Yanfei Jiang

Department of Biochemistry and
Molecular Biophysics,
School of Medicine,
Washington University,
St. Louis, MO 63110

Kenneth M. Pryse

Department of Mechanical Engineering
and Materials Science,
Washington University,
St. Louis, MO 63110

Srikanth Singamaneni

Department of Mechanical Engineering
and Materials Science,
Washington University,
St. Louis, MO 63110

Guy M. Genin¹

Department of Mechanical Engineering
and Materials Science,
Washington University,
St. Louis, MO 63110;
NSF Science and Technology,
Center for Engineering Mechanobiology,
Washington University,
St. Louis, MO 63110
e-mail: genin@wustl.edu

Elliot L. Elson¹

Department of Biochemistry and
Molecular Biophysics,
School of Medicine,
Washington University,
St. Louis, MO 63110
e-mail: elson@wustl.edu

Atomic Force Microscopy of Phase Separation on Ruptured, Giant Unilamellar Vesicles, and a Mechanical Pathway for the Co-Existence of Lipid Gel Phases

Phase separation of lipid species is believed to underlie formation of lipid rafts that enable the concentration of certain surface receptors. However, the dynamics and stabilization of the resulting surface domains are unclear. We developed a methodology for collapsing giant unilamellar vesicles (GUVs) into supported bilayers in a way that keeps membrane nanodomains stable and enables their imaging. We used a combination of fluorescence and atomic force microscopy (AFM) of this system to uncover how a surprising phase separation occurs on lipid vesicles, in which two different gel phases of the same lipid co-exist. This unusual phase behavior was evident in binary GUVs containing 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC) and either 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) or 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC). The approach showed that one of the phases is stabilized by lipid patches that become ejected from the membrane, thereby enabling the stabilization of what would otherwise be a thermodynamically impossible coexistence. These results show the utility of AFM on collapsed GUVs, and suggest a possible mechanical mechanism for stabilization of lipid domains. [DOI: 10.1115/1.4043871]

Keywords: giant unilamellar vesicles, lipid domains, fluorescence microscopy, atomic force microscopy, supported lipid bilayers, GUV rupture, lipid rafts

1 Introduction

The heterogeneity of cell membranes is generally believed to play important roles in a variety of cellular processes, although very little is known regarding the underlying physical basis by which the membranes accomplish these functions [1]. A current and popular hypothesis is that cell membranes contain dynamic submicrometer “lipid raft” domains that are enriched in certain kinds of lipids and protein species and phase separated from the rest of the membrane [2]. Since the earliest studies of phase separation on membranes, binary mixtures of lipids having long saturated acyl chains together with lipids having short or unsaturated chains have proven to be important model systems [3,4]. Upon cooling these binary systems from a state in which the two lipid components are uniformly mixed in a fluid phase, two phases emerge.

The lipid with the higher transition temperature appears as an ordered solid (gel) while the lipid with the lower transition temperature remains in a disordered liquid state. Ternary mixtures containing the above two lipid species and cholesterol or other sterols can allow the emergence of a new phase: a liquid-ordered

phase. The liquid-ordered phase is believed to have lateral order similar to that in a liquid disordered phase, but configurational order of the hydrocarbon chains more similar to that in a gel phase due to the lipid–cholesterol interaction [5]. Lipid rafts are generally believed to be in the liquid-ordered state [2,6,7].

In studying these systems, we observed an unusual set of phases that emerged in a binary lipid system: a binary mixture that permitted what appeared to be two coexisting gel phases. This is a thermodynamic impossibility from the standpoint of mixture theory, which prompted us to search for a mechanical explanation. By developing a technique for forming these phases in a model lipid system known as a giant unilamellar vesicle (GUV) and then preserving them while collapsing them on to a supported lipid bilayer, we were able to use atomic force microscopy (AFM) of the supported bilayer to infer the topography of the phases in a GUV, and propose a mechanism for mechanical stabilization of two separate gel phases in a binary lipid system.

1.1 Background. Motivated in part by the supposition that micron-sized domains in binary and ternary lipid systems serve as models for these nanoscopic lipid rafts, micron-sized lipid domains have long been studied in model membranes [6,8–11]. These can persist in a stable or metastable state over times sufficiently long to be relevant to physiologic processes. However,

¹Corresponding authors.

Manuscript received December 10, 2018; final manuscript received May 26, 2019; published online June 13, 2019. Assoc. Editor: Victor H. Barocas.

lipid rafts, if they exist, are nanoscopic. It is not clear whether simple lipid nanoscopic phase domains can persist for times long enough to be relevant physiologically and if so, what forces stabilize the domains at the nanoscopic range. This motivates a broad range of experimental and theoretical studies of model lipid membrane systems [12–15]. Several potential factors have been proposed, including entropic force and mechanical repulsion caused by the difference of the intrinsic curvature of different phases [1,16,17].

