Morphology and Kinematics of Langmuir–Blodgett Monolayers

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With fluorescence microscopy, we have found that monolayers of dipalmitoylphosphatidylcholine with a small concentration of dye lipids at an air/water interface always contain a number of membrane defects and domains in the liquid expanded phase. These features range in sizes from several micrometers to several millimeters. Furthermore, the fluorescence intensity of these monolayers exhibits a maximum at the full transition from the liquid expanded phase to the liquid condensed phase. At low surface pressures, monolayers of pure fluorescence dye lipids have a large number of circular defects. It was also found that lipid motion is a common phenomenon with average speeds much larger than the rate of diffusion.

Introduction

Langmuir–Blodgett (LB) monolayers have been extensively studied in the past several decades for their unique two-dimensional (2-D) characteristics. They are ideal model systems for studies of some kinetics and dynamic processes in membranes systems.1–9 Furthermore, LB monolayers on a trough have been transferred to various solid substrates, forming supported layers, with applications ranging from modifying surface properties,10 developing various biosensors,5,11,12 and high-resolution structural characterization of 2-D crystals.13–15 Supported molecular layers have also been obtained through self-assembly for surface morphological studies.16,17

LB monolayers of amphiphile molecules on an air/water interface display a variety of cooperative characteristics. Normal phases of LB monolayers are gaseous (G), liquid expanded (LE), liquid condensed (LC), and solid condensed (SC). Phase coexistence regions are also notable under certain conditions.1,2 Some monolayers have shown chiral domains with handedness directly related to their enantiomorphic configurations.18 Chirality related molecular orientational ordering and the corresponding mechanisms have also been explored on other monolayer systems.19,20 In short, LB monolayers have displayed rich structures and phases.

Scattering and spectroscopic studies have probed orientational order and revealed some information about intermolecular interactions of composite molecules in LB monolayers.1,3,6,21 Surface pressures vs area isotherms have been used to assist in phase identification.1 Imaging with optical microscopy has shown monolayer structural characteristics with the help of fluorescent dyes. All these methods and their complementary results have led to current knowledge of LB monolayers formed by a great number of molecules, including lipids, fatty acids, organic molecules, and polymers.22

In this report, we present our results of studies of LB monolayers formed with dipalmitoylphosphatidylcholine (DPPC). Two kinds of fluorescent dyes were used, 1-palmitoyl-2-{[7-nitro-2,1,3-benzoxadiazol-4-yl]amino)dodecanoyl]-sn-glycero-3-phosphocholine (NBD-PC) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N(7-nitro-2,1,3-benzoxadiazol-4-yl) (NBD-PE). The PC headgroup has been found at high concentrations in most biological membranes.23,24 Defects and domains have always been present for all of our LB monolayers, with decreased number at higher surface pressures. Their sizes range from several micrometers to several millimeters as seen under the microscope. At higher pressures, where the LB monolayers are in the LE or LC phase, linear shapes are common. The fluorescence intensity for DPPC monolayers containing the dye lipids exhibits a maximum at the full transition from the LE to the LC phase. At very low surface pressures, LB monolayers with pure fluorescence dye lipids have a large number of 2-D circular defects, like soap bubbles or slices of Swiss cheese. For all of our LB monolayers, lipid motion is a common phenomenon with...
average speeds much larger than the rate of diffusion. The speed of motion slows down dramatically as the surface pressure increases.

**Experimental Section**

**Materials and Instruments.** The dipalmitoylphosphatidylcholine (DPPC) lipids in the powder form, 1-palmitoyl-2-[12-
(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl-sn-glycero-
3-phosphocholine (NBD-PC) and 1,2-dipalmitoyl-sn-glycero-3-
phosphothanolamine-N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (NBD-
PE) in chloroform, were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. A Nima
610D Langmuir—Blodgett (LB) trough was used in our experi-
ments. The optical microscope system includes a Nikon Optiphoto
2 microscope, a PTI IC-110 CCD camera, a Pinnacone DC30Pro
video capture card, and a video recorder. Images were continu-
ously recorded with videotape and later were digitized with the
video card.

**Sample Preparation.** DPPC lipids with either NBD-PC or
NBD-PE were mixed at a ratio of 19:1 by weight, approximately
22:1 in molar ratio. The mixture was dissolved in chloroform at
a concentration of 1 mg/mL and spread onto the air/water
interface of the LB trough. The trough was inside a black cage to
avoid any stray light getting into the microscope objective.
After the initial spread, the system was usually left to stand for
at least 90 min, enough time to let the chloroform evaporate
and for the monolayer to reach equilibrium. Typical initial
pressures after the waiting period were below 1 mN/m.

