Lipid Membrane Reorganization Induced by Chemical Recognition

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ABSTRACT Nanoscale structural reorganization of a lipid bilayer membrane induced by a chemical recognition event has been imaged using in situ atomic force microscopy (AFM). Supported lipid bilayers, composed of distearylphosphatidylcholine (DSPC) and a synthetic lipid functionalized with a Cu^{2+} receptor, phase-separate into nanoscale domains that are distinguishable by the 9 Å height difference between the two molecules. Upon binding of Cu^{2+} the electrostatic nature of the receptor changes, causing a dispersion of the receptor molecules and subsequent shrinking of the structural features defined by the receptors in the membrane. Complete reversibility of the process was demonstrated through the removal of metal ions with EDTA.

INTRODUCTION

The dynamic association and aggregation of receptors and lipids within the cell membrane dictates numerous events (Alberts et al., 1994), including signaling, adhesion, raft formation (Brown and London, 1998), and cell motility. By monitoring the movement and positioning of membrane components, and the subsequent nanoscale structures they produce, we may begin to understand the supramolecular constructs that are responsible for specific cellular responses. The complexity of the cell membrane and the difficulties encountered in labeling or identifying surface features, however, makes nanometer level characterization a formidable task. Simple models of cell membranes coupled with analytical techniques that allow near-molecular-scale resolution can aid in revealing the simple to complex interactions that dictate membrane organization.

Spectroscopic and microscopic techniques are powerful analytical tools to evaluate membrane reorganization. For example, fluorescence microscopy was used to visualize the dynamic motions of T-cell receptors following activation by the major histocompatibility complex (MHC) (Grakoui et al., 1999). In spectroscopic experiments, fluorescence resonance energy transfer (FRET) revealed details about the preassociation and heterodimerization of a dopamine receptor and a somatostatin receptor (Rocheville et al., 2000). Synthetic lipid bilayer systems have also been used to gain insights into the complex dynamic processes occurring within the cellular membrane in response to chemical recognition events. These synthetic systems were designed to utilize fluorescence or colorimetric changes as reporters of the receptor aggregation or dispersion that occurs due to hydrogen bonding interactions (Ariga and Kunitake, 1998), protein (Song et al., 1998; Maloney et al., 1996, 1999) or polypeptide (Ng et al., 1995) binding, virus recognition (Reichert et al., 1995), or metal ion chelation (Singh et al., 1992; Sasaki et al., 1995; Sasaki and Padilla, 1998).

Although optical techniques, such as those described above, provide valuable information on either averaged molecular associations through fluorescence (e.g., FRET, excimer formation) or the positioning of receptors in a membrane at the micron scale, the nanoscale structures these molecular associations define are unknown. Such information would yield deeper insights into the physical and chemical forces that cause phase separation or receptor aggregation, and the supramolecular assemblies they produce and enable. Scanning probe techniques can provide the means to visualize reorganizational processes in these bilayer systems at the nanometer level to monitor and evaluate these intermolecular associations. Atomic force microscopy (AFM) has been used previously to visualize biological molecules and processes, including phospholipid bilayer formation (Singh and Keller, 1991; Egawa and Furusawa, 1999; Reviakine and Brisson, 2000), the assembly of proteins into crystalline structures (Yip et al., 2000), the stability of phospholipid bilayers (Hui et al., 1995), and the binding of antibodies and receptors (Shibata-Seki et al., 1996; Weisenhorn et al., 1990). The advantages of this technique include the ability to image biological systems in their native form under physiological conditions using very small forces (nanoNewtons) to probe the sample. AFM could also be used to monitor structural changes occurring in lipid membranes as they respond to physical or chemical stimulation. Such studies of dynamic reorganization of molecular assemblies could aid in understanding fundamental processes in cell biology and provide a platform for the development of unique nanostructured synthetic materials with addressable features.