Several model systems are in widespread use to study membrane heterogeneity and phase separation. These include supported monolayers or bilayers, and synthetic vesicles, all of which can be synthesized with controlled compositions [6,18]. Among these, giant unilamellar vesicles (GUVs) are particularly promising. First, compared to other vesicle models, their size range falls into that of mammalian cells (tens of micrometers). This is important because one driving force of phase separation relates to differences between the curvature of a membrane and the curvature that a lipid species would adopt in the absence of mechanical constraint [12,13,17]. Second, compared to supported lipid monolayers and bilayers, lipid molecules diffuse more freely on vesicles due to the absence of lipid–substrate interactions. Indeed, phase separation behavior observed on supported bilayers differs from that observed on GUVs [19].

As reviewed elsewhere [1], a broad range of characterization technologies have been applied to image micro- and nanodomains on model membranes. Phase separation can be visualized directly with either AFM [20,21] or fluorescence microscopy [8,9]. AFM has the advantages of nanoscale resolution and the detection of thickness differences among membrane phases. Fluorescence microscopy, which involves lipid probes with different affinities to the different phases, allows for color rendering of phase behavior over large regions and, in principle, for application of statistical fluorescence fluctuation technologies to characterize nanodomain dynamics [6,8,9,22,23].

However, each of these methods and models has limitations. Phase separation in GUVs has been rarely studied using AFM due not only to routine challenges associated with probing compliant structures [24] such as a lipid bilayer on a GUV, but also to additional challenges associated with thermal fluctuations of the GUV [1]. Fluorescence fluctuation methods can reveal much about phase domain dynamics, but do not have the combination of spatial and temporal resolution needed to provide details sufficient to image mechanisms that might contribute to stability of nanodomains [1]. We, therefore, explored whether these limitations could be overcome by more straightforward AFM characterization of phases on GUVs that have been collapsed into a lipid bilayer.

1.2 Overview. In this study, we found that the patterns and shapes of gel domains in phase separated GUVs, as observed using fluorescence microscopy, persisted in the membrane adherent to a glass surface after the GUVs were ruptured. This enabled further AFM measurements on these domains. We applied this method to two binary systems: GUVs consisting of a mixture of 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC) and either 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) or 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC). These two systems are unusual in that they present two different gel phases that appear to exist in equilibrium with one another. One of these gel phases recruits a high concentration of the fluorescent probe 1,1'-diicosanyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, also known as DiI-C₂₀ (DiI), but excludes the fluorescent probe Bodipy-HPC, while the other gel phase excludes both of these probes. The DiI labeled phase appears as an interphase enclosing the second gel phase and separating domains of the second gel phase both from each other and from the outside liquid disordered phase.

Because standard mixture theory rules out the possibility of two solid phases co-existing in a binary system in equilibrium via the Gibbs phase rule [25], we sought a mechanical explanation. We

used a combination of AFM, fluorescence microscopy, and fluorescence photobleaching recovery to arrive at the conclusion that the co-existence of two gel phases can be made possible by mechanics.

2 Materials and Methods

2.1 Lipid and Giant Unilamellar Vesicle Preparation.

Giant unilamellar vesicles were synthesized from lipids (Avanti Polar Lipids, Alabaster, AL), Bodipy-HPC fluorescent dye (Molecular Probes, Eugene, OR, number D3803), and DiI-C₂₀ (Molecular Targeting Technologies, West Chester, PA). Lipids were stocked at 10 mM in chloroform, DiI-C₂₀ in ethanol at 0.1 mM, and Bodipy-HPC in ethanol at 0.5 mM. GUVs were electroformed [10]. Briefly, 3 μ l DSPC (or DPPC), 7 μ l DLPC, 1 μ l Bodipy-HPC, and 1 μ l DiI-C₂₀ stock solutions were mixed and deposited onto two platinum wires by dragging the lipid solution along the wires back and forth until the chloroform and ethanol dried. The two wires were then placed into a homemade Teflon cylinder chamber filled with 300 mM sucrose solution. The chamber cap had two holes to hold the wires and separate them at a distance of 2 mm. The chamber was next placed upon a heating stage which maintained a temperature of 70 °C. The wires were then connected to a function generator providing a 10 Hz/1.5 V square waveform for 1–2 h.

2.2 Fluorescence Confocal Microscopy.

After the GUV solution cooled to room temperature, it was transferred from the Teflon chamber to an eight-well chamber slide (Lab-Tek) or glass cover slip (Fisher No. 0) with 300 μ l distilled water or 300 mM glucose. Fluorescence confocal microscopy was performed on a LSM 510 ConfoCor 2 Combination System (Carl Zeiss, Germany) based on a Zeiss Axiovert 200 M inverted microscope. A C-apochromatic 40 $\times$ water immersion objective (numerical aperture 1.2) was used.

