Heterogeneous rotational diffusion of a fluorescent probe in lipid monolayers

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The rotational correlation time of the lipid probe 1-palmitoyl-2-\(\{6-[(7\text{-nitro}-2-1,3\text{-benzoxadiazol-4-yl})\text{amino}]\text{hexanoyl}\}\)sn-glycero-3-phosphocholine (NBD-PC) is measured using fluorescence anisotropy for two lipid species. We measure the rotational diffusion in a monolayer of 1,2-Didecanoyl-sn-glycero-3-phosphocholine (DPPC) which displays a phase transition at room temperature from the liquid-expanded to the liquid-condensed phase. The constant rotational diffusion of the probe throughout the phase transition reflects the measurement of dynamics in only the liquid-expanded phase. We contrast the dynamic changes during this phase coexistence to the continuous density increase observed in 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) at room temperature. We observe a non-exponential decay of the probe diffusion consistent with heterogeneity of the orientational dynamics.

I. INTRODUCTION

Biological lipid membranes are inherently dynamic. These dynamics are essential to their ability to adapt to new particle insertion, for the sorting of membrane proteins, and for recycling material within the structure. Dynamic models, such as the lipid raft model, suggest that the nanoscale heterogeneity of lipid structures and dynamics function to drive protein sorting and self-assembly. The mechanism for complex dynamic heterogeneity in cellular membranes remains controversial, although the observation of critical fluctuations in lipid compositions near physiological temperatures may represent one possible mechanism. However, a complete understanding of the interplay between membrane constituents and self-assembly is still lacking. Living lipid structures are extremely complex due to a myriad of lipid species, high concentration and diverse population of membrane proteins, and the variation of composition amongst different lipid structures. This complexity makes identifying universal features of dynamic behavior a significant challenge. Therefore, in this study, we consider a model lipid system that can provide a baseline for describing the distribution of dynamic clustering in free-standing lipid structures. To this end, we measure the distribution of rotational correlation times for lipids at the air-water interface to characterize the degree of dynamic heterogeneity in single constituent lipid monolayers. Such observations have implications for the heterogeneity for more complex lipid structures and biological membranes.

Lipid monolayers formed at the air-water interface have been studied by the Langmuir technique for many decades. The evolution of lipid phase and domain structure at the air-water interface has been well characterized by Brewster-angle microscopy, fluorescence microscopy, x-ray scattering, and vibrational sum frequency generation spectroscopy. In particular, the large-scale equilibrium phase behavior of single-constituent lipid monolayers are well understood and phase diagrams exist for many lipid species.
1,2-Didecanoyl-sn-glycero-3-phosphocholine (DPPC) is the most abundant lipid component in pulmonary surfactant. DPPC exhibits a phase transition at room temperature from liquid-expanded (LE) phase to liquid-condensed (LC) phase, which is characterized experimentally by a plateau in the pressure-area isotherm, see Figure 1(a). Phase changes in lipid monolayers are accompanied by changes in long-range order which impacts local packing and dynamic mobility. In particular, the condensed phase of DPPC exhibits a tilted phase in which the tails are highly ordered; however, the head groups remain disordered and rotationally mobile. The phase coexistence between the LE and LC phases occurs for molecular area \( \approx 60-80 \, \text{Å}^2 \) (and corresponding pressures between \( \approx 3 \, \text{mN/m} \) and \( 10 \, \text{mN/m} \)). In this region, fluorescently labeled lipids which preferentially partition to the LE phase can be used to image phase coexistence. Within the phase coexistence regime, the fractional area of the LE phase is reduced at the expense of the increased area of the LC phase as pressure increases. However, the average density of the LE phase remains uniform through the phase coexistence regime. Fluorescent microscopy images of the phase coexistence are displayed in the supplementary material. In contrast, a monolayer of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) near room temperature displays a continuous isotherm, indicating a single liquid phase (Figure 1(b)), and no observable partitioning of the probe in fluorescence microscopy images.

