A Close Look at Domain Formation in DPPC Monolayers

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Insoluble monolayers of dipalmitoylphosphatidylcholine (DPPC) exhibit a phase transition resulting in the formation of domains with interesting shapes. We studied the features of DPPC domain shapes throughout the coexistence region. We find that the basic domain shape is an asymmetric "bean" with a flattened lobe and a distinct cavity. The cavity is a locus for growth, in the form of either a terminal nub or a projection which grows to form a new lobe. Multilobed domains thus grow from beans via a specific process; however, these are not stable and transform over time back to beans. We also find that atypical compression schemes can be used to generate domain shapes that stray markedly from the norm, such as multilobed complexes or toruses.

Introduction

Insoluble monolayers of phospholipids at the air−water interface have long been of interest, both because of their intriguing behavior and because of their biomimetic applications. Phospholipid behavior in this two-dimensional environment can include phase transitions, some of which display a remarkable heterogeneity. The presence of phospholipids in biological systems has been well established; most significantly, they constitute the backbone of the cellular membrane. Monolayers of phospholipids have thus been applied as a simple model for the cellular lipid bilayer.[1] In an even more realistic application, monolayers serve as a model for the alveolar interface in the lung. Phospholipids are a main component of alveolar fluid, lowering the surface tension of the fluid−air interface and thus increasing the efficiency of breathing.

The power of a monolayer, particularly when compared to a bilayer, lies in its controllability. A monolayer's properties can be carefully tuned, allowing the ability to define molecular density by varying the area per molecule on a Langmuir film balance. In addition, the planar geometry of this experiment makes it accessible to several optical techniques. Fluorescence microscopy was the first technique used to study the phase transitions in monolayers;[2−5] more recently, Brewster angle microscopy was developed as a complimentary technique.[6,7]

Dipalmitoylphosphatidylcholine (DPPC) has frequently been the phospholipid of choice for many monolayer studies. Because phosphatidylcholines are the primary phospholipids in the mammalian cell membrane and a significant component of alveolar fluid, DPPC is a natural focus for study. In addition, DPPC exhibits a phase transition at room temperature from what is called a liquid-expanded (LE) phase to a liquid-condensed (LC) phase. The LC phase appears as domains in a field of LE phase; this heterogeneity can be imaged using the techniques named above. DPPC domains exhibit remarkable shapes, the study of which provides a unique method for probing the interfacial film.

The inherently interesting behavior and applications of DPPC have motivated diverse studies of its monolayers. Studies of the DPPC pressure−molecular area isotherm have revealed information about its phase transitions[8−11] and the effects of film compression rate.[12] As models for membranes, mixtures of DPPC and biologically-relevant species have been examined, particularly in the case of pulmonary surfactant,[13−19] cholesterol,[20−25] and other lipids.[26−29] Hydrolysis of DPPC by the protein phospholipase A_2 has been observed using fluorescence microscopy.[30−32] Penetration of DPPC monolayers by proteins has also been studied.[33] The surface rheology of DPPC films has been examined.[34−36] With respect to long-range

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order within the monolayer, AFM images of Langmuir–Blodgett films of DPPC have been acquired,[37,38] and textures within DPPC domains have been visualized with polarized fluorescence microscopy[2] and Brewster angle microscopy.[39,40]

Even amidst a seemingly large body of work, there exist in the literature surprisingly few images of DPPC domains tracked from nucleation through the entire LE/LC transition region. In fact, most pictures of pure DPPC domains have been presented as a reference case in studies of multicomponent systems. And because the images shown are not displayed in the context of general DPPC behavior, images from one study rarely match those from others.[20,32,38,39,41–43]

Indeed, a systematic study of domain shape is valuable from both physicochemical and biological perspectives. Shapes formed are ultimately related to the structure of the constituent molecules and their packing and orientation within a domain; characterization of shapes is the initial step in making this connection. It is important to appreciate that different phospholipids form distinct domain shapes in the LE/LC coexistence region. For example, DPPC behavior differs greatly from that of DMPE,[44] DMPA,[45] and DLPE,[46] despite only subtle differences in lipid structure. One approach taken to predict domain shapes is based on the competition between line tension and electrostatic repulsion (arising from a field of oriented dipoles making up the domain).[47–51] The advancement of these models depends on a solid understanding of the experimental phenomena, which can only be gained by an in-depth study of domain shape and growth. From the biological perspective, one may better understand how other species (proteins, cholesterol, membrane perturbants) interact with phospholipids in a membrane by examining the effects of these substances on DPPC domains. Again, full characterization of pure DPPC domains is a prerequisite, so that the influence of additional species can be ascertained.