Fluorescence photobleaching recovery (FPR/FRAP) experiments were conducted to determine the stability and mobility of lipid species. An image was first taken with 5% laser power. Then, a predetermined region was photobleached by continuous scan with 50% laser power for 30 s before switching the system back to the normal imaging mode. Fluorescence intensity under 5% laser power was then recorded every 2 s over the predetermined region. Control regions adjacent to these predetermined regions were also monitored every 2 s over the course of fluorescence recovery.

2.3 Atomic Force Microscopy.

Atomic force microscopy scans of soft lipid bilayers are now straightforward to perform, thanks to a strong body of literature on this subject (e.g., see Ref. [20]), which builds upon the body of knowledge for AFM of soft matter [26–30]. The goal of this work was to collapse GUVs into lipid bilayers that preserved gel phases and enabled study of the structures of these phases by AFM. To achieve this, an asylum Research MFP-3D-BIO (Asylum Research, Goleta, CA) device housed in our laboratories was employed for AFM imaging. The imaging was performed in light tapping mode (set point ratio was maintained above 0.9).

The cantilever used had a resonant frequency of 10 kHz, a spring constant of 0.02 N/m, and a four-sided pyramidal tip with a radius of 40 nm (iDrive Magnetic Actuated Cantilever, Asylum Research, Goleta, CA). Note that although it is fully appropriate to report the free oscillation amplitude of the cantilever as well, it is typically not reported for measurements such as this because accurate measurement requires performing force–distance measurement against a hard surface. Especially for cantilevers appropriate for interrogating soft matter, the likelihood of compromising subsequent measurements by damaging the cantilever typically outweighs any benefit derived from precise measurement of the free oscillation amplitude of the cantilever. Scanning parameters were adjusted dynamically to obtain the best quality

images, but typically began with the following values: drive amplitude 1.44 V; drive frequency: 7.63 kHz; integral gain: 9.36; feedback filter: 1.00 kHz. The scanning rate was set to 1 Hz.

The AFM head was mounted on an Olympus X711 microscope housed in our laboratories. Fluorescence images were taken using a 60× Olympus oil objective. To image the lipid layers, a cover glass (Fisher No. 0) was first cleaned using Fisher lens cleaning paper and then placed onto the microscope. Next, 200 μ l 300 mM glucose solution was pipetted onto the cover glass. A 50 μ l GUV solution was then transferred from the Teflon chamber to the glucose solution on the cover glass. After 15–30 min, the cover glass was gently rinsed using water. About 200–400 μ l of water were left after the rinsing step. The collapsed GUVs were thus imaged under conditions of aqueous immersion.

Finally, the AFM head was mounted on the microscope stage for AFM measurements, and imaging procedures were adopted that followed long-established, published protocols [31]. Optical focus was first achieved before lowering the AFM cantilever. The AFM cantilever was then lowered in three steps. First, the cantilever was lowered into the water covering the collapsed GUVs. Second, it was further lowered until it approached sufficiently close to the surface to appear in the field of view of the microscope. Thereafter, the cantilever was lowered to touch the sample while monitoring the sum and deflection readings.

3 Results and Discussion

3.1 Lipid Domain Structures on Giant Unilamellar Vesicles Remained Stable During and Following Giant Unilamellar Vesicle Rupture. Upon cooling binary GUVs, confocal fluorescence microscopy revealed that the lipids on the GUVs formed two distinct gel phases in addition to the liquid phase (Fig. 1). The Gibbs phase rule indicates that this should not be possible. These two gel domains shared many similarities with patch and stripe domains reported previously and which we also observed in DLPC/DPPC membrane systems [11,21]. As reported by Li et al. [11], the patch domains excluded both Bodipy-HPC and fluorescent labels of DPPC, while the stripe domains attracted high concentrations of the fluorescent labels for DPPC and low concentrations of Bodipy-HPC. The DLPC/DPPC patch and stripe domains differed from those observed on DLPC/DSPC GUVs: the domains that accumulated labels for DPPC in the DLPC/DPPC GUVs were stripe-like and had smooth edges [11], while the red

domains in the DLPC/DSPC GUVs were irregular with rough edges (Fig. 1(a)). Therefore, we termed the dye-rich gel domains “bright domains” in the following to distinguish them from the patch-like “dark domains.” Bright domains tended to form on the edges of dark domains in DLPC/DSPC GUVs, potentially affecting the latter both thermodynamically and kinetically.

The GUVs studied contained 300 mM sucrose (342 g/mol) solution and therefore sedimented to the glass slide when transferred to a lower density solution of 300 mM glucose (180 g/mol) or water. There, some of these GUVs ruptured after contacting the glass and spread over the glass surface. We found that the patterns and shapes of the gel domains in DLPC/DSPC GUVs were preserved after the GUV ruptured (Fig. 1(b)). The bright domains remained around the edges of the dark domains and retained their irregular shapes.