Dynamic studies of lipid films have been performed using a variety of physical techniques. Most of these studies focus on lateral translation in model membranes. Translational diffusion appears only weakly dependent upon the chemical nature of the probe. In contrast, rotational diffusion is more sensitive to the probe properties, including labelling site, polarity, and probe size and shape. We have carefully chosen a probe which strongly orients itself in the polar headgroup region of the monolayer and does not change orientation or position within the monolayer over the pressure range reported in this study. Thus, the rotational diffusion of this probe is governed by the nearest-neighbor interactions and dictated by the parent liquid phase, and can be used to assess the uniformity of dynamic freedom experienced by the probes within various lipid phases. The rotational diffusion of fluorescent probes in single-constituent lipid structures has been previously reported. For example, the average rotational correlation time of 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphocholine (NBD-PC) in a vesicle of pure DPPC with a diameter of 100 nm has been measured to be 5.04 ns at 20°C, or 2.5 ns for a vesicle diameter of 100 nm at 49.8°C.
However, the uniformity of the probe dynamics within single component lipid models has not been explored. The distribution of vesicle diameters and the presence of an inner and outer leaflet would create a distribution of membrane curvatures which would compound any interpretation of observed non-exponential behavior in vesicle models.

This study takes advantage of the adjustable pressure in a Langmuir monolayer to characterize the change in dynamic distributions of lipids as the ordering of the film is altered. Here, we describe the dynamic changes associated with rotational diffusion and nearest neighbor interactions for the LE to LC phase change associated with DPPC; we contrast these dynamic changes to the continuous LE phase of DMPC compressed over the same pressure range. We report the pressure dependent diffusive behavior of lipids in free-standing films, describing the degree of heterogeneity in the measured dynamics for these structures. This work offers insight into the local organization of lipid structures which has important consequences protein self-assembly in biological membranes.

II. EXPERIMENTAL METHODS

Lipid monolayers were prepared via the Langmuir method, and their pressure-area isotherms were monitored using a Wilhelmy film balance. DPPC; DMPC; and NBD-PC were purchased from Avanti Polar Lipids and were used without further purification. Lipid solutions were prepared in chloroform, with a 1 mol. % NBD-PC fluorescent label concentration. Monolayers were prepared on a subphase of ultra-pure water (18 MΩ). 35 µl of solution was spread on the water surface, and 15 min is allowed for solvent evaporation and layer stabilization.

NBD-PC was chosen as the lipid probe due to its location in the polar head-group region of the monolayer. The highly polar nature of the probe anchors it to the lipid-water interface, which allows the probe to remain in the same location and orientation through the experimental conditions probed in this study. We verified a uniform orientation and hydration state by monitoring the steady state fluorescence spectrum of NBD upon excitation using an Ocean Optics Spectrofluorimeter and fiber optic collection. This allowed us to collect sample fluorescence in-situ. The fluorescence maxima of NBD—excited with 463 nm light, and collected for a monolayer at the air-water interface—was measured to be 544 ± 1 nm for all samples and pressures. We have also monitored the lifetime of the probe which was measured to be 6 ns and invariant to changes in pressure, see supplementary material. These data give us confidence that the orientation and location of the probe within the monolayer is constant, and that the relative size and shape of the probe is not changing during the compression. Thus, the observed changes in rotational dynamics are due to the microviscosity of the local environment, not due to the probe properties.

A. Langmuir system

Our Langmuir trough was milled from a solid piece of Teflon, and has a maximum working area of ~400 cm². The temperature of the subphase is controlled by circulating water bath through the aluminum base of the trough. The surface pressure-area isotherm was monitored by controlling the positions of two computer-controlled DC motors which position Delrin barriers to provide symmetric film compression. The rate of compression and position of the barrier are controlled using a Labview feedback program, which continuously monitors the pressure of the film by the Wilhelmy plate method (Nima PS4 Pressure Sensor). Films were compressed at the rate of 5 cm²/min to the target pressure and then held constant for the duration of the time-resolved fluorescence anisotropy (TRFA) experiments. Our trough and microscope are enclosed to minimize the effects of layer drift and subphase evaporation.