Normally, a desired pressure was obtained over a 2–5 min
period by using the trough barrier slowly compressed or expanded for a
pressure change of greater than 5 mN/m at a time. Afterward, the
monolayer was again allowed to settle for 10 min. The
monolayer was then viewed, and the images were recorded. When
necessary, a shutter was employed to minimize fluorescence
quench. The whole setup, including the black cage, was placed
on a vibration isolation table.

**Average Fluorescence Intensity.** To obtain readings of
average fluorescence intensity, we used a capacitor, a diode, and
two resistors to build a low-pass rectified filter that outputs a
signal proportional to the average amplitude of the image
brightness. The signal was sensed via an analog-to-digital
converter and recorded by a computer. Therefore, we could obtain a
continuous fluorescence intensity reading vs time.

**Results**

**Monolayer Morphologies.** Both NBD-PC and NBD-
PE dye lipids are miscible with DPPC in our experi-
ments, which was supported by our surface area vs
pressure isotherms. However, completely uniform fluo-
rescence was not found for most cases with our LB
monolayers. There were often dark areas or spots within
the microscope image window. Sometimes bright regions
were observed as well.

Figure 1 shows several typical images of DPPC/NBD-
PC monolayers. At pressures less than 5 mN/m, dark
rounded objects with average diameters from several tens
to several hundreds of micrometers were seen. Part A
shows a dark area with an average diameter of about 200
μm. As the surface pressure was increased to 10 mN/m, more
features became evident. In part B we see dark
stripes with widths of several tens of micrometers.
Typically the lengths of stripes extend well over several
millimeters. In addition, we also observed regions of porous
area characterized by many small dark spots as can be
seen in part C, with the size of dark spots ranging from
several to several tens of micrometers. Part D shows
irregular dark spots and areas of various darkness and
sizes. One regular dark area is observed here with an
average diameter of about 110 μm. As the pressure was

Figure 2. Images DPPC monolayers containing NBD-PE at
surface pressures of (A) 4.0 mN/m, (B) 4.8 mN/m, and (C–D)
12.8 mN/m. Scale bar: 200 μm.

Figure 3A is a picture of a NBD-PE monolayer at 0.3 mN/m. Round dark defects,
bright islands, and ribbon stripes are prevalent. Similar
features are also present with the monolayer at 4 mN/m
(Figure 3B). Fewer features were seen at higher pressures.
However, parts C and D of Figure 3, taken at 16 mN/m, show images containing several irregular and small lipid
islands, indicating that the monolayer was still far from
optically uniform.

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However, NBD-PC monolayers displayed similar features only at surface pressures below 0.3 mN/m. Figure 4 contains examples. There are lipid islands of various sizes, with diameters ranging from tens to several hundreds of micrometers. Inside these islands, there are smaller circular dark defects devoid of lipids. These defects range in diameter from several to about a hundred micrometers. There are also line-shaped lipid islands with widths less than 100 µm extending beyond the observation window, possibly several millimeters in length. These dark circular structures are similar to some earlier results reported elsewhere. However, the existence of those irregular shapes as marked by arrows in the figures has not been shown before. Moreover, these islands did not show any significant structural change for more than an hour, in contrast to an earlier report of the evolution of the bubble-like structures over minutes for monolayers of a different lipid species.

There is a marked difference between these two dye lipids. For NBD-PE monolayers, uniformity was never observed for pressures up to about 20 mN/m. This was not the case with NBD-PC monolayers, where the monolayers were optically uniform with the exception of a few bright objects above 5 mN/m.

In addition to those monolayer features already shown in the previous section, there were also a variety of distinct patterns. Figure 5A shows two bright regions of about 100 µm in diameter, indicated by black arrows. Figure 5B shows a very bright region too but also reveals an irregularly shaped lipid island containing many small defects while its two neighboring islands are much more uniform. Figure 5C has a very dark area at the upper right corner and a number of smaller and irregular not-so-dark spots and areas covering the whole imaging area. Figure 5D has essentially similar features as those seen in Figure 5C. An interesting feature here is that a relatively darker area of a diameter about 150 µm contains a central bright region of about 50 µm in diameter. We note that these structures were obtained after approximately 2 h of an initial spread. Thus, they represent structures of some metastable states as opposed to a transient intermediate state. All these features were constantly in motion without any noticeable shape change.

Figure 6 shows more examples of monolayer patterns. The two regions in part A of apparent different structural features, marked p1 and p2, were joined together with a clear boundary. Part B shows a darker area surrounded by an even darker border. Part C shows a defect with spiral characteristics. This feature was rare in our monolayers, unlike earlier reported cases with different monolayers. Parts D and E show stripes of lipid islands with circular defects. Note in part E there is a very bright lipid island containing several lipid defects. In contrast with these features that depict clear borders of lipid islands and dark regions, part F demonstrates a gradual change in fluorescence intensity in the central region. Likewise, part G shows an area with a gradual variation of the concentration of dark spots over a range.