Fluorescence photobleaching recovery experiments (FPR/FRAP [32]) revealed that the domains were stable after collapse of the GUVs (see Fig. S1 available in the [Supplemental Materials](#) on the ASME Digital Collection).

3.2 Atomic Force Microscopy Studies Revealed Structural Differences Between the Two Gel Phases. We compared AFM scans of DLPC/DSPC GUVs conducted in tapping mode to fluorescence images to search for structural differences between these two different gel domains (Fig. 2). For DLPC/DSPC GUVs, the two were broadly similar. However, AFM scans revealed what appeared to be “nanodomains” in the liquid phase area that did not appear in fluorescence images (Figs. 2(b) and 2(c), line 1). These nanodomains, several hundred nanometers in diameter and about 2 nm thicker than the surrounding membrane (Fig. 2(c), line 1), were also observed in pure DLPC membranes (see Fig. S2 available in the [Supplemental Materials](#) on the ASME Digital Collection), suggesting that these were not composed of DSPC. To control against the possibility of the nanodomains being contaminants, we scanned the glass surfaces under identical conditions. These scans showed no contaminants of such uniform height (see Fig. S3 available in the [Supplemental Materials](#) on the ASME Digital Collection). Taken together, the results supported the hypothesis that the nanodomains were DLPC bilayers embedded in a surrounding monolayer membrane. These could arise from the curvature of the glass, or possibly from inhomogeneity of the hydrophilicity of the glass surface.

Fig. 1 Confocal images of a binary DLPC/DSPC GUV and ruptured GUV on a cover slip. GUVs were labeled with Bodipy-HPC (dark gray; green online) and DiI-C₂₀ (light gray; red online). The pictured GUVs, made of 30% DSPC and 70% DLPC, show unusual phase behavior: although the GUVs have only two components, three phases are visible in this image, which is an apparent violation of the Gibbs phase rule.

Fig. 2 AFM measurements on ruptured GUVs: (a) fluorescence image of domains on which AFM scanning was performed. Images were taken using a standard fluorescence microscope instead of the confocal microscope used for Fig. 1, resulting in a lower resolution; (b) height profiles from the domains shown in panel A; (c) height profiles along the four lines shown in panel B. The origins of the curves (left) correspond to the numbered ends of lines shown in panel B. Cartoons of colored lipid molecules correspond to the hypothesized stacking of lipid layers (lightest gray (orange online): DLPC monolayer; second lightest gray (pink online): DLPC bilayer; second darkest gray (red online): DSPC bilayer, dark domain; darkest gray (blue online): DSPC bilayer, bright domain). (d) Magnified image from panel B, with contrast changed to better illustrate the topography. (e) Color rendering of the topography in panel D. Colors match those of panel C.

We next proceeded to characterize the topographies of the two unusual gel phases. The height profile of a line drawn from the monolayer liquid phase to the inside a dark domain revealed a stair topography of two steps (line 2 in Figs. 2(b) and 2(c)). The first step was about 2 nm high, and the second step was about 1.8 nm high. From comparison to the fluorescence images, the first step was interpreted as a progression from the DLPC monolayer to a bilayer, and the second step as a progression from a DLPC bilayer to a DSPC bilayer. The height difference of 1.8 nm is consistent with published thickness differences between DLPC and DSPC bilayers studies via AFM [20].

In contrast to the dark domains that were connected directly to the DLPC liquid phase, the bright domains were found to consist of an extra layer of membrane residing atop the remainder of the membrane. This surprising conclusion was reached based upon three observations. First, the height difference between the DLPC monolayer and the bright domains was around 8 nm, which was too thick to be explained by a new phase of a bilayer (Figs. 2(b) and 2(c), line 3). Second, the bright domains could be knocked off of the dark domain by progressively applying a scanning AFM tip in tapping mode (Fig. 3(a)). The loss of the bright domains was much faster with contact mode scanning (data not shown). This was confirmed by fluorescence microscopy before and after experiments designed to knock off the bright domains (Fig. 3(b)). Third, the height measured via AFM changed with that of the lipids supporting the bright domains. For example, as a bright domain extended from a DLPC monolayer to a DLPC bilayer (second blue point in Figs. 2(b) and 2(c)), its thickness remained at 8 nm (third blue point) while the overall height rose 2 nm. This

further suggested that the bright domains could retain their cohesion over height steps greater than those found at the interface between DSPC and DLPC bilayers, and that, although they existed in the gel phase, they were sufficiently compliant in shear to follow the contour of the lipids beneath them.

The thickness of the bright domains (8 nm) in our study was large but consistent with thickness measurements reported in the literature for bilayers supported atop other bilayers. We note a broad range of membrane thicknesses reported from AFM experiments in the literature, and propose that they can be reconciled as follows. AFM measurements are reported for both membrane layers supported directly by a mica substrate and for additional membrane layers atop such membrane layers. We found that the thicknesses reported for second layers of membrane are between 6 and 9 nm and much thicker than those reported for a first layer, which are between 3 and 5 nm [20,33,34]. Although the reasons for this are not clear, we suspect different water layer thicknesses between membrane/membrane and membrane/substrate interfaces, and note that our measurements are consistent with the former.