In this report we describe the first direct observation of a reversible molecular reorganization in a lipid membrane actuated by a chemical recognition event. In situ AFM provided the means to view changes of nanoscale features in a supported lipid bilayer as receptor molecules moved in response to the binding or removal of a specific chemical agent (i.e., metal ion). A two-component bilayer composed of lipids functionalized with a metal ion receptor

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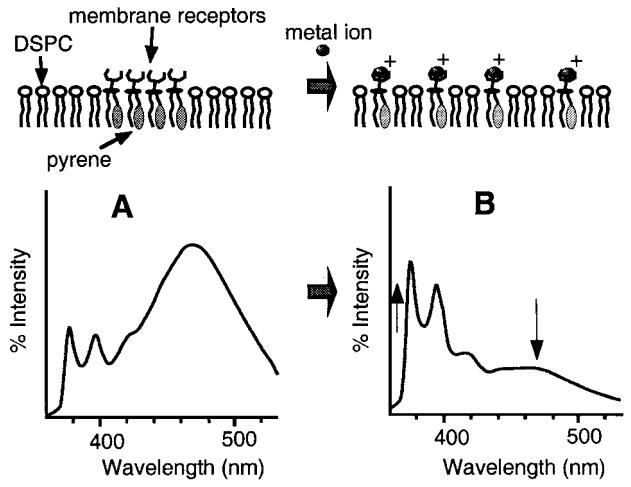
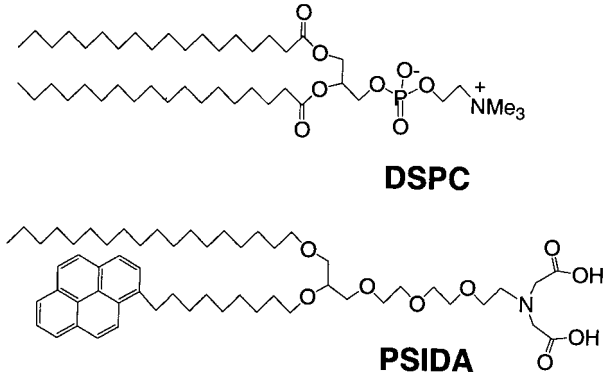


FIGURE 1 *Top:* A schematic illustrates the dispersion of the receptor molecules in one leaflet of a lipid bilayer (*A*) before and (*B*) after metal ion binding. *Bottom:* The corresponding fluorescence spectra of 5% PSIDA/DSPC bilayers (λ_{ex} = 346 nm) showing the changes in excimer (470 nm) and monomer (375 nm) intensities upon metal ion recognition.

pyrenenonyl stearyl-glycero-imido diacetic acid (PSIDA) and a phospholipid, distearylphosphatidylcholine (DSPC), served as our receptor imbibed membrane. Although the system is quite primitive compared to a cellular membrane, it does provide a unique platform to readily study chemical recognition-induced membrane reorganization. In previous work we found that the aggregational state of receptors was dictated by selective metal ion chelation at the membrane surface (Sasaki et al., 1995; Sasaki and Padilla, 1998; Sasaki and Waggoner, 1999). A pyrene label on the receptor provided information on the aggregational state of the lipids through the monitoring of relative intensities of the pyrene excimer (λ_{max} = 470 nm) and monomer (λ_{max} = 375 nm) fluorescence emissions (Fig. 1). However, the nanoscale details of the aggregated structures were unknown. In the current work we show that chemical recognition can controllably alter the size of nanoscale domains of lipid aggregates, and do so with high chemical selectivity and reversibility.



Scheme 1

MATERIALS AND METHODS

Liposome preparation

DSPC was purchased from Avanti Polar Lipids (Alabaster, AL). The synthetic preparation of the PSIDA receptor-lipids is described in detail elsewhere (Ng et al., 1995). The liposomes were prepared by dissolving DSPC and PSIDA in chloroform at various mole ratios (e.g., 5, 10, and 20% PSIDA/DSPC) with total lipid concentrations of ~1.5 mM. The solution was evaporated to a thin film on the walls of a glass centrifuge tube using a rotary evaporator. Residual solvent was removed from the film by further drying overnight under high vacuum. The film was then hydrated in 3.0 ml MOPS buffer solution (0.02 M 4-morpholino-propanesulfonic acid, 0.1 M NaCl, in 18 MΩ water, pH 7.4) at 65°C with vortex stirring, producing a total lipid concentration of 3.5 mM. The completely suspended film was then degassed with N_2 gas for several minutes, followed by sonication with a 3-mm probe tip at 25 W power under N_2 gas. Sonication was performed in 4-min cycles with 1 min resting between each cycle, for a total of 20 min at room temperature. The translucent solution was centrifuged for 20 min at 15,000 × *g* to remove large bilayer aggregates. The supernatant was then filtered through a 0.2 μm filter. Liposome sizes range between 400 nm and 700 nm in diameter as determined by transmission electron microscopy (TEM) (Sasaki and Waggoner, 1999).