We have examined in detail the shapes of DPPC domains throughout the coexistence region. In this paper, we point out their important and interesting features and also demonstrate methods that cause divergence from the fundamental shape.

Experimental Section

L-α-1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was obtained from Avanti Polar Lipids (Birmingham, Alabama), as was the fluorescent probe, 1-palmitoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC). Both were at purities >99% and used without further purification. The phospholipid was spread from a chloroform solution (Fisher HPLC grade), and unless otherwise noted, all experiments were carried out with a probe concentration of 0.5 mol %. The subphase for all experiments was Millipore water (18.2 MΩ cm resistivity), maintained at 20 °C with a Neslab circulating unit to an accuracy of 0.1 °C.

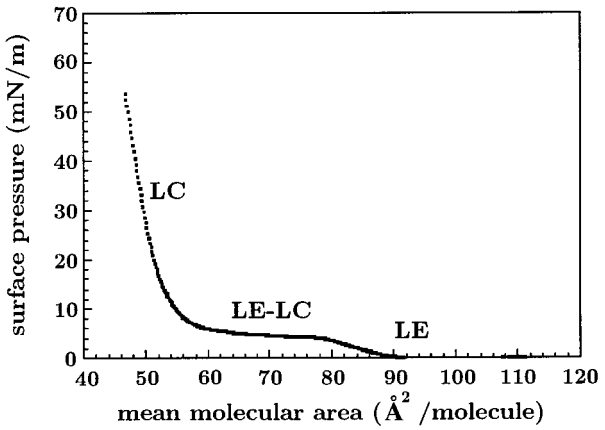

Figure 1. DPPC isotherm at 20 °C.

Lipids were spread on a Langmuir film balance (R & K Ultrathin Organic Film Technology RK1, Germany), and time was allowed for the spreading solvent to evaporate (at least 10 min). Unless otherwise noted, films were compressed at a rate of 0.86 Å^2 molecule^{-1} min^{-1}. The surface pressure was measured with a platinum Wilhelmy plate with an accuracy of 0.1 mN/m. The entire trough was enclosed by a Plexiglas housing and several layers of clear plastic, all serving a dual purpose: to keep the convection caused by air currents to a minimum and also to aid in keeping the film balance free from airborne contaminants. The film balance was mounted on a vibration isolation table (MOD-2, JRS, Switzerland), which was in turn placed on a large optical table (Newport, Irvine, CA).

Fluorescence microscopy was conducted with a Zeiss Axiotron epifluorescence microscope (Germany). A mirror was placed on the bottom of the film balance under the microscope objective to enhance picture quality by eliminating scatter from the bottom of the balance. Fluorescence images were monitored with an intensified CCD camera (Quantex QC-200, Sunnyvale, CA) and videotaped throughout compression of the film (NEC PC-VCR). Images were acquired from videotape with an image-grabbing card (PIXCI, Epix Inc., Buffalo Grove, IL), and analysis was performed using NIH Image (developed at the U.S. National Institutes of Health). All images presented appear as taped without image enhancement.

Brewster angle microscopy images were acquired using a home-built apparatus modeled after the design of Hönig and Möbius.[6] An argon ion laser (Innova 304, Coherent, Santa Clara, CA) was used to illuminate the film. The remainder of the imaging apparatus (camera, monitor, VCR, etc.) matches the fluorescence configuration.

Results and Discussion

We present our results in several sections. In the first section, we describe the characteristics of DPPC domains with the "bean" as the fundamental shape. We then describe the specific mechanism by which beans can develop into multilobed shapes. Finally, we discuss how atypical compression schemes affect domain shape.

Fundamental Shape Characteristics. A DPPC pressure–mean molecular area isotherm is shown in Figure 1, with the LE/LC coexistence region labeled. Domain nucleation occurs at the kink in the isotherm (typically at 3.6–3.8 mN/m). Initially, domains appear completely round; whether this is the case in reality or due to limits in the resolution of the microscope is unclear. Indeed, it is only as they grow that they take on their fundamental shape.