It can also be noted in Figs. 2(b) and 3(a) that there are some patches sitting on the top of dark domain that appear to be the same as the edge domains. However there are three differences that we have found. First, they do not have as much of both dyes as the edge domain. This is especially evident in Fig. 3(a). Although they cover a significant area over the bottom domain, the fluorescence image still shows this area is dark. Second, the height of these patches is about 6 nm, which is about 2 nm smaller than the edge domains (Fig. 2(b), line 4). Third, these patches

Fig. 3 Bright domains on DLPC/DSPC collapsed GUVs could be knocked off by an AFM tip. (a) Height images from a continuous scanning. The scale bar is for the first four images. For the last two images, showing the domain that appears on the left side in the first four images, the scale bar indicates 6 μm . Note that these figures have been modified using interpolation tools in the software package Gwyddion (Department of Nanometrology, Czech Metrology Institute); unmodified images can be found in Fig. S4 available in the Supplemental Materials on the ASME Digital Collection. (b) Fluorescence images (DiI-C₂₀) of the domains before and after the AFM scanning. The scanning area is indicated by the black square, which is 15 μm by 15 μm .

progressively change to a round shape under continuous tapping mode scanning (Fig. 3(a)); in contrast, the bright edge domains remained stable under tapping mode scanning.

3.3 The Edge Domains are Not Artifacts Produced by the Fluorescent Lipid Probes. The bright domains have a high concentration of DiI, posing a question of whether the edge domains are simply aggregated DiI around the dark domains surrounding DSPC gel. To control for this possible artifact, GUVs were made without DiI and only labeled with Bodipy-HPC. AFM experiments performed on these ruptured GUVs showed that the 8 nm edge features were still present around the dark domains (Fig. 4). This suggested that the edge domains were neither aggregations of DiI nor artifactual phases induced by DiI. Instead, the two-gel-phase separation appears to be intrinsic to the DLPC/DSPC membrane.

One alternative hypothesis to explain these data is that patches of lipid that exist above the main bilayer can fluoresce measurably only if larger than a critical size. Indeed, several patches in the height profile image of Fig. 2 between profile scan lines 2 and 4 have the same height as those of the DiI labeled phase, but do not emit measureable fluorescence. These patches are similarly visible

in phase imaging (cf. Ref. [35]). However, the domains in the dashed box on the left-hand side in Fig. 2(b) are of the same size as the domains in the dashed box on the right-hand side in Fig. 2(b), but the domains in the left-hand dashed box do not emit measureable fluorescence in Fig. 2(a) while those in the

Fig. 4 Height image of a dark domain on a ruptured DLPC/DSPC GUV membrane. The GUV was labeled only with Bodipy-HPC. The image shows that the second gel phase was not dependent upon DiI-C₂₀.

right-hand dashed box do. This observation falsifies that alternative hypothesis and instead supports the hypothesis illustrated in Fig. 2(c).

This conclusion extends that of Loura et al. [22] who performed fluorescence lifetime and Förster resonance energy transfer measurements on DiI in a DLPC/DSPC membrane. Their results indicate the segregation of DiI into the gel/fluid interface, but they were not able to test this hypothesis on the small vesicles they employed. By showing interfacial domains with a high concentration of DiI, our confocal images of GUVs provide direct support of their conjecture that DiI might segregate into the gel/fluid interface. However, the dimension of interfacial domains we have observed is wider than a single molecular interface layer between the phases.

The application of AFM to collapsed GUVs enabled this to be explored more deeply and showed that instead of being just a single boundary layer of DiI, there is in fact a distinct gel phase that is rich in DiI.

3.4 The 1,2-Dilauroyl-sn-Glycero-3-Phosphocholine/1,2-Distearoyl-sn-Glycero-3-Phosphocholine System Differs Fundamentally From the 1,2-Dilauroyl-sn-Glycero-3-Phosphocholine/1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine System. Although this is the first report of two gel phases coexisting in the DLPC/DSPC system, a similar phenomenon has been reported in a DLPC/DPPC system in both GUVs [11] and supported lipid bilayers [21]. However, several differences were evident between these two membrane systems. As discussed above, the bright domains in DLPC/DPPC GUVs were stripe-like and had smooth edges, with the width of the bright domains homogeneous along the stripes. Bright domains were readily observed for DLPC/DPPC GUVs with a branching/turning pattern having a characteristic turning angle of 60 deg [21]. In contrast to this highly ordered pattern, bright domains in DLPC/DSPC GUVs were more irregular and their edges were rougher. Combined AFM and fluorescence microscopy experiments showed that ruptured DLPC/DPPC GUVs also preserved their domain patterns following rupture and flattening, with smooth edges of constant width evident both before and after rupturing of GUVs (Fig. 5). Scans analogous to those performed above showed that, in contrast to the DLPC/DSPC system, there was only one step observed, which corresponded to the transition from the green liquid phase to the red strip domains. The dark and bright gel domains in a DLPC/DPPC membrane showed no height difference (Fig. 5(b)), and no