Atomic force microscopy

AFM experiments were performed with a Nanoscope IIIa Multimode scanning probe microscope (Digital Instruments, Santa Barbara, CA). The images were acquired in tapping mode in solution using a commercially available liquid cell (Digital Instruments) with 120-μm oxide-sharpened silicon nitride V-shaped cantilevers. The nominal spring constant of the cantilever was 0.35 N/m. Images were collected with the J scanner, which has a maximum range of 120 × 120 μm, operating at a scan rate of 2 Hz. The images were collected with 256 data points per line.

Supported lipid bilayers were prepared via vesicle fusion on freshly cleaved mica substrates. The clean mica was first imaged in MOPS buffer to establish the baseline. The liposome solution was then injected into the AFM liquid cell and the mica surface imaged subsequently. The liposome solution was incubated with the mica at room temperature for ~1 h to allow optimal time for vesicle fusion, resulting in full bilayer coverage of the substrate.

Fluorescence spectroscopic studies

Fluorescence spectra of PSIDA/DSPC liposomes were obtained on a SPEX Fluoromax II (Instruments S.A., Edison, NJ) spectrophotometer using an excitation wavelength of 346 nm and 5-nm slit widths. All sample solutions were analyzed at 20 ± 0.1°C using a water-jacketed cell. The fluorescence excimer to monomer (E/M) response curve of 20% PSIDA/DSPC bilayers to increasing $CuCl_2$ concentration is shown in Fig. 2. The curve is about a factor of 100 less sensitive to Cu^{2+} ion than that observed for the 5% PSIDA/DSPC bilayer. Such lipid mole fraction-dependent behavior is consistent with studies conducted previously (Sasaki et al., 1995).

RESULTS AND DISCUSSION

Flat, homogeneous bilayers of PSIDA and DSPC were formed on freshly cleaved mica via the vesicle fusion technique (Egawa and Furusawa, 1999; Reviakine and Brisson,

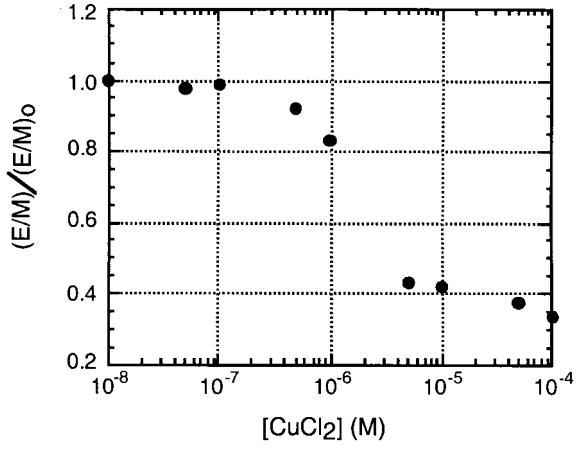


FIGURE 2 Fluorescence E/M response curve of 20% PSIDA/DSPC liposomes (in MOPS buffer, pH 7.4) versus increasing $CuCl_2$ concentration in solution.

2000; Brian and McConnell, 1984; Lipowsky and Seifert, 1991) and imaged by in situ AFM. Bilayer compositions of 5, 10, and 20 mol % PSIDA/DSPC were prepared and imaged. All compositions exhibited similar features; however, the 20% PSIDA/DSPC bilayer composition provided the clearest features and contrast by AFM, due primarily to the larger lateral features formed with this mole ratio. The AFM images suggest a phase-separated system (Fig. 3 *A*), consistent with the fluorescence spectral data (Fig. 2). Three predominant areas representing different heights were observed. The darkest regions in Fig. 3 were assigned to the mica surface. The medium gray regions were measured as 52 ± 4 Å taller than the mica surface, commensurate with the combined expected height of a phospholipid bilayer (47 Å) sitting above a water layer (10 Å) on the mica surface (Lis et al., 1982; Johnson et al., 1991). The brightest regions were measured as 10 ± 3 Å taller than the DSPC bilayer surface. Molecular modeling studies found that the iminodiacetic acid headgroup of PSIDA extends up to 9 Å further into solution relative to the DSPC bilayer surface, and upon examination of bilayers prepared with various mole fractions of PSIDA to DSPC, the total area of these brightest regions was found to change proportionately with the PSIDA concentration. Thus, the brightest regions could be assigned to the aggregates of PSIDA. The images observed are assumed to reflect the structural features of the outer leaflet of the bilayer only; that is, we do not expect transference of structural information from the lower leaflet because the topological features of the lower leaflet are accommodated by the ~10 Å water layer existing between the mica and the bilayer.

