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Lipid domain pixelation patterns imposed by e-beam fabricated substrates

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Abstract

This work describes a technique for forming nanometer-scale pixelated lipid domains that are self-organized into geometric patterns residing on a square lattice. In this process, a lipid multibilayer stack is deposited onto a silica substrate patterned with a square lattice array of bumps, hemispherical on their sides, formed by electron beam lithography. Domain patterns are shown to be confined to the flat grid between the bumps and comprised of connected and individual domain pixels. Analysis of lattices of varying sizes shows that domain pattern formation is driven by mechanical energy minimization and packing constraints. We demonstrate single lattice sizes and a gradient in lattice size varying from the micrometer to the 100 nm scale applicable to precise arraying, patterning, and transport of biomolecules that partition to lipid domains.

Introduction

Lipid mixtures self-assemble to form planar bilayers containing coexisting lipid phases in many compositional regimes.^{1,2} Coexisting lipid phases each have distinct compositions that selectively partition optically active dyes, ligands, and membrane proteins.^{3,4,5,6,7,8} With the exception of compositions near a critical point, coexisting lipid phases exist as microscopic domains owing to high line energy, and their size is non-uniform.^{1,9} Therefore, a significant challenge exists in forming uniformly sized lipid domains existing as single addressable elements that could be arrayed at high density and provide compatibility with current nanometer-scale device elements. Formation and arraying of these domain “pixels” in a high throughput manner could be used to create optical devices, biosensor arrays, drug delivery devices, and artificial organelles where the major advantage is that the positioning of the domain pixels could be dynamically controlled by applied forces, e.g. electric fields. In contrast, most current lipid bilayer patterning techniques form compositionally well-defined, but disconnected, patches of lipid bilayers that lack positional flexibility.^{10,11,12,13,14,15}

Imposition of membrane curvature provides a means to guide the growth (both position and size) of phase separated lipid domains according to differences in bending moduli of the coexisting phases and line tension. Curved substrates formed through various means such as standard photolithography, polymerization of PDMS on a template, and wrinkling of PDMS have been demonstrated to guide and pattern the growth of lipid domains of supported lipid bilayers.^{16,17,18,19} In addition, these studies strengthen the argument that curvature plays an

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Supporting Information: Detailed methods, calculations, and figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

important role in lipid sorting and localization to the membranes of cellular organelles.²⁰ However, these methods for forming the curvature patterns are limited in resolution and control of the pattern. Electron beam (e-beam) lithography, on the other hand, has been used to make curved features in the nanometer range that can be highly repetitive and/or vary spatially.^{21,22} An additional challenge is the requirement for the phase separated lipid bilayer to be removed by several nanometers from the substrate, to limit the strong non-curvature related influence of the solid substrate on lipid phase behavior. Parsatharthy developed a low throughput technique to form a double bilayer, where the upper bilayer is decoupled from the substrate by rupture of giant vesicles on an oppositely charged supported bilayer.¹⁶ Simonsen has shown that similar results can be obtained through high throughput spin-coating of a lipid film followed by rehydration, without the requirement for a charged lipid.²³

Here we demonstrate the formation of patterns comprised of lipid domain pixels in supported lipid bilayers. These domain patterns reflect the underlying curvature patterns in the substrate, square lattices of hemispherical features “bumps” formed by standard e-beam lithography of a poly(methyl methacrylate) (PMMA) layer on silica. We show that nanometer-scale resolution in the domain patterns can be achieved when the lattice spacing of the bumps is in the nanometer-scale, a scale routinely achievable using e-beam lithography. In addition, we demonstrate that the resolution of the domain patterns can be varied in the same continuous lipid bilayer by varying the lattice spacing. Our lipid bilayers were formed by spin coating followed by hydration to implement a high throughput, broadly applicable method for forming lipid bilayers removed from the substrate by several nanometers. The lipid domains were of the well-studied, and biologically relevant, liquid ordered (L_o) phase, where the lipids have high lateral mobility but their acyl chains are ordered, coexisting with a liquid disordered (L_d) phase, where the acyl chains are disordered.² To move this technology toward the applications mentioned in the first paragraph would require the incorporation of active molecules (e.g. dyes and membrane proteins) as well as design features to drive the precise arrangement of the domain pixels such as an underlying array of nanoelectrodes or an optimized curvature pattern, and sensing elements such as nanophotodiodes – all possible with current e-beam lithography technology.

Materials and Methods

Details of the sample preparation and experimental measurements are provided in the Supporting Information.