Fluorescence Intensity Variation and Fluctuation. To know the state of our DPPC monolayers, we also obtained their corresponding isotherms. The two isotherms in Figure 7A are consistent with an earlier report. For the DPPC/NBD-PE monolayer, we can assign the LC/LE coexistence region between 10 and 15 mN/m. For the

Figure 3. Images of NBD-PE monolayers at surface pressures of (A) 0.3 mN/m, (B) 4.0 mN/m, and (C–D) 16.0 mN/m. Scale bar: 200 µm.

Figure 4. Images of NBD-PC monolayers at a surface pressures of approximately 0.3 mN/m. Scale bar: 200 µm.

DPPC/NBD-PC monolayer, the LC/LE coexistence region is between 8 and 11 mN/m. These coexistence regions are not perfect plateaus, indicating that our systems are nonpure according to the currently accepted notion.\textsuperscript{1}

It was found that the average fluorescence intensity for DPPC monolayers containing both dye lipids decreased with an increase of the surface pressure and recovered at the reverse of the surface pressure change. Figure 7B shows our results, with DPPC/NBD-PC monolayers exhibiting a larger variation. The recovery of fluorescence intensity was not a hundred percent, possibly due to degradation of dye lipids over time. More interestingly, the fluorescence intensity showed a maximum around 15 mN/m for the DPPC/NBD-PE monolayer and 10 mN/m for the DPPC/NBD-PC monolayer. Note these maxima occurred roughly at the full transition from the LE to the LC phase. The average fluorescence intensity for monolayers of pure dye lipids did not show much variation, despite their interesting patterns.

We have also found the fluorescence intensity of our monolayer sometimes showed large fluctuations over time at a constant surface pressure. Figure 8 shows plots of fluorescence intensity vs time for four cases, with parts A and B for a monolayer DPPC with NBD-PE lipids at pressures of 8.6 and 12.8 mN/m, respectively. Parts C and D depict a monolayer of DPPC with NBD-PC at 5 mN/m. In contrast to A, B, and C, part D exhibits roughly the same intensity over the 10-min period. Since there is constant motion of the monolayer, the intensity changes signify the movement of very large areas of varying intensity passing through the image window.

**Monolayer Motion.** All features observed in the monolayers showed movement within the window of our microscope. When a droplet of lipid solution was initially applied to the trough surface, rapid movement of patterns persisted for a couple of hours before finally settling down to a moderate motion, consistent with an earlier report.\textsuperscript{2,18} In our experiments, we gave an initial waiting period of 90 min after applying a lipid droplet onto the trough surface. Then, for each pressure change, a 10-min waiting period was implemented. A relatively slow and steady
motion was always observed at pressures less than 20 mN/m. To get some quantitative analysis of the motion, we measured the speed of dark objects for several pressures. Table 1 summarizes our results. The numbers in parentheses after the standard deviations are the numbers of objects used for the measurements.

In our measurements, the image window was stationary, so we waited for dark objects to pass by as images were constantly recorded. At greater surface pressures, the speed of lipid motion was relatively slow so that the fluorescence quenching caused more dramatic image intensity decrease. This led to lower image quality, and therefore fewer objects could be resolved. We closed the shutter during our waiting period to avoid unnecessary fluorescence quenching.

At higher surface pressures, features remained at the same position yet became darker and darker as a result of fluorescence quenching. This gave us the opportunity to record a fluorescence intensity decay curve. Figure 9 shows the fluorescence intensity vs time plot for a DPPC with NBD-PE monolayer at 20.6 mN/m. With a simple exponential fitting, we obtain a time constant of 1.9 min. Other quenching curves have revealed time constants ranging from 1.5 to 2 min (data not shown). We note that typical molecular scale fluorescence quenching has a time constant on the order of nanoseconds. For fluorescence recovery on bilayer systems, the typical time constant is on the order of seconds. Thus, our observed time constant of minutes indicates that the average fluorescence signal decay within the microscope window actually measured an averaging effect of a combination of quenching and recovery.

Table 1. Speed of Domains and Defects Observed in Lipid Monolayers Several Hours after Application

<table>
<thead>
<tr>
<th>pressure (mN/m)</th>
<th>DPPC/NBD-PC speed (μm/s)</th>
<th>DPPC/NBD-PE speed (μm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \pi &lt; 4.5 )</td>
<td>227 ± 40 (5)</td>
<td>227 ± 23 (6)</td>
</tr>
<tr>
<td>4.5 &lt; ( \pi &lt; 8 )</td>
<td>72 ± 15 (10)</td>
<td>102 ± 12 (3)</td>
</tr>
<tr>
<td>8 &lt; ( \pi &lt; 16 )</td>
<td>53 (1)</td>
<td>23 ± 0.2 (2)</td>
</tr>
</tbody>
</table>

* The numbers in parentheses after the standard deviations are the numbers of objects used for the measurements.