The AFM images indicate that the liquid-phase PSIDA molecules ($T_c < 20$°C) (Sasaki et al., 1995) tend to aggregate in regions between the crystalline domains of DSPC ($T_c = 55$°C). These nanoscale structures were completely

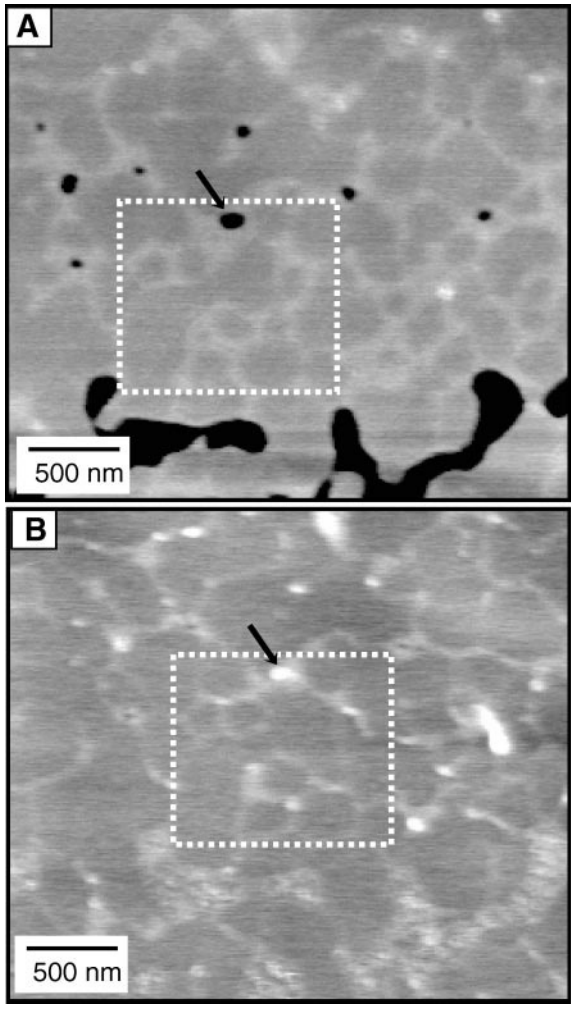


FIGURE 3 AFM tapping mode images in solution of 20 mol % PSIDA/DSPC (*A*) before and (*B*) after addition of 1 μM $CuCl_2$. The white boxes identify identical 1 μm^2 areas of the two images. The arrows indicate a hole in the bilayer that fills with PSIDA molecules after addition of $CuCl_2$.

stable under the imaging conditions. Features as fine as 20 nm (~30 molecules wide) are clearly resolved. A network of PSIDA aggregates extends throughout the bilayer with widths that range from tens to hundreds of nanometers.

Upon the addition of $CuCl_2$ to the bilayers, a molecular reorganization of receptor-lipids in the phosphatidylcholine matrix was observed as a response to the chemical recognition event between the iminodiacetic acid headgroup and Cu^{2+}. Metal ion recognition changes the physical properties of the receptors (e.g., electrostatic charge) causing them to repel each other and disperse. Previously, we reported evidence suggesting the change in electrostatic charge of the receptor, from neutral to positive, as the cause of the dispersion of lipid aggregates (Sasaki and Waggoner, 1999). The present experiment was performed by adding a solution of 1 μM $CuCl_2$ in MOPS buffer to the solution cell followed by subsequent imaging of the bilayer (Fig. 3 *B*). In going