B. Wide-field TRFA microscope

We have built a time-resolved fluorescence microscope (Figure 2) that makes measurements at the air-water interface. There are two modes of operation: layer imaging and
measurement of time-resolved dynamics. The combination of our imaging capabilities, tunable and highly selective ultrafast excitation, wide-field excitation, and use of reflective objective (which minimizes chromatic aberration and dispersion) are a novel and flexible combination. We use layer imaging to quantify the phase separation and probe partitioning of the sample, and TRFA for measuring probe dynamic distributions. See supplementary material\textsuperscript{18} for fluorescent images of the DPPC phase coexistence and probe partitioning.

TRFA is used to characterize the rotational diffusion of the probe. The experimental apparatus consists of a tunable ultrafast oscillator (Coherent Chameleon Ultra II, 140 fs), pulse selector (Conoptics model 305), frequency doubling crystal (LBO Type 1, 5 × 5 × 2 mm), and Cassegrain focusing objective (15×, Infinite BFL). Our excitation beam is tuned to 463 nm and has a power of 20 mW at 10 MHz. We employ a wide-field excitation at the air-water interface with a beam diameter of ~500 μm. By avoiding collinear confocal excitation, we avoid depolarization caused by large NA focusing objectives. In order to measure TRFA signal, we employ traditional time correlated single photon counting (TCSPC) techniques.\textsuperscript{34} We use two single photon avalanche photodiodes (SPAD, id100 ID Quantique), a router (HRT-41), and TCSPC module (SPC-1300 Becker-Hickl). Our instrumental response time is 94 ps FWHM determined by measuring the Raman scattering of water. For TRFA, probe signal fluorescence is collected using a Cassegrain objective, and focused through a Wollaston polarizing beam splitter (Thorlabs, WP10). Simultaneous detection of the two polarization channels is accomplished by focusing each beam through a monochromator and onto the SPAD detector using achromatic lenses.

Most measurements of time-resolved dynamics in membrane structures are investigated using confocal microscopy or epifluorescence microscopy, both of which are limited to collinear excitation/detection. This restricts the observation of dynamics to molecules whose chromophores have an excitation and emission dipole parallel to the plane of the monolayer. Our system employs a Cassegrain objective which allows for a significant layer gap between the sample and the objective. This allows us tremendous flexibility in excitation and collection angle. Our system can switch between a perpendicular excitation using a small right angle mirror mounted to the face of the objective, or a grazing angle excitation which allows us to probe fluorophore subpopulations which may have significant mobility out of the plane of the membrane.

Upon excitation with polarized light, the emission from fluorophores is also polarized. The extent of the emission polarization alignment is described in terms of the anisotropy. The ability to excite probe molecules with excitation dipole orientation both parallel and perpendicular to the monolayer represents a significant advantage of our TRFA instrument. This allows the user to monitor changes in molecular orientation or characterize the degree of molecular
freedom such as wobbling perpendicular to the membrane surface. The rotational diffusion and mobility of the fluorescent probe is monitored by calculating the anisotropy

$$r(t) = \frac{I_{\|}(t) - GI_{\|}(t)}{I_{\|}(t) + 2GI_{\perp}(t)};$$

where $I_{\|}$ is the signal intensity with polarization parallel to the excitation polarization, $I_{\perp}$ is the signal intensity with polarization perpendicular to the excitation polarization direction, and $G$ represents the correction factor for the detector and monochromator sensitivity to different polarization orientations. The $G$-factor is determined experimentally by the tail matching method using fluorescein in pure water. A value of $145 \pm 16$ ps was obtained for the rotational correlation time of fluorescein consistent values in the literature. Time-resolved anisotropy represents the rate of reorientation of the excitation dipole moment with respect to the excitation polarization vector, assuming that the angle between the excitation absorption dipole and the emission dipole moment is approximately zero.