Fig. 5 AFM of ruptured DLPC/DPPC GUVs: (a) fluorescence image, (b) AFM height image, (c) height profile along the red line in figure B, revealing that the DLPC/DPPC membrane shows no height difference between the dark gel domain and the bright gel domain surrounding it, and showed no evidence of the bright layer residing atop other membrane components

evidence of the bright layer residing atop other membrane components.

3.5 Results Provide Insight Into Mechanisms for Lipid Domain Stability. From the perspectives of both lipid domain stability and biomechanics, the results are surprising and interesting. Results highlight that mechanical energy can play a significant role in the energetics of phase separation. For the DLPC/DSPC GUVs, a seemingly impossible combination of two different lipid gel phases were observed in a binary system because mechanical terms affected the structure of the phases. From the perspective of biomechanics and mechanobiology, results highlight that mechanical factors can affect lipid domains sufficiently to alter their phase behavior. The newly identified structural mechanism for phase stability represents a tool that cells might possibly use in membrane trafficking.

The fact that the DSPC and DPPC GUVs have different morphologies is not surprising given that their spontaneous curvatures have been estimated to be different by approximately a factor of two [36]. However, a question that we could not answer is whether the bright domain exists as an external layer on DLPC/DSPC GUVs or whether this can occur only on collapsed GUVs. In other words, were the DSPC lipid molecules pushed out of the GUVs during phase separation, or did this occur during rupture? Differences in spontaneous curvature and area changes associated with phase demixing are potential sources of strain energy that could drive either buckling or ejection of a gel phase.

Alternatively, a structural instability in a bright phase might stay connected to the mother membrane before the GUV ruptures, but become disconnected and form a second layer of membrane as the GUV ruptures and spreads over the substrate. This happens with the center dark domains too, with some patches observed atop the dark region that did not provide measurable fluorescence. We note that we never observed a bright edge domain that could not be removed by the AFM tip. With the assumption that adhesion depends upon curvature, we suggest that the bright edge domains have curvature differing from that of the dark domains. Our AFM results showed that the width of the bright domain was usually below 1 μm on the ruptured membrane, so the radius of budding in GUVs would be close to or below the optical resolution limit (around 250 nm), making such bulges difficult to observe optically. Therefore, it is difficult to draw conclusions about the topography in the unruptured GUVs using current optical techniques.

Bulges, buckles, or ejected lipid could all lead to high local curvature capable of inducing a repulsive interaction between domains [17]. This would have consequences important to the study of membrane domain stability because a repulsive interaction of this character could increase the energetic barrier to domain coalescence and thereby serve to slow enlarging of domains. This provides motivation for extrapolating our findings from the microscopic level to the nanoscopic level and for investigating how the bright interphase impacts the stability of nanodomains.

4 Conclusions

Our results show that two distinct gel phases can exist simultaneously and interact on GUVs. The so-called bright domains can exist as an extra layer of membrane on ruptured GUVs possibly indicating a highly curved structure on unruptured GUVs, which might have important implications for nanodomain stability. We propose that this was caused by mechanical effects associated with a highly curved structure of the DiI-labeled interphase. The extra layer of membrane was not found on the collapsed DLPC/DPPC membrane, suggesting that mechanical effects sufficient to block coalescence of microdomains do not require an out-of-plane membrane domain.

Collapsing both DLPC/DPPC and DLPC/DSPC GUVs onto glass slides enabled the application of AFM to the study of phase behavior in a way that could be related to behavior of GUVs.

Although questions arise about the disconnection of bright domains from the lipid bilayer, the overall shape and patterns of domains were preserved after GUV rupture. Our findings provide a basis for further AFM investigations of the nanoscopic phase separation in GUVs, which has proven difficult to study due to the lack of powerful techniques that can break through the optical limit in a highly diffusive environment.

Funding Data

- National Institutes of Health (Grant No. R01GM084200; Funder ID: 10.13039/1000000002).
- NIH Neuroscience Blueprint Interdisciplinary Center Core (Grant No. P30 NS057105; Funder ID: 10.13039/100000135).
- NIH Shared Instrumentation (Grant No. S10 RR024733; Funder ID: 10.13039/1000000002).
- The National Science Foundation through the Science and Technology Center for Engineering Mechanobiology (Grant No. CMMI 1548571; Funder ID: 10.13039/501100008982).