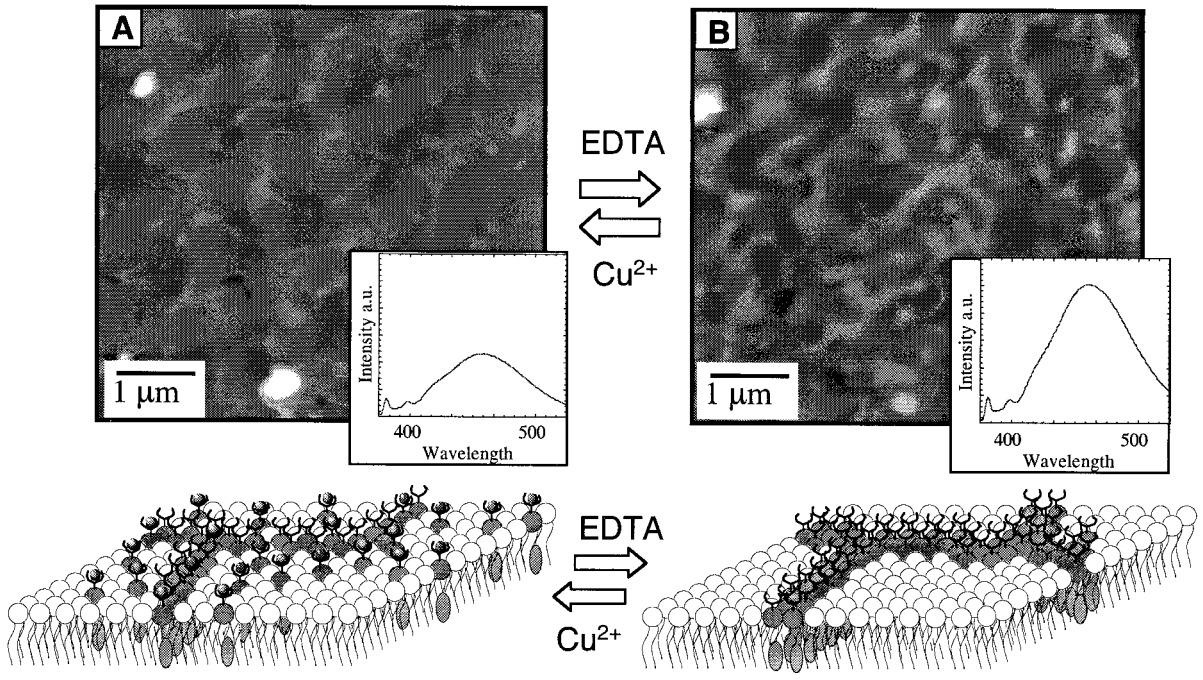


FIGURE 4 Reversible aggregation and dispersion of receptor molecules (PSIDA) induced by the addition or removal of Cu^{2+} as imaged by AFM. *Top:* Time sequence AFM tapping mode images are shown of 20 mol % PSIDA/DSPC bilayers (*A*) after the addition of 1 μM $CuCl_2$ and (*B*) following the subsequent addition of 10 μM EDTA. Insets in the AFM images show fluorescence spectra of liposomes of identical composition under the same solution conditions. *Bottom:* A schematic illustration of the Cu^{2+}-recognition induced reorganization of receptor molecules. (*Left*) Recognition and binding of the metal ion causes a dispersion of receptors into the DSPC matrix. (*Right*) Removal of Cu^{2+} by EDTA allows the receptor molecules to phase-separate from the DSPC matrix, returning to their initial aggregated state.

from Fig. 3 *A* to 3 *B*, the brightest areas (PSIDA aggregates) have decreased in size, although the general shapes remain the same. Molecular reorganization in the lipid bilayer was not observed with the addition of $MnCl_2$ or $CaCl_2$ up to 10 μM concentrations. A notable difference between the images before and after the addition of Cu^{2+} is the filling of holes in the bilayer with what appears to be material containing the taller receptor-lipids. The holes, which remain as stable features in the bilayer over long periods of imaging, filled immediately upon addition of Cu^{2+}. This rapid and reproducible actuation of the film indicated that the filling was due to the receptor-lipid dispersion and not a result of repeated scanning with the AFM tip. Closer examination of these regions finds a low packing density of receptor-lipids and no observable mica surface. Dispersion of PSIDA molecules into the DSPC domains may alter the fluidity of the membrane, allowing both lipids to subsequently flow into the holes.