Figure 2 shows a progression of fluorescence images through the coexistence region. The surface pressures are noted in the caption. The domains formed are chiral,

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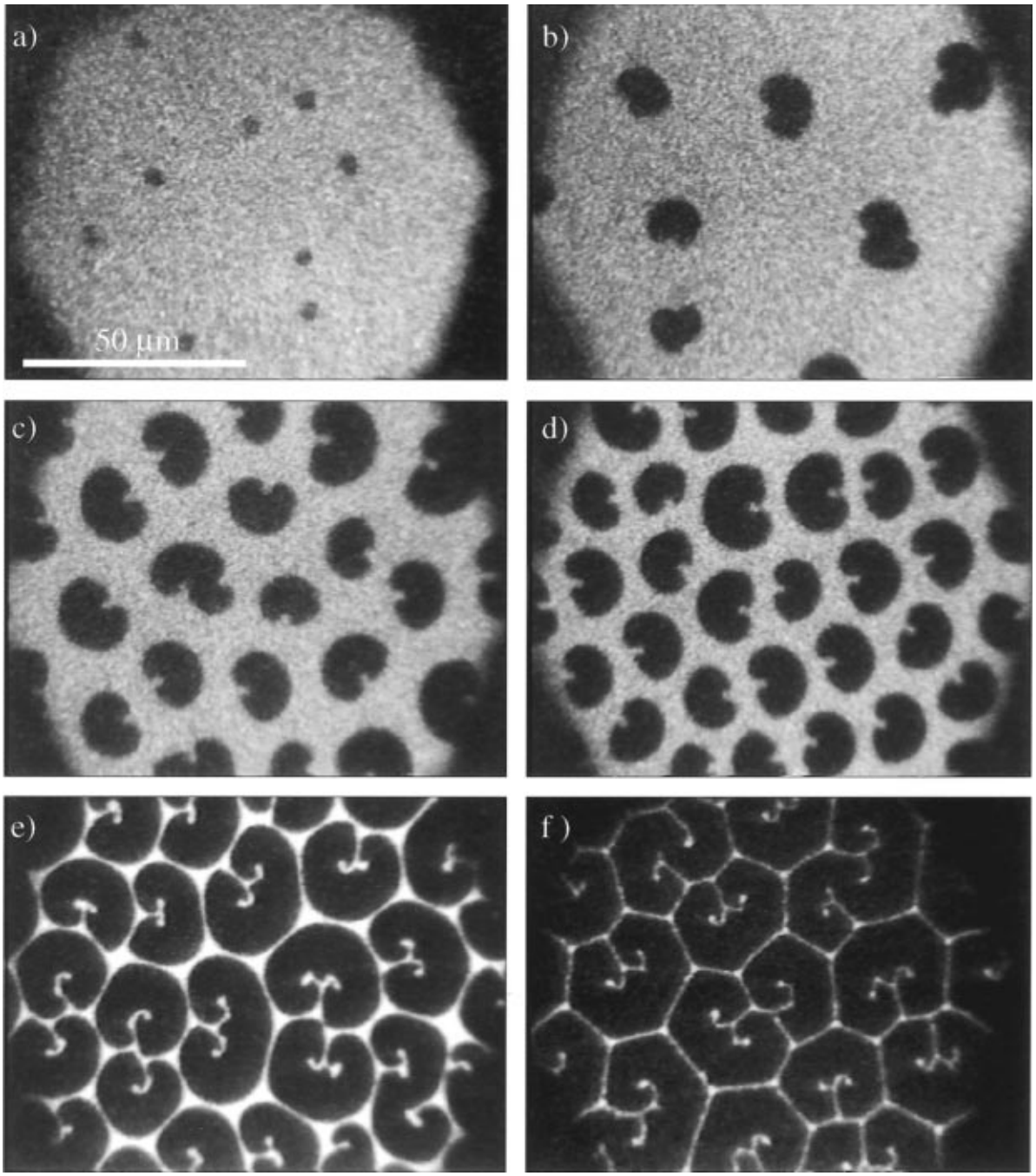

Figure 2. DPPC domain growth with a compression rate of 0.86 Å^2 molecule^{-1} min^{-1}: (a) 3.8 mN/m; (b) 3.9 mN/m; (c) 4.2 mN/m; (d) 4.3 mN/m; (e) 5.0 mN/m; (f) 7.5 mN/m.

an expression of the chirality of the DPPC molecule. As would be expected, the enantiomer forms mirror images of the domains shown, and a racemic mixture yields nonchiral domains.[52] As is most evident in Figure 2, the predominant domain shape is a bean with a distinct cavity. Multilobed shapes can also be observed, but these originate as beans and over time transform back to beans; this will be discussed in detail in the following section.