Discussion

Our results show that at low surface pressures, LB monolayers are generally not homogeneous on a microscopic scale. This is consistent with other reports. Specifically, our monolayers contain various structural features hours after the initial spread. These features are very different in the monolayers well into the LC phase. It is evident that some of the areas are very dark, indicating a lack of lipids or an extremely wide distribution of lipids so that they are effectively in a gaseous phase. We refer to these areas as membrane defects since they are regions apparently devoid of highly packed lipids.

For pure dye lipids, the dark areas are mostly of this type. We also note that for those monolayers of pure dye lipids, only at extremely low pressures are circular defects such as 2-D soap bubbles observed. These defects have diameters ranging from several micrometers to ~100 μm. More interestingly, they mostly reside in isolated islands of dimensions ranging from tens to hundreds of micrometers. The bubble-like features are very similar to those of earlier reports. However, we observe no evolution of these features for several hours. This, along with the existence of some irregular islands, indicates a different mechanism of their formation. A clear boundary between two types of islands shown in Figure 6A also indicates that the structure within each island is stable.

For DPPC monolayers containing dye lipids, in addition to defect areas, there are also regions of various darkness. The varying intensity indicates that these regions must contain dye lipids at different concentrations than their surroundings, yet it is possible that the concentration of total lipids in these regions is not much different. Not totally devoid of lipids, we refer to these structures as membrane domains. Most domains show irregular structures without any particular shape. The existence of domains in different LB monolayers was also conjectured via nonuniform surface potential readings.

The stability of the defect and domain structures indicates that the system must be at a lower free energy state than if it were uniform. For most defects, the boundary energy is likely positive so we see rounded features that minimize the contribution of the boundary to the total free energy. For domains, we see mostly irregular shapes, indicating that the corresponding boundary energies may not be significant. However, the formation of domains must be favored since they persist for all of our monolayers.

Within the microscope window, we observed membrane domains with sizes ranging from several micrometers to several millimeters. It is conceivable that there might exist domains outside this range as well. Domains of a micrometer or smaller in diameter would not be large enough to resolve with the light microscope. Furthermore, there were apparently larger domains that were too big to be clearly differentiated with the microscope. The large fluctuations in the average fluorescence intensity must have been due to the movement of these large membrane domains beneath the objective. Constant UV light irradiation onto the LB monolayers would cause fluorescence quench but would not lead to signal recovery. According to the speed of the domains, the intensity fluctuation over time indicates that the corresponding membrane domains can be as large as 3 cm in size.

The persistent motion of membrane domains indicates that monolayers rely on convection, rather than diffusion, to reach an equilibrium (for a typical diffusion constant of \( 10^{-8} \text{ cm}^2/\text{s} \), the average diffusion rate after 10 min is below 0.1 μm/s). The lipids we observed were still in...
constant motion even after hours of standing. Therefore we can generally conclude that monolayers, even at the relatively compact LE phase, are in constant motion at equilibrium.

The fluorescence intensity has been known to depend on the molecular orientation.\textsuperscript{10,31} Our results show that there is a maximum fluorescence emission at pressures around the LE to LC phase transition. This phenomenon indicates that at the transition, the molecular arrangement more likely favors the optimum fluorescence orientation. However, on the basis of our observations that monolayer lipids are constantly in motion, it is not likely that the dye lipids actually take a particular molecular orientation. It is likely that these dye lipids undergo a large degree of orientational fluctuations and that in these fluctuations there is a certain probability of them taking the optimum orientation for fluorescence. Our results suggest that the larger the fluctuation, the larger the probability that the dye lipids take the optimum orientation for fluorescence. In this scenario, the fluorescence intensity should be generally weaker in the LC phase compared to the LE phase, because the LC phase is more closely packed, reducing the degree of fluctuations as a result of a stronger intermolecular interaction. We note that in magnetic systems the orientations, or the quantum states of magnetic moments, undergo large fluctuations around the phase transition.\textsuperscript{32} Consistent with accepted theories of phase transitions,\textsuperscript{32} our observed maximum fluorescence intensities indicate a larger fluctuation at the full LE to LC phase transition.

In conclusion, we have shown that membrane defects and domains are common for LB monolayers. Furthermore, lipids in LB monolayers are constantly in motion. The motion slows down dramatically only near the SC phase but is still much faster than the rate of diffusion.

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