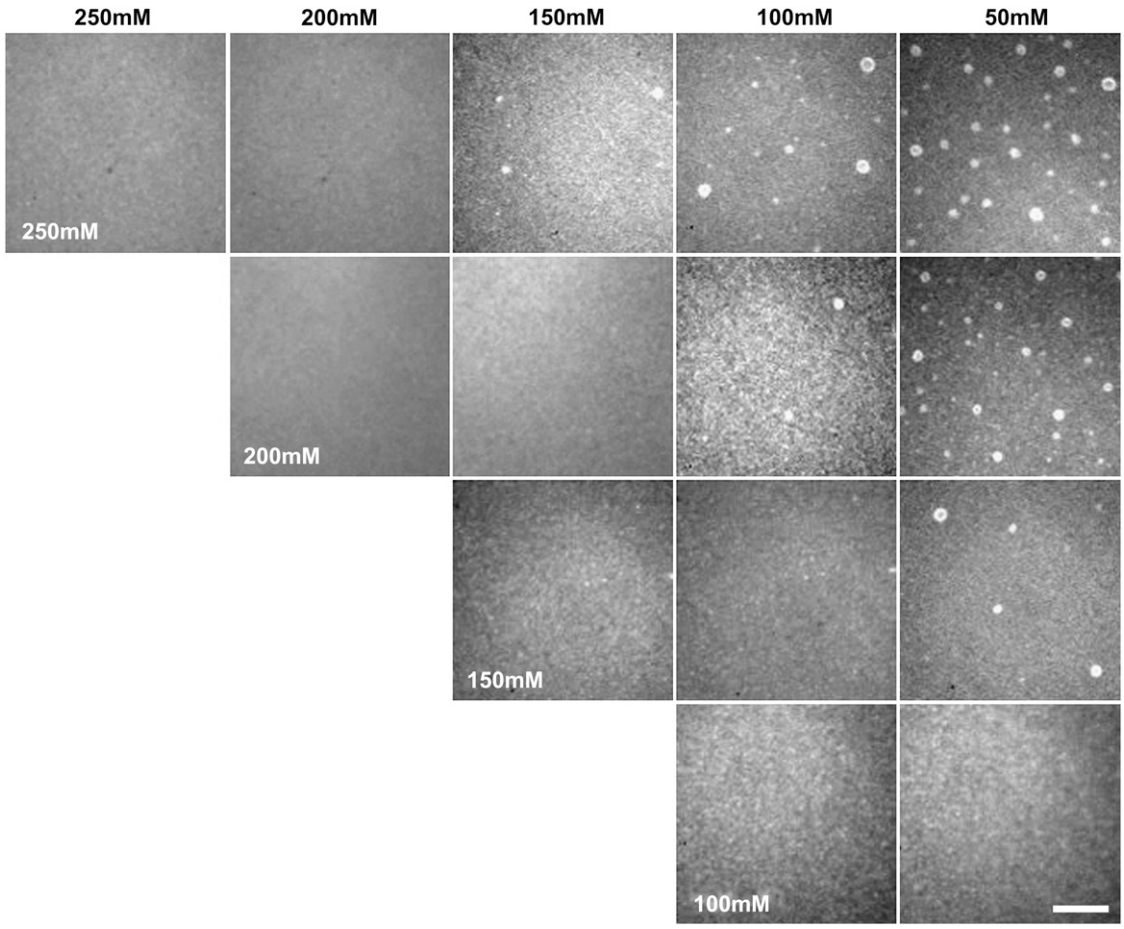

FIGURE 6 Effect of asymmetry on the size and number of the caps was examined using epifluorescence microscopy. Bilayers of 69 mol % DOPC/30 mol % DOPA/1 mol % NBD-PC were formed by vesicle fusion in buffers containing varying amounts of KCl, 250 mM (*top row*) to 100 mM (*bottom row*), with 50 mM MES, 0.1 mM EDTA, pH 5. After the excess vesicles were rinsed away, the bulk outside solution was exchanged to varying amounts of KCl, 200 mM (*left side*) to 50 mM (*right side*), with 50 mM MES, 0.1 mM EDTA, pH 5. The bilayers were rapidly imaged after solution exchange. A 100×, 1.3 NA objective was used to acquire the images. The scale bar represents 10 μm.

50 mol % POPG in POPC. None of these bilayers were observed to bend away from the support. The effect of ionic strength on the shape of a lipid is difficult to determine; however, PA is thought to be particularly sensitive (7). Given that we need at least 20 mol % of PA and a significant asymmetry, it is not unsurprising that other anionic membranes do not show shape transformations into three dimensions. Nevertheless, it should be possible to find other lipid mixtures that will bend away from the surface.