In addition to being chiral, the domains are asymmetric: if the bean is oriented with its cavity facing upward, the left lobe has a flattened edge. This was noted by Flörscheimer and Möhwald, who also proposed a model for the orientations of the molecular tilt within the domain.[53] We find that this flattened edge plays a role in the domain's growth process at higher pressures (i.e., in a more condensed film). In particular, the flat edge extends into the interior of the cavity, as is illustrated in Figure 2e. Upon further compression, these domains can undergo an irreversible shape transformation which will be described in the last section.

The extent to which the flattened edge grows into the cavity depends on domain size. Only smaller beans exclusively undergo this process; larger beans can also develop a nub at the cavity. As shown in Figure 2e, this nub grows outward, forcing the lobes of the bean to spread. Interestingly, as the nubs grow larger, they also exhibit chirality, curving counterclockwise. At higher pressures, beans thus display two distinct growth modes: larger

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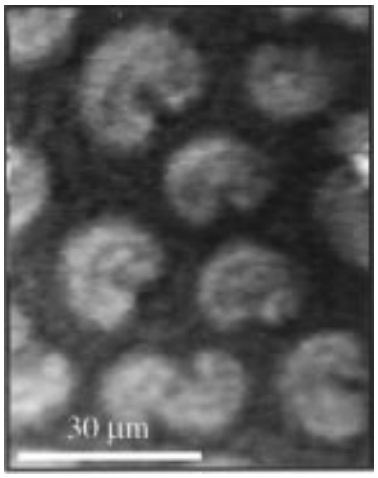

Figure 3. DPPC domains at 4.0 mN/m imaged by Brewster angle microscopy.

beans have a larger cavity and can accommodate a nub (growth from the inside out), while smaller beans with smaller cavities can only grow from the outside in.

As the monolayer is compressed and the domains have less room to grow, the repulsive nature of the domains becomes strikingly apparent. Domains do not fuse readily and will, in fact, grow into all available area. This is seen clearly in Figure 2f, where domain growth has proceeded into the interstitial region between groups of domains and the normally-curved domains become polygonal. Domain repulsion has been studied, as has the ability of DPPC domains to deform under stress.[53,54] We do note that domain fusion can ultimately be achieved at high enough pressures, evidenced by aggregates that persist upon expansion.

The domain shapes shown in Figure 2 are stable over periods of days. Over the first 8−10 h, the domains relax to a slightly smaller size, but their shape remains unchanged. As such, we do not see the behavior previously reported, where all domains transform to nearly circular shapes over time.[42] In agreement with that study, however, we do find that any multilobed domains convert to a more compact shape: in our case, beans. From this, we conclude that the bean is the stable domain shape for DPPC.

In fluorescence experiments, one must be careful to interpret the results because of the potential influence of the fluorescent probe. Any possible effects will be even more exaggerated at higher surface pressures, due to enrichment of the relative probe concentration in a shrinking LE phase. We decreased the concentration of the probe by a factor of 20 (to 0.025 mol %) to determine if this enrichment affects domain shape. The domain shapes found in this experiment match those in Figure 2, indicating that the fluorescent probe does not perceptibly affect domain shape.

We also used Brewster angle microscopy—which requires no fluorescent probe—to further confirm the negligible influence of probe on DPPC domain shapes. BAM images, such as the one shown in Figure 3, closely match our fluorescence images. We do note, however, one difference between the two experiments. Domains in a fluorescence experiment remain dispersed at a higher pressure than those in a BAM experiment; the fluorescent probe thus delays the onset of domain fusion. This phenomenon is confirmed by a fluorescence experiment using a probe that is soluble in both LE and LC phases (headgroup-labeled NBDPE) instead of an LC-insoluble probe (tail-labeled NBDPC). In this case, with the probe distributed in both phases, the barrier to fusion is expected to be lower. Indeed, the images seen match those of BAM in that domains fuse more readily at lower pressures. Apart from this effect, the fluorescent probe does not influence the shapes of DPPC domains. Thus, we are confident that the shape characteristics we have presented are intrinsic to pure DPPC.