Nomenclature

AFM = atomic force microscopy
 DLPC = 1,2-dilauroyl-sn-glycero-3-phosphocholine
 DPPC = 1,2-dipalmitoyl-sn-glycero-3-phosphocholine
 DSPC = 1,2-distearoyl-sn-glycero-3-phosphocholine
 GUV = giant unilamellar vesicle
 SLB = supported lipid bilayer

References

- [1] Elson, E. L., Fried, E., Dolbow, J. E., and Genin, G. M., 2010, "Phase Separation in Biological Membranes: Integration of Theory and Experiment," *Annu. Rev. Biophys.*, **39**(1), pp. 207–226.
- [2] Pike, L. J., 2006, "Rafts Defined: A Report on the Keystone Symposium on Lipid Rafts and Cell Function," *J. Lipid Res.*, **47**(7), pp. 1597–1598.
- [3] Hinz, H. J., and Sturtevant, J. M., 1972, "Calorimetric Studies of Dilute Aqueous Suspensions of Bilayers Formed From Synthetic L- α -Lecithins," *J. Biol. Chem.*, **247**(19), pp. 6071–6075.
- [4] Melchior, D. L., Morowitz, H. J., Sturtevant, J. M., and Tsong, T. Y., 1970, "Characterization of the Plasma Membrane of Mycoplasma Laidlawii—VII: Phase Transitions of Membrane Lipids," *Biochim. Biophys. Acta*, **219**(1), pp. 114–122.
- [5] Harder, T., and Sangani, D., 2009, "Plasma Membrane Rafts Engaged in T Cell Signalling: New Developments in an Old Concept," *Cell Commun. Signal. CCS*, **7**, p. 21.
- [6] Simons, K., and Vaz, W. L. C., 2004, "Model Systems, Lipid Rafts, and Cell Membranes," *Annu. Rev. Biophys. Biomol. Struct.*, **33**, pp. 269–295.
- [7] Mouritsen, O. G., 1991, "Theoretical Models of Phospholipid Phase Transitions," *Chem. Phys. Lipids*, **57**(2–3), pp. 179–194.
- [8] Koralch, J., Schwillie, P., Webb, W. W., and Feigenson, G. W., 1999, "Characterization of Lipid Bilayer Phases by Confocal Microscopy and Fluorescence Correlation Spectroscopy," *Proc. Natl. Acad. Sci.*, **96**(15), pp. 8461–8466.
- [9] Feigenson, G. W., and Buboltz, J. T., 2001, "Ternary Phase Diagram of Dipalmitoyl-PC/Dilauroyl-PC/Cholesterol: Nanoscopic Domain Formation Driven by Cholesterol," *Biophys. J.*, **80**(6), pp. 2775–2788.
- [10] Morales-Pennington, N. F., Wu, J., Farkas, E. R., Goh, S. L., Konyakhina, T. M., Zheng, J. Y., Webb, W. W., and Feigenson, G. W., 2010, "GUV Preparation and Imaging: Minimizing Artifacts," *Biochim. Biophys. Acta*, **1798**(7), pp. 1324–1332.
- [11] Li, L., and Cheng, J.-X., 2006, "Coexisting Stripe- and Patch-Shaped Domains in Giant Unilamellar Vesicles," *Biochemistry*, **45**, pp. 11819–11826.
- [12] Embar, A., Dolbow, J., and Fried, E., 2013, "Microdomain Evolution on Giant Unilamellar Vesicles," *Biomech. Model. Mechanobiol.*, **12**(3), pp. 597–615.
- [13] Maleki, M., and Fried, E., 2013, "Multidomain and Ground State Configurations of Two-Phase Vesicles," *J. R. Soc. Interface R. Soc.*, **10**(83), p. 20130112.
- [14] Brewster, R., and Safran, S. A., 2010, "Line Active Hybrid Lipids Determine Domain Size in Phase Separation of Saturated and Unsaturated Lipids," *Biophys. J.*, **98**(6), pp. L21–L23.
- [15] Palmieri, B., and Safran, S. A., 2013, "Hybrid Lipids Increase the Probability of Fluctuating Nanodomains in Mixed Membranes," *Langmuir ACS J. Surf. Colloids*, **29**(17), pp. 5246–5261.
- [16] Frolov, V. A. J., Chizmadzhev, Y. A., Cohen, F. S., and Zimmerberg, J., 2006, "Entropic Traps' in the Kinetics of Phase Separation in Multicomponent Membranes Stabilize Nanodomains," *Biophys. J.*, **91**(1), pp. 189–205.
- [17] Ursell, T. S., Klug, W. S., and Phillips, R., 2009, "Morphology and Interaction Between Lipid Domains," *Proc. Natl. Acad. Sci.*, **106**(32), pp. 13301–13306.
- [18] Ulrich, A. S., 2002, "Biophysical Aspects of Using Liposomes as Delivery Vehicles," *Biosci. Rep.*, **22**(2), pp. 129–150.