Distinct features within the lipid bilayer allowed a tracking of specific areas to monitor and quantify the structural changes that occurred following chemical recognition. Dashed-line boxes in Fig. 3, *A* and *B* show a 1 μm^2 area, chosen for its unique features, before and after addition of Cu^{2+} to the 20% PSIDA/DSPC membrane. A pixel analysis was used and the lightest, medium, and darkest intensities were assigned to PSIDA, DSPC, and the mica surface, respectively. Over large areas (>25 μm^2) of the bilayer, the pixel ratio and the molar ratio of PSIDA to DSPC lipids were equivalent. Locally, however, the lipid ratios can vary. Within the 1 μm^2 box of Fig. 2 *A*, for example, 36% of the image was attributed to the aggregated PSIDA receptor molecules. Following the addition of Cu^{2+}, these regions of PSIDA in the 1 μm^2 box decreased to $\sim$15%. This represents a dispersion of 60% of the aggregated PSIDA molecules into the DSPC matrix. The fluorescence E/M response of the same bilayer composition of liposomes at the same Cu^{2+} concentration, however, produces only a third of a maximum response (Fig. 2). A maximum response, produced at $[CuCl_2] \geq 10$ μM, is associated with the highest possible dispersion of PSIDA. Full dispersion of the receptor lipids, which can be induced at concentrations >10 μM $CuCl_2$, results in a complete loss of the nanoscale features defined by the PSIDA lipid aggregates in the bilayer. A partial dispersion of PSIDA aggregates, as was used above, allowed us to maintain some of the initial aggregate structure and follow specific areas in the AFM images.

Under partial dispersion conditions, PSIDA molecules not remaining in their aggregates after Cu^{2+} addition have either moved to holes in the bilayer or dispersed into the DSPC domains. Examination of other areas far removed (>10 μm) from holes showed similar Cu^{2+}-induced behavior, suggesting that PSIDA dispersion into the DSPC do-

mains occurs readily. The limit of our resolution, however, fails to identify the position of single molecules of PSIDA. Assuming that the behavior of the bilayer in liposomal form is nearly identical to that supported on a mica surface, we can couple the fluorescence data of Fig. 2 with the AFM images to infer detailed information on the positioning of molecular species relative to the lipid aggregate structures. The combined data indicate that although the aggregate structures have thinned considerably, the PSIDA molecules remain at high local concentration. This suggests that the dispersed receptors must exist within close proximity of the remaining aggregated receptors.

The membrane reorganization observed above can be reversed through the removal of metal ions from the membrane receptors. Fig. 4 shows a 20% PSIDA/DSPC bilayer that was switched between dispersed (4 *A*) and aggregated (4 *B*) states using Cu^{2+} to induce dispersion and EDTA (Cu^{2+} scavenger) to return the bilayer to its original phase-separated state. The corresponding fluorescence spectra of the bilayers, as liposomes, are shown in the insets. The addition of Cu^{2+} and EDTA was executed by slowly flushing the solution cell with aqueous solutions of $CuCl_2$ (1 μM) or EDTA (10 μM). This process could be repeated several times with nearly identical results. Further flushings usually gave AFM images that indicated degradation of the supported bilayers from turbulent fluid flow. This observed reversibility of phase separation suggests that the self-assembled structures have some size and shape memory for their aggregated states. It is quite plausible that the solid phase domains of the DSPC serve as a pseudo-skeletal structure that sets the coarse structural features of the membrane. The DSPC domains define the PSIDA aggregate shape and size during phase separation, yet can act as a "sponge" to absorb dispersing PSIDA molecules following activation via chemical recognition of Cu^{2+}. The DSPC domains thus act as reservoirs of receptor lipids, storing them for ready release at any time by removal of the chemical signal (i.e., Cu^{2+}). The Cu^{2+}-free receptor lipids are then free to reassemble into nanoscale structures in the membrane.

We have shown here that chemical recognition-induced molecular reorganization can rapidly and reversibly alter nanoscale structures in self-assembled materials. The phenomenon observed above demonstrates how a simple change in the physical properties of a receptor, through host-guest complexation, can actuate a rapid change in molecular assembly structure. Although cellular membranes can perform such tasks in fluidized bilayers with considerably higher fidelity and specificity, our phase-separated films may at least provide some insight into the imaging capabilities and possible chemical phenomena that can control the specific and reversible molecular reorganization within lipid bilayers. Beyond biological models, functionalized lipid bilayers may also provide a unique, chemically addressable material that can yield novel structures for nanoscale engineering and architecture.

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