Development of Multilobed Domain Shapes. Although the bean is the fundamental shape, multilobed shapes can sometimes develop from this origination. This typically occurs early in the coexistence region and will not happen once domains have grown to occupy most of the monolayer. The influences that can cause a bean to transform into a multilobed structure are quite subtle—changes in compression speed or slight variations in subphase conditions are sufficient to drastically affect the fundamental domain shape. In all cases, however, multilobed domains relax over time to reform the original bean, as described above. So although it is important to understand the process by which multilobed domains are formed because of their predominance in the literature, it is also important to recognize that the bean is the truly stable domain shape for DPPC.

The appearance of multilobed shapes and their inherent metastability are consistent with predictions of domain shape based on a simple energy model, as proposed by McConnell and co-workers.[55,56] The model takes into account the energy costs associated with line tension and with repulsive interactions arising from intermolecular dipole forces. Using a variational approach, Mayer and Vanderlick[49] showed that the only stable shapes are either circular or bilobed. However, multilobed shapes of only slightly higher energy are also predicted. Using a Monte Carlo simulation, Mayer and Vanderlick[57] showed that metastable, sometimes highly-branched, shapes occur readily as a result of thermal fluctuations; once formed, they are long-lived. The simple energy model employed does not take into account molecular tilt and the associated energy costs of splay and bend orientations, nor does it account for molecular chirality. Hence only symmetric, defect-free, shapes are predicted. While DPPC shapes are clearly more complicated, the appearance of multilobed shapes and the ultimate stability of the more compact bean shape are in general agreement with the results of these theoretical studies.

We have identified a specific process by which multilobed domains are formed. Figure 4 shows a series of different domains caught at different stages of their growth development. We highlight with a small line the flattened edge discussed earlier. A bean, shown in Figure 4a, develops a lobe from its cavity, shown in Figure 4b. This lobe grows counterclockwise out from the domain. The lobe grows toward the asymmetric flat edge and torques this edge about the cavity (Figure 4c and d). The resulting bilobed shape is shown in Figure 4e; it resembles a mirrored S but without symmetry. As the domain grows, the two lobes increase in size, and the newer lobe may become larger than the original. The original bean can always be recognized by the position of the preserved flat edge. An arrow in Figure 4e points to the original bean.

Trilobed domains are formed from bilobed domains in the following fashion. A new projection develops at the flat edge of a bilobed domain and grows counterclockwise around the domain (Figure 4f and g). The domain develops

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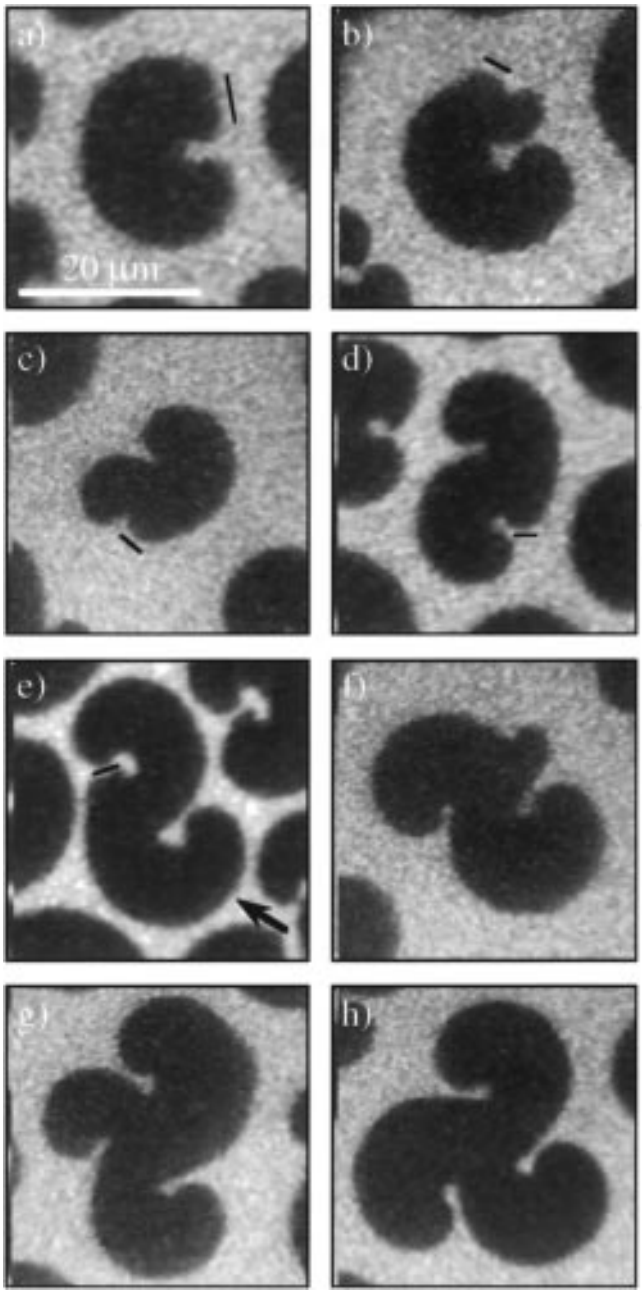