Results and Discussion

50 $\mu\text{m} \times 50 \mu\text{m}$ arrays of features with hemispherical sides, “bumps”, arranged on a square lattice, were fabricated by electron beam lithography of PMMA coated silicon wafers. In this procedure, which is described in detail elsewhere,²¹ the pattern emerges through selective removal of PMMA from the silicon wafer surface. Therefore, the bumps consist of PMMA and the flat surface between the bumps is silica. A lipid multibilayer stack was deposited on the nanofabricated substrates using spin coating and subsequently hydrated. The protocol for spin coating lipids was the one developed by Simonsen et al.^{23,24} The chosen lipid bilayer composition for this study was (6:4, mol:mol) 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC): 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 20 mol% cholesterol. This lipid mixture has a well understood phase diagram² where below the miscibility temperature of approximately 32 °C²⁵ it forms coexisting liquid-ordered (L_o) and liquid disordered (L_d) bilayer phases. A fluorescent dye was included, 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphocholine (NBD-

PC, 2 mol %), that partitions strongly into the DOPC-rich L_d phase. Fluorescence microscopy was used to characterize the L_o phase domain patterns identified by their dark appearance in comparison to the DOPC-rich L_d phase. The sample temperature was $22\text{ }^\circ\text{C} \pm 1\text{ }^\circ\text{C}$ unless noted otherwise. Overlapped L_o phase domains within the multi-bilayer stack appear as the darkest regions using fluorescence microscopy, and those that do not overlap show a lighter gray scale as previously observed.^{23,24,17} Cohen-Simonsen and coworkers have shown that the proximal domains of a DOPC/DPPC/cholesterol supported bilayer, in an aqueous environment, are sub-microscopic, while those in subsequent bilayers coarsen quickly to form microscopic domains.²⁴ Therefore, the domains observed here are most likely in the 2nd and 3rd bilayers distal from the substrate as there are two shades of grey primarily observed. Preliminary experiments (Supporting Information Figure S1) confirmed that hydrated lipid multilayers formed on side-by-side flat PMMA and flat silica surfaces behaved virtually identical with respect to their phase behavior. In addition, there appeared to be no domain partitioning between the two materials as evidenced by no observable depletion or enrichment of domains near the interface of the PMMA and silica. This is not surprising; the domains that are microscopically visible are probably in the layer(s) distanced from the substrate and therefore are directly adjacent to a compositional similar proximal bilayer, not two different substrates.

The e-beam formed arrays imposed defined patterns of curvature that were reflected remarkably well in the domain patterns of L_o domains that emerged in the lipid multilayer. Figure 1 (a and b) shows AFM images from a square lattice array of $5\text{ }\mu\text{m}$ and 100 nm projected radius bumps with a spacing between bumps of approximately $10\text{ }\mu\text{m}$ and 200 nm respectively. See Figure S2 and S3a-c for AFM section analysis and scanning electron microscopy (SEM) images of the substrates. The $5\text{ }\mu\text{m}$ projected radius bumps were flat on top as shown in the AFM image in Figure 1a. The sides of these bumps were curved at $0.200 \pm 0.002\text{ }\mu\text{m}^{-1}$ (see calculations in Supporting Information), within the curvature threshold (0.04 to $.4\text{ }\mu\text{m}^{-1}$) for sorting of lipid domains found by Parthasarathy et al¹⁶ by using a microfabricated trough pattern. Therefore, as shown in Figure 1c, the darker L_o phase domains are sorted to the central flat regions on top of the bumps and flat regions between bumps, thereby avoiding the curved sides of the bumps. The two shades of grey in the interdomain regions as well as the bright rings around the bumps are due to the gradual detachment of the uppermost bilayer which probably did not contain enough material to be formed completely, as has been shown previously in spin coated multi-bilayers.²³ This overall process appears to have resulted in the removal of the uppermost bilayer from the area of the bumps, such that each bump contains one non-overlapping micron-scale L_o phase domain.

Much higher curvature, similar to the membrane curvature of cellular organelles and small vesicles can be achieved by e-beam lithography. The sides of the 100 nm projected radius bumps in the pattern shown in Figure 1b were curved at $9.69 \pm 0.83\text{ }\mu\text{m}^{-1}$. This is well above the curvature threshold range necessary for sorting. The bumps were arranged on the lattice points of a square lattice with approximately 200 nm spacing between the bumps. Therefore in a mixed lipid bilayer, the L_o domains should be confined to an interconnected flat “grid” that exists in the bump-free space forming domain patterns made up of lines and rectangular shapes. As can be observed in Figure 1d, L_o phase domains do appear to be composed of connected lines and rectangular shapes.