III. RESULTS AND DISCUSSION

We obtained time-resolved fluorescence anisotropy for DPPC and DMPC at different surface pressures. Figure 3 shows a typical experimental measurement of TRFA signal fluorescence using perpendicular excitation. The anisotropy decay is fit with stretched exponential by model

$$r(t) = r_\infty + r_1 \exp \left( -\frac{t}{\tau_1} \right)^\beta.$$
The stretched exponential model represents the model with the fewest parameters which can accurately describe the dynamic distributions observed for all of our samples. However, the data have also been analyzed using a Maximum Entropy Model (MEM). Figure 3, right panel. The MEM analysis fits the decay with no assumptions about the number of decay processes or the shape of the distribution, thus it represents an un-biased fit. However, the analysis can provide additional decay components without a significant improvement in the quality of the fit. An extended discussion of the fitting procedure and MEM results are given in the supplementary material. The MEM analysis clearly displays a growing dynamic distribution for DMPC and a more uniform distribution for DPPC, and the results are consistent with the distributions described by the stretched exponential model which is discussed in detail below.

We evaluated the rotational diffusion of NBD-PC in two different physical systems; one that undergoes a phase transition from the LE to LC phase, and another that remains in a single disordered phase throughout the compression. For DPPC, we describe the changes to rotational reorientation starting from the LE phase and compressing though coexistence until we reach the LC phase. In the coexistence region, the liquid phase has a rotational correlation time of 2.4 ns (see Figure 4 and Table 1). Essentially, the observed behavior of DPPC is insensitive to pressure since the probe always partitions to the LE phase. This measured value is smaller than rotational correlation times observed for a fluorescent probe a DPPC vesicle at 20 °C, which is consistent with the finding that lipid diffusion in a Langmuir monolayer is significantly enhanced, and is about two times faster than that of the plasma membrane.

Within the LC phase of DPPC, our probe excitation dipole moment becomes highly ordered with an orientation perpendicular to the plane of the monolayer. This is observed by a large loss in the fluorescence intensity under perpendicular illumination and measured anisotropy times that are significantly reduced (τ ~ 0.7 ns). We interpret the reduction of reorientation time to the observation of a small subpopulation of probes with a wobbling component parallel to the plane of the membrane such that this subset represents a non-equilibrium population of probe orientations. We are unable to accurately measure the rotational diffusion of this probe at these pressures due to the ordering of the excitation dipole moment out of the plane of the membrane. However, the use of an alternate probe, such as a headgroup labeled molecule that partitioned to the ordered phase, may allow one to measure dynamics in the LC phase.

FIG. 4. Rotational correlation time as a function of pressure for 1% NBD-PC labeled in a DPPC monolayer which exhibits phase coexistence of the LE and LC phase for surface pressures of 3.5–10 mN/m (the probe preferentially partitions to the LE phase); and a DMPC monolayer which represents the compression of a uniform liquid phase. Both data sets are taken at a temperature of 20 °C.
The pressure independent rotational dynamics observed for NBD-PC in the phase coexistence regime of DPPC are to be contrasted with the dynamics observed for the compressed liquid phase of DMPC. For all pressures observed in this study, DMPC exhibits a single LE phase with uniform distribution of fluorescent probe. As we increase the pressure on this liquid phase, we observe an increase in the rotational correlation time (Figure 4) and a decreasing half-cone angle obtained from 40° to 30°, assuming a wobbling in cone model. This behavior is consistent with the expected increase in nearest neighbor interactions due to increasing density and correlated motions.

The values of the stretched exponential fitting parameters are shown in Table I. The values of β in the stretched exponential fitting demonstrate a distribution of dynamics exhibited by the probes in our monolayer, for DMPC the distribution grows as we compress the film toward a condensed state. Such stretched-exponential relaxation is characteristic of systems that exhibit a broad range, or heterogeneity, of relaxation. A value for β < 1 is typical, and is observed for systems ranging from glass-forming fluids to proteins. The weak variation of β in DPPC suggests that the distribution of dynamics remains unchanged for the LE phase of DPPC as pressure is increased, which is expected for phase coexistence. The departure from purely exponential behavior is indicative of a range of probe microenvironments and could represent the presence of nano-scale density fluctuations similar to those observed for many liquid condensed matter systems.