Figure 4. DPPC domains at different stages of maturation: (a) fundamental bean; (b) bean with projection from cavity; (c) projection developing into a lobe; (d) lobe growing; (e) mature bilobe; (f) bilobe with projection; (g) projection maturing; (h) mature trilobed domain.

into the familiar trilobed shape published frequently for DPPC (Figure 4h). The new growth must originate from the flat edge because it is absent in any domain with more than two lobes.

We distinguish the projections that develop into lobes from the nubs described in the previous section, even though both orginate in the bean cavity. Lobe growth begins at lower pressure; nubs only appear at higher pressure, and the nub remains confined in the cavity. It is possible that the nub is a frustrated lobe, unable to mature due to space limitations in the more condensed film. We do not think this is the case. Unlike lobes, which only appear in a subset of domains, nubs appear predictably, growing simultaneously in all domains with a large enough cavity. One thing, however, is certain: the domain cavity is a locus for growth, either in the form of a terminal nub or a new lobe.

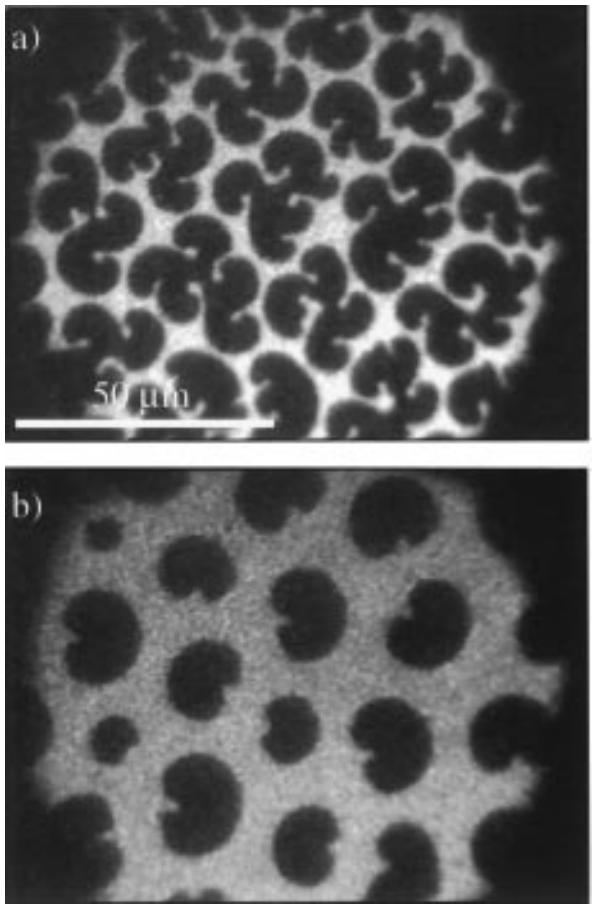

Figure 5. Domains formed at a compression rate of 17.2 Å^2 molecule^{-1} min^{-1}: (a) film immediately after compression, 5.0 mN/m; (b) same film after 4 h, 4.8 mN/m.