To develop an understanding of how the underlying curvature pattern directs domain pattern formation, we used e-beam lithography to create individual arrays that varied in lattice spacing of the bumps, while keeping their curvature roughly constant by using 100 nm projected radius bumps (see Figures S3c-e for SEM images). As shown in Figure 2, the domain patterns differ for 200 nm (Figure 2a) 400 nm (Figure 2c) and 600 nm (Figure 2e)

square lattice spacing. Qualitatively, the interconnectedness (or “percolation”) of the pattern can be seen to decrease as the lattice spacing increases. An interesting aspect of this is that isolated circular domains that are of a similar scale to the unit cell of the square lattice emerge in the pattern as shown in Figure 2(c and e). These are essentially the “pixels”, or smallest addressable elements, that can be positioned by the energetics of the system. By overlaying the lattice pattern on higher magnification images shown in Figure 2d and 2f it can be confirmed that most isolated domain pixels are centered on the position of a unit cell. Larger domains are formed of adjacent circular domain pixels and therefore their edges have a wavy appearance in the largest scale pattern shown in Figure 2f. The troughs of the waves as shown in Figure 2e and maxima in a line intensity profile shown in Figure 3 correspond to the positions of bumps, confirming that the L_o phase avoids them. Similarly, for the smallest scale 200 nm lattice pattern, the overlaid lattice in Figure 2b shows that the linear and rectangular domain shapes are confined by the lattice pattern, consistent with domain formation from adjacent domain pixels.

We analyzed the formation of domain patterns in two ways to determine the underlying patterning mechanism. First, we investigated the role of line energy and bending energy²⁶ in achievement of a pixilated domain pattern. As shown in the schematic in Figure 4, extra domain perimeter, in comparison to a circular domain, exists at the wavy outer perimeter and around the perimeter of each bump contained within the domain pattern. The unfavorable line energy created by the additional perimeter is balanced by the favorable decrease in bending energy created because the L_d phase is bent by the high curvature bumps rather than the stiffer L_o phase. Equation (1) equates the energy of an observed domain pattern to the energy of a circular domain of the same area as shown in Figure 4 (derivation in Supplemental Information).

$$N \left[A \left(\frac{K_{L_d}}{2} \right) C^2 \right] + P_{pd} \sigma = N \left[A \left(\frac{K_{L_o}}{2} \right) C^2 \right] + P_{cd} \sigma \quad (1)$$

Where N is the sum of the number of bumps, including fractions, covered by the circular domain placed randomly on the bump lattice; A is the surface area of a bump; P_{pd} is the sum of the outer perimeter and inner perimeters of the patterned domain; P_{cd} is the perimeter of the circular domain including a correction due to domain area that is out of plane. This data was obtained from analysis of domain patterns on 600 nm, 400 nm, and 200 nm lattices as shown in Figure 2. K_{L_d} (1×10^{-19} J)²⁷ is the bending modulus of the liquid disordered phase, and K_{L_o} (6×10^{-19} J)²⁸ is the bending modulus of the liquid order phase; C is the average curvature of each bump ($9.69 \pm 0.83 \mu\text{m}^{-1}$); σ is the line tension at the interface of the L_o and L_d phases. Solving equation (1) for the line tension, we obtain an average line tension of 3.23 ± 0.70 pN, 3.40 ± 0.62 pN, and 4.50 ± 1.07 pN for the 600 nm, 400 nm, and 200 nm lattices respectively. The value of 4.50 pN should be viewed with caution; we relied solely on image analysis for that particular calculation which underestimates P_{pd} and thus overestimates σ . See Supporting Information for details of the calculations. The line tension at the interface of the L_o and L_d phases was previously determined by Baumgart et al.⁹ who found values of 3.3 pN and 2.3 pN for similar compositions containing 16 mol% and 23 mol % cholesterol respectively. This good agreement with the measured line tension indicates that mechanical energy minimization plays an important role in determining the distribution of domain pattern geometries.

Second, we investigated the role of percolation in the interconnectedness of the domain patterns. At a domain area fraction above the critical area for percolation (A_c), we would expect to observe domains that appear to form pathways of infinite length and very few, if any, isolated domains. The critical area fraction for percolation (A_c) on a square lattice is

given by $f p_c$, where f is the filling factor of the lattice, i.e. the maximum fraction of space that can be occupied and p_c is 0.59, the critical probability, above which the mean cluster size goes to infinity. With domains confined to the flat areas between bumps, the filling factors are 0.803, 0.908 and 0.954 for the 200 nm, 400 nm and 600 nm lattices respectively. The critical area fractions are 0.47, 0.54, and 0.56 compared to actual area fractions of 0.43 ± 0.01 , 0.48 ± 0.01 and 0.45 ± 0.03 for the 200 nm, 400 nm and 600 nm lattices respectively. Qualitatively, the patterns agree with this analysis where the 200 nm system, with the domain area fraction closest to A_c , appears to be nearly fully percolated as evidenced by the largest domain in Figure 1d which forms a pathway of at least 100 μm . However, the appearance of isolated domains, isolated domain pixels, and shorter pathways in the 200 nm and 400 nm lattices shown in Figures 2(d and e) are indicative of their area fractions being further from A_c . Therefore the area fraction, relative to A_c , plays an important role as well in the interconnectedness of domain pixels that are confined to lattice sites.