- [19] Tokumasu, F., Jin, A. J., Feigenson, G. W., and Dvorak, J. A., 2003, "Nanoscale Lipid Domain Dynamics Revealed by Atomic Force Microscopy," *Biophys. J.*, **84**(4), pp. 2609–2618.
- [20] Lin, W.-C., Blanchette, C. D., Ratto, T. V., and Longo, M. L., 2006, "Lipid Asymmetry in DLPC/DSPC-Supported Lipid Bilayers: A Combined AFM and Fluorescence Microscopy Study," *Biophys. J.*, **90**(1), pp. 228–237.
- [21] Bernchou, U., Midtiby, H., Ipsen, J. H., and Simonsen, A. C., 2011, "Correlation Between the Ripple Phase and Stripe Domains in Membranes," *Biochim. Biophys. Acta*, **1808**(12), pp. 2849–2858.
- [22] Loura, L. M., Fedorov, A., and Prieto, M., 2000, "Partition of Membrane Probes in a Gel/Fluid Two-Component Lipid System: A Fluorescence Resonance Energy Transfer Study," *Biochim. Biophys. Acta*, **1467**(1), pp. 101–112.
- [23] Pryse, K. M., Rong, X., Whisler, J. A., McConnaughey, W. B., Jiang, Y.-F., Melnykov, A. V., Elson, E. L., and Genin, G. M., 2012, "Confidence Intervals for Concentration and Brightness From Fluorescence Fluctuation Measurements," *Biophys. J.*, **103**(5), pp. 898–906.
- [24] Tsukruk, V. V., and Singamaneni, S., 2011, *Scanning Probe Microscopy of Soft Matter*, Wiley-VCH, Hoboken, NJ.
- [25] Gibbs, J. W., 1961, *The Scientific Papers*, Vol. 1, Dover Publications, Mineola, NY.
- [26] Tsukruk, V. V., and Singamaneni, S., 2012, *Scanning Probe Microscopy of Soft Matter: Fundamentals and Practices*, Wiley, New York.
- [27] McConney, M. E., Singamaneni, S., and Tsukruk, V. V., 2010, "Probing Soft Matter With the Atomic Force Microscopies: Imaging and Force Spectroscopy," *Polym. Rev.*, **50**(3), pp. 235–286.
- [28] Singamaneni, S., McConney, M. E., and Tsukruk, V. V., 2010, "Swelling-Induced Folding in Confined Nanoscale Responsive Polymer Gels," *ACS Nano*, **4**(4), pp. 2327–2337.
- [29] Tian, L., Fei, M., Kattumenu, R., Abbas, A., and Singamaneni, S., 2012, "Gold Nanorods as Nanotransducers to Monitor the Growth and Swelling of Ultrathin Polymer Films," *Nanotechnology*, **23**(25), p. 255502.
- [30] Tian, L., Chen, E., Gandra, N., Abbas, A., and Singamaneni, S., 2012, "Gold Nanorods as Plasmonic Nanotransducers: Distance-Dependent Refractive Index Sensitivity," *Langmuir ACS J. Surf. Colloids*, **28**(50), pp. 17435–17442.
- [31] Richter, R. P., and Brisson, A. R., 2005, "Following the Formation of Supported Lipid Bilayers on Mica: A Study Combining AFM, QCM-D, and Ellipsometry," *Biophys. J.*, **88**(5), pp. 3422–3433.
- [32] Axelrod, D., Koppel, D. E., Schlessinger, J., Elson, E., and Webb, W. W., 1976, "Mobility Measurement by Analysis of Fluorescence Photobleaching Recovery Kinetics," *Biophys. J.*, **16**(9), pp. 1055–1069.
- [33] Leidy, C., Kaasgaard, T., Crowe, J. H., Mouritsen, O. G., and Jørgensen, K., 2002, "Ripples and the Formation of Anisotropic Lipid Domains: Imaging Two-Component Supported Double Bilayers by Atomic Force Microscopy," *Biophys. J.*, **83**(5), pp. 2625–2633.
- [34] Simonsen, A. C., and Bagatolli, L. A., 2004, "Structure of Spin-Coated Lipid Films and Domain Formation in Supported Membranes Formed by Hydration," *Langmuir ACS J. Surf. Colloids*, **20**(22), pp. 9720–9728.
- [35] Kollmitzer, B., Heftberger, P., Rappolt, M., and Pabst, G., 2013, *Monolayer Spontaneous Curvature of Raft-Forming Membrane Lipids*, Soft Matter, **9**(45), pp. 10877–10884.
- [36] Melcher, J., Carrasco, C., Xu, X., Carrasco, J. L., Gomez-Herrero, J., Jose de Pablo, P., and Raman, A., 2009, "Origins of Phase Contrast in the Atomic Force Microscope in Liquids," *Proc. Natl. Acad. Sci.*, **106**(33), pp. 13655–13660.