Heterogeneous dynamics has been previously reported for lipid diffusion in membrane systems. The two experimental demonstrations include a measure of steady-state single molecule anisotropy distributions of fluorescent probes in the liquid-ordered state of ternary lipid mixtures and polymer lateral diffusion in a supported lipid bilayer in the disordered phase. The added complexity of phase separation in ternary mixture can cause small compositional variations around the probe molecules which complicate interpretation of observed heterogeneity. Such added complexity is not an issue in the present study. In the second experimental demonstration, the diffusion of polymers in the fluid state of a single-constituent lipid film is monitored. The presence of a large perturbing molecule can influence heterogeneity in liquids; however, it has been found that guest molecules larger than the host molecules actually underreport the heterogeneity of the host liquid. Other measurements of lipid diffusion in vesicle models have not discussed the heterogeneity of rotational dynamics. Perhaps, this is due to complicating geometric factors such as; the presence of two leaflets which could possess distinct rotational freedom, as well as a distribution of vesicle size which can impact membrane curvature, and thus compound the interpretation of these results. The current study does not suffer these limitations, and the broad distribution of observed diffusion times can be viewed as intrinsic to the lipid dynamic freedom.

The presence of a non-exponential relaxation in the rotational correlation of the lipid probes indicates heterogeneity in the probe microenvironments. The variability of local dynamic

| Lipid | Pressure (mN/m) | $r_1$ | $t_1$ (ns) | $\beta$ |
|-------|----------------|------|------------|--------|
| DMPC  | 3.5            | 0.18 ± 0.01 | 1.71 ± 0.19 | 0.98 ± 0.12 |
| DMPC  | 5              | 0.2 ± 0.01  | 2.04 ± 0.17 | 0.86 ± 0.07 |
| DMPC  | 7              | 0.19 ± 0.01 | 2.3 ± 0.23  | 0.92 ± 0.1 |
| DMPC  | 10             | 0.18 ± 0.01 | 2.84 ± 0.21 | 0.93 ± 0.08 |
| DMPC  | 15             | 0.19 ± 0.01 | 3.11 ± 0.12 | 0.84 ± 0.03 |
| DMPC  | 20             | 0.18 ± 0.01 | 4.34 ± 0.27 | 0.81 ± 0.06 |
| DPPC  | 3.5            | 0.18 ± 0.01 | 2.42 ± 0.22 | 0.87 ± 0.08 |
| DPPC  | 5              | 0.15 ± 0.01 | 2.33 ± 0.27 | 0.94 ± 0.12 |
| DPPC  | 7              | 0.16 ± 0.01 | 2.56 ± 0.35 | 0.9 ± 0.11  |
| DPPC  | 10             | 0.1 ± 0.01  | 2.23 ± 0.49 | 0.88 ± 0.2 |
freedom can be caused by dynamic density fluctuations which result in regions of enhanced mobility. Such dynamic clusters have been observed for a large range of strongly interacting condensed matter systems. These dynamic fluctuations are very small (~10 nm) and short lived (10 μs). Dynamic clustering in lipid membranes could have a large impact on membrane recognition, transport, and protein self-assembly in biological systems. Though not a direct observation of dynamic clusters; the measurement of dynamic distributions in lipid diffusion, as presented in this study, warrants much closer examination. Langmuir monolayers are known to display increased dynamic freedom, thus we might expect that the added constraints within the cellular membrane might significantly enhance the dynamic heterogeneity of lipid motion. Specifically, the equivalent pressure within the lipid bilayer is reported to be about 32 mN/m, thus we might expect local density fluctuations to play a significant role in membrane lateral density according to our data. Combining the results of rotational and translation relaxation in lipids offers a route to examine the heterogeneity and possible decoupling of translational and rotational relaxation mechanisms; which may play an important practical role in the biological function of living lipid systems.

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