Next, we varied the bump lattice spacing within each $50 \mu\text{m} \times 50 \mu\text{m}$ array by patterning adjacent one-thirds of the area with 75 nm, 200 nm and 400 nm lattices while keeping the bump curvature roughly constant ($9.69 \pm 0.83 \mu\text{m}^{-1}$ corresponding to the 100 nm projected radius bumps) as shown in Figure 5a (see Figures S4 for a $50 \mu\text{m} \times 50 \mu\text{m}$ SEM image). The adjacent 75 nm, 200 nm, and 400 nm lattices created a gradient in domain pixel size in the same continuous bilayer as can be observed in the increase in domain pattern line-width between the 200 nm and 400 nm lattice pattern demonstrated by the arrows in Figure 5b (domain pixels in the 75 nm lattice are sub-microscopic and could not be resolved). To slow the flux of domain lipids to the PMMA that surrounds the $50 \mu\text{m} \times 50 \mu\text{m}$ arrays, three walls (100 nm in height and 250 nm apart), that serve as mechanical energy barriers, were patterned around $50 \mu\text{m} \times 50 \mu\text{m}$ pattern (see arrow in Figure 5b). The placement of the barriers slowed the time for the miscibility transition to drop below $22^\circ\text{C} \pm 1^\circ\text{C}$, when domains are no longer visible, from approximately 1 day to 3 days. The area fraction of domain patterns made on curvature arrays that included walls initially matched the domain area fraction (~ 0.58) on the surrounding flat PMMA, indicating that domain patterns are formed from the Lo phase and not from dewetting the substrate. Domains could be reformed by dropping the temperature below the new miscibility temperature as was the case for the image in Figure 5b that was taken at $15^\circ\text{C} \pm 1^\circ\text{C}$. Note that the bright spots in the pattern are evidence of vesicle budding. Budding and growth of vesicles is an additional mechanism to escape the high curvature environment. Long time-scale dynamics of these systems will be the subject of a subsequent manuscript.

Conclusions

In summary, we have demonstrated that it is possible to form pixilated patterns of lipid domains in lipid bilayers with domain pixel sizes controllable from the micrometer to the nanometer scale via the lattice spacing of underlying curved features. We found that patterns emerged from both mechanical energy minimization and packing constraints, with the most connected pattern occurring near the percolation threshold of the domain pixels. The straight forward sample preparation approach, involving standard electron beam lithography and lipid spin coating, combined with the pattern formation mechanism presented here suggest a way forward to integrate lipid domains with nanometer-scale device elements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Demonstration of two different bump arrays and domain patterns of lipid bilayers deposited onto these arrays. AFM images of (a) 5 μm projected radius bumps with 10 μm lattice spacing (b) 100 nm projected radius bumps with 200 nm lattice spacing. (c and d) Fluorescence microscopy images of corresponding domain patterns.

Figure 2.

Fluorescence microscopy images of domain patterns imposed by increasing lattice spacing between the 100 nm projected radius bumps. (a) 200 nm lattice spacing (c), 400 nm lattice spacing and (e) 600 nm lattice spacing. Drawing of bump lattice (to scale) has been overlaid on zoomed images showing that the domain patterns are formed of individual and connected domain pixels scaled by the unit cell. (b) 200 nm lattice spacing (d), 400 nm lattice spacing (f) and 600 nm lattice spacing.

Figure 3.

Line intensity profile of a domain pattern formed on the 100 nm projected radius bumps with 600 nm lattice spacing (800 nm center to center distance of the bumps). The location of the intensity maxima appear approximately 800 nm apart confirming that the L_o phase avoids the bumps and that L_d phase occupies the area covering each bump. The inset shows the line of image pixels that was analyzed. Also note the lighter appearance of the bumps.

Figure 4. Schematic representation of a liquid ordered domain (left) avoiding the high curvature of the bumps by taking on a higher perimeter. (right) A continuous circular domain of the same area is curved at the location of the bumps and has a lowered perimeter.

Figure 5.

Lattice spacing varied within the same $50\ \mu\text{m} \times 50\ \mu\text{m}$ bump array to vary the domain pixel size within the same continuous lipid bilayers deposited on the array. (a) SEM images showing the variation in lattice spacing from top to bottom of the array used to pattern the domains in b. (b) Fluorescence microscopy image of domain patterns formed on this $50\ \mu\text{m} \times 50\ \mu\text{m}$ array, budding vesicles (bright spots), and three walls associated with the underlying e-beam patterned substrate.