Divergence from the Fundamental Shape. We have found that, by manipulating the manner in which the film is compressed, we can drastically change the shapes of domains that form. For example, multilobed domains are clearly deviations from the fundamental bean shape, and we will describe two procedures by which multilobed domains can be deliberately generated. We will also show how even more intriguing shapes can be produced.

It is known that multilobed domains can be intentionally created by increasing the compression rate.[39] Figure 5a shows domains formed at a compression rate 20 times greater than that used to form the shapes shown in Figure 2. Domains with many lobes are evident, some of which appear to be a domain complex made up of two mature, but joined, domains. This may be the result of domains that nucleate in close proximity and form a bridge that persists through the compression. In any case, we do not observe, even at higher compression rates, the dendritic behavior reported by others.[39] As discussed previously, multilobed domains are not stable and transform to beans over time, as shown in Figure 5b.

The effects of repetitive compression−expansion cycles are particularly important in pulmonary applications, as this is the process involved with each breath. We examined the reversibility of the isotherm and domain shapes over a series of sequential compressions, each extending further and further into the coexistence region and beyond. The cycles were performed at the same speed used to generate the shapes shown in Figure 2. In all cases, we find the isotherm perfectly reversible with no hysteretic behavior. Domain shapes, however, are only reproducible until the cycles reach a region in the isotherm

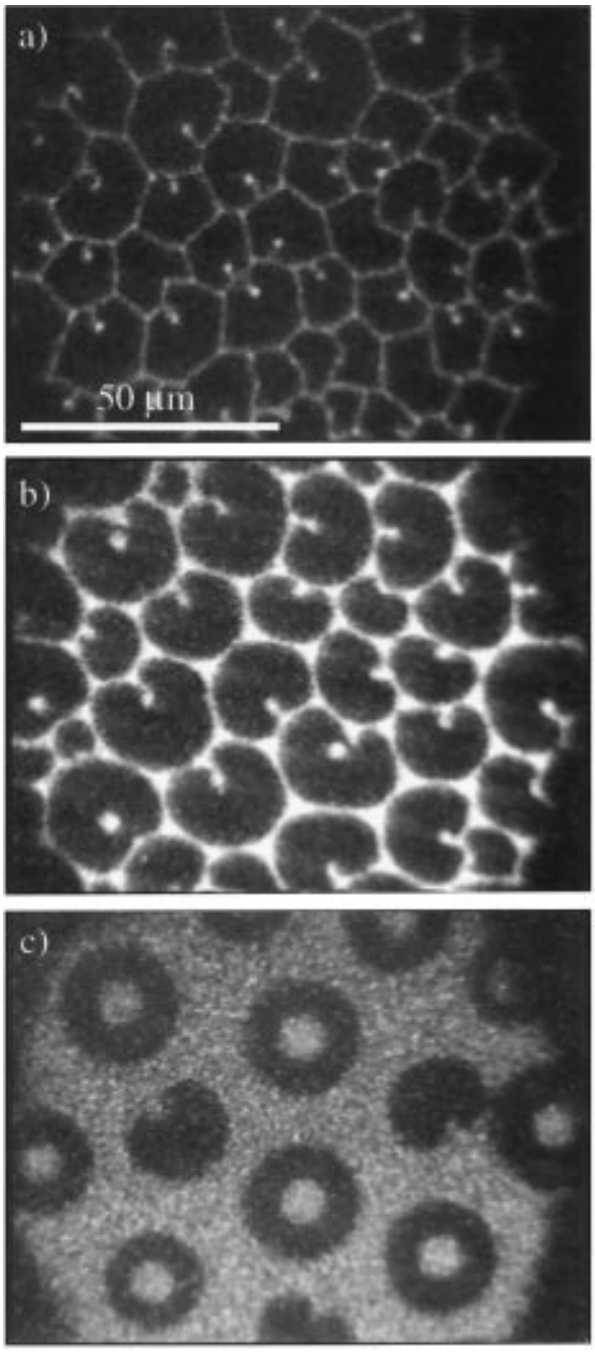

Figure 6. Development of DPPC toruses: (a) compressed film just prior to expansion, 7.5 mN/m; (b) film after expansion, 4.5 mN/m; (c) same film after 10 h, 4.3 mN/m.

above the plateau. Once the monolayer is compressed to this state (approximately >7−8 mN/m), any subsequent compression yields multilobed domain shapes (similar to the kinetic case above). Even when an annealing step is inserted between cycles (heating the gaseous film to 33 °C and cooling back to 20 °C), multilobed domains continue to be formed upon compression.