Bilayers are able to expand in the fluid phase but only by a few percent (12). To confirm that the expansion observed in these experiments is within an expected range, the extent of area expansion was calculated. In all cases it was found to be a few percent at most. In Fig. 5 *d*, for example, a ~3% expansion has occurred. The details of this calculation can be found in the Supplementary Material. When considering the area expansion, the question arises as to why discrete caps form instead of the entire bilayer bending slightly. As discussed, the drop in ionic strength increases the headgroup area of the PA in the upper leaflet (7,15,16), which in turn increases the asymmetry in the area of the monolayers. The area asymmetry is ''global'' in nature (17): it averages over the entire bilayer and would not be expected to result in the formation of discrete regions of curvature. A local spontaneous curvature, however, can be created if there is any inhomogeneity in the composition—either within a leaflet or between leaflets, as long as the different lipids have different geometries (1). The conditions applied in this work will certainly create an inhomogeneity in PA geometry between the leaflets. As the bilayers are fluid, an inhomogeneity within a leaflet could also be created. The NBD-PC appears uniform in the caps; however, due to the limits of optical microscopy it is impossible to say for certain. An interesting question for future work would be to determine whether the composition of the caps is different from the composition of the flat area.

In Fig. 1, the PA lipids are shown to be evenly distributed between the leaflets. This may not be the case; the glass

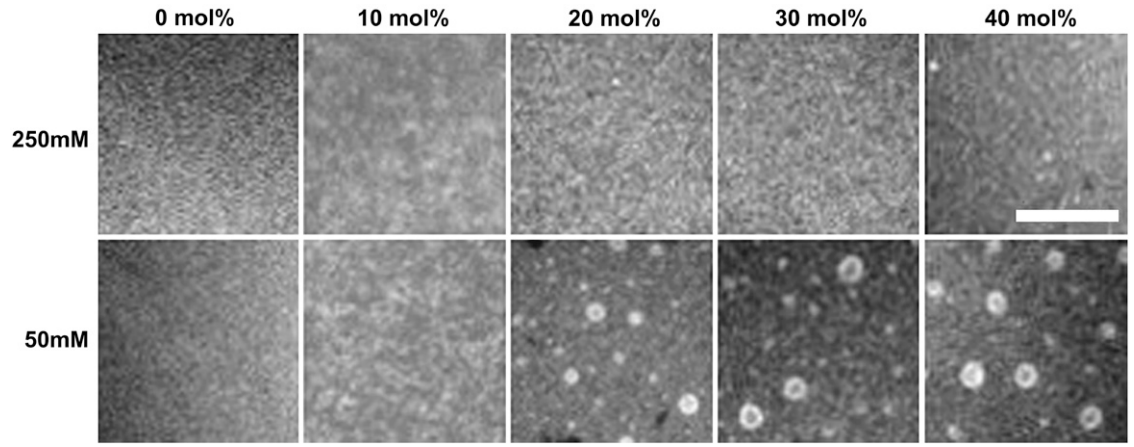

FIGURE 7 Effect of composition on cap formation was examined using epifluorescence microscopy. Bilayers containing 0–40 mol % DOPA in DOPC with 1 mol % NBD-PC were formed by vesicle fusion in a 250 mM KCl, 50 mM MES, 0.1 mM EDTA, pH 5 solution (*top row*). To form the caps the outside bulk solution was exchanged to a 50 mM KCl, 50 mM MES, 0.1 mM EDTA, pH 5 solution (*bottom row*). A 100×, 1.3 NA objective was used to acquire the images. The scale bar represents 10 μm.

surface is negatively charged, which may result in there being more PA in the top leaflet than the bottom (18). Even if this is the case, the bilayer will still want to bend when the external ionic strength is lowered. It may, however, be less likely that the bilayer-support interaction will be overcome since there is less PA in the bottom leaflet to induce the curvature. The pK_a of PA is sensitive to ionic strength (19). As the ionic strength is lowered, some of the PA^- may become $PA^{\varnothing}$, in which case their shape will not change. Again, this may make it less likely the bilayer will overcome the bilayer-support interaction; but as long as there are more PA^- in the lower leaflet than $PA^{\varnothing}$ in the upper leaflet, the bilayer will want to bend away from the support.