Despite the predisposition of DPPC to form lobed, chiral domains, we have developed a specific process by which some domains can be made circular. The monolayer is compressed slowly so that beans are the predominant shape. The compression proceeds until the flattened edge wraps around to the interior of the domain cavity. Then, upon re-expansion, some of the domains exhibit self-fusion, creating a pocket in the domain. The pocket slowly migrates toward the center of the domain (over time scales of hours), and the domain itself becomes circular. Several

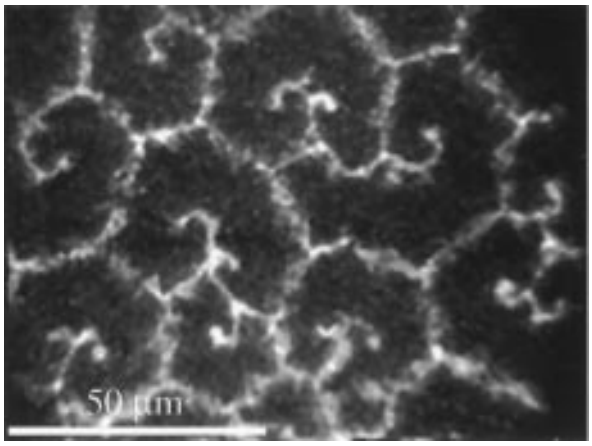

Figure 7. Probe-induced shape instability in DPPC domains, 12.3 mN/m.

domains in different stages of this process are shown in Figure 6. After long times (10 h), we find mixtures of toruses coexisting with beans. The pocket of the torus is frequently brighter than the exterior, reflecting the higher probe concentration in the LE phase at the time the pocket was formed. Although the connection between domain internal structure and macroscopic shape is not yet clear, the cavity most likely arises from a packing defect pinned at the boundary. Internalization of the defect (in the form of a pocket) allows the domain to become circular.

Lastly, we report on a peculiar shape instability in highly-compressed DPPC monolayers, as observed only by fluorescence microscopy. Figure 7 shows the result of this instability: the domains appear to be cut in a very specific fashion with tiny slices proceeding clockwise into the domains. Interestingly, no such transition is seen using BAM, suggesting that this shape change is associated with the presence of the fluorescent probe. We found that the onset of this phenomenon is independent of probe concentration in a range from 0.02 to 4 mol %. We cannot establish the specific pressure at which the instability occurs but find that it does occur in a reproducible window of pressures ranging from 11 to 15 mN/m at a compression rate of 0.86 $Å^2$ $molecule^{-1}$ min^{-1}. At compression rates of 2.58 $Å^2$ $molecule^{-1}$ min^{-1} and higher, however, the transition is suppressed. Although we do not yet fully understand this instability, the findings above suggest that the phenomenon is kinetic rather than thermodynamic in nature. Regardless of its origin, the shape change is an interesting manifestation of the internal structure of a DPPC domain. The slices reflect the chirality of L-DPPC, evidenced by slices curving in the opposite direction in films composed of D-DPPC. It is as though the probe acts to cleave the domain along intrinsic pathways. Interestingly, the domain cavity remains immune to this attack.

Conclusions

We have conducted a detailed study of DPPC domain shape. We find that the fundamental domain shape is an asymmetric "bean" with a distinct cavity. Multilobed domains develop from beans via a specific growth process but transform back to beans over time. Markedly different domain shapes (such as multilobed complexes and toruses) can be generated using atypical compression schemes.

DPPC domain shapes provide an interesting challenge to those seeking to predict domain shape on the basis of models incorporating line tension, oriented dipole interactions, and molecular chirality. It is only with a solid understanding of the experimental shapes that the success

of predictive theories may be evaluated. This work also serves as a springboard for study of DPPC interactions with other biologically-relevant species. Domain shapes perturbed by other substances provide a unique window for examination of these interactions. With a clear understanding of pure DPPC behavior, the effects of other species can be better understood.

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