If a solution is separated from pure solvent by a semipermeable membrane, more solvent will move in one direction across the membrane than the other until equilibrium is reached. Closed membranes, vesicles, can withstand an osmotic pressure difference that depends on the material properties and inversely on the radius of the vesicle. For example, small vesicles, ~50–100 nm, can withstand osmotic pressure differences of hundreds of millimolar before the net flux of water across the membrane is no longer zero (20–23). When this pressure is exceeded, up-shocked vesicles shrink, whereas down-shocked vesicles expand slightly before a pore opens (24). A free-standing bilayer (radius of infinity), in contrast, will have a nonzero net flux of solvent no matter how small the concentration of solute. Supported bilayers are more complicated. For one, they are held to the support through a variety of interactions. Additionally, the water layer between the bilayer and the solid support contains both ''fluid-like'' and ''ice-like'' water (25): some of the ions will be in the ''ice-like'' region, whereas others will be in the ''fluid-like'' region. It is unclear then whether, like closed lipid bilayers, supported lipid bilayers can withstand a significant solute concentration difference.

Measurements of the thickness of the water layer between the bilayer and support places it at ~5–15 nm (25–29). Assuming, for a moment, that a supported bilayer is similar to a free-standing bilayer (in that it can withstand zero osmotic pressure), it might be expected that the DOPC bilayer in Fig. 7 would have lifted off the surface. The fusion buffer was 250 mM and the outside buffer was 50 mM KCl; to equilibrate, the volume of water between the bilayer and support would need to increase fivefold. This increase in volume would significantly disrupt the bilayer-support interaction. That the bilayer is not observed to disappear or dramatically alter in any way suggests that such a large increase in volume does not occur. Whether this volume change does not occur because the bilayer can withstand the concentration difference or because pores opened in the membrane is unclear at this time.

In the case where caps form, there is clearly a net flux of water from the bulk to the inside. The caps form with 1 s—a reasonable time given the permeability of the membrane to water, the concentration difference, and the area. A significant dilution occurs when a cap forms. Likewise, when the external concentration is raised, a net flux of water is again created, this time outward, causing the caps to shrink. In Fig. 5, it was observed that on short timescales small caps disappear at the expense of large ones; this is simply Ostwald ripening (the pressure inside the small caps is larger than the pressure in the large caps, making them thermodynamically unstable).

A few notes on Fig. 6, which we have not discussed in detail. First, the observed asymmetry required for cap formation is between 51–100 mM KCl. Changes in lipid headgroup area are expected to scale with the Debye screening length; therefore, we would not initially have predicted a consistent range of values. Whereas curvature effects provide the reason for bending, osmotic effects are what cause the caps

to grow. It is possible that this ionic strength asymmetry is reporting on the extent of osmotic pressure difference a supported bilayer can handle. Above this value, a net water flux is created. Second, the greater the asymmetry, the more numerous the caps. If the caps arise because of a transient compositional inhomogeneity, then the larger the curvature asymmetry between the leaflets, the less pronounced the inhomogeneity needs to be to form a cap. Consequently, there are more locations on the surface where a cap can form.

The shape transformation process described in this work is a highly complex problem. We have presented a simple model that captures the essential aspects of the observed results. A more detailed model would require several pieces of information that we do not currently have: the strength of the bilayer-support interaction, the osmotic pressure that a supported bilayer can withstand, the precise curvature of PA at the different KCl concentrations, and the asymmetry in the PA distribution between leaflets. Work is ongoing to determine these factors and develop a more detail model.

Supported lipid bilayers with flat and curved regions may prove useful for studies probing the effects of curvature on protein binding/function. They provide a unique surface where direct, single sample comparisons can be made between flat and curved regions. Although the asymmetry induced curvature shown in this work is not long lived, $\sim$20 min, for some experiments it may be enough. By further increasing the asymmetry, larger caps can be created that are stable over longer periods of time. Additionally, the short-lived samples might be of utility to assess the ability of certain proteins to stabilize curvature.

CONCLUSIONS

It has been shown that supported lipid bilayers containing DOPA and DOPC can be induced to bend away from the solid support when exposed to asymmetric screening environments. The lipids in the curved region are connected to those in the flat regions and exchange freely. Small caps disappear rapidly, due to Ostwald ripening, and then over many minutes the large caps disappear due to reequilibration. In the conditions examined here, DOPA is required for cap formation. When bilayers contain 30 mol % DOPA, an asymmetry of 51–100 mM KCl is required for cap formation. The bilayers bend due to the asymmetry in the leaflet curvatures that arise from each leaflet being exposed to different screening environments. Osmotic pressure differences across the bilayer help to fill the caps.

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

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