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Metastability in pixilation patterns of coexisting fluid lipid bilayer phases imposed by e-beam patterned substrates

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Abstract

We study the dynamic evolution of pixilation patterns of the liquid-ordered (L_o) phase in coexistence with the liquid-disordered phase in lipid multibilayers. The pixilation patterns were formed by imposing lattice patterns of localized high curvature on phase-separating multibilayers using curvature-patterned regions of an underlying support. The projected radius of underlying hemisphere-like features, that provided the local curvature, was varied from 60 nm to 100 nm and the square lattice spacing between the features was varied between 200 nm and 400 nm using standard electron (e) -beam lithography. Over time, the area fraction of the L_o phase on the patterned regions of the substrate decreased toward zero at room temperature. This apparent metastability of the pattern derives from the high line energy of a pixilation pattern where a Boltzmann distribution shows near zero equilibrium partitioning of the L_o phase in the patterned regions. Kinetic rate analysis identifies two pattern-dependent mechanisms that dominate the transition to zero L_o area fraction; diffusion limited dissolution of the L_o phase driven by an Ostwald ripening-type process or the cooperative formation of vesicles containing L_o phase lipids. Interestingly, we observed the spontaneous formation of tubules in the corners of the array due to the high local curvature applied to the membrane. Furthermore we show that it is possible to regenerate pixilation patterns on the curvature-patterned regions by cooling below room temperature. Regenerated area fractions are in agreement with a room-temperature composition of primarily L_d phase and the high degree of overlap with the original patterns is suggestive of fixed nucleation sites.

Introduction

Lipid molecules self-assemble into a dual leaflet form of smectic liquid crystal with fascinating properties. These fluid lipid bilayers play host to a two-dimensional assembly of molecules in cell membranes in addition to providing biological cells with compartmentalization and transportation in the third dimension. Model membrane studies that impose curvature on lipid membranes^{1,2,3,4,5} have concluded that when heterogeneous lipid compositions form in the membrane, lipid heterogeneities will localize to regions with curvatures that are energetically favourable. These studies have strengthened the argument that curvature plays an important role in lipid sorting and localization to the membranes of cellular organelles. Relationships between geometry, energy, and time scale given by soft matter physics have been used to give insight into how dynamic processes, such as lipid

[†]Electronic Supplementary Information (ESI) available: derivation of equation 3 and supporting SEM and fluorescence microscopy images. See DOI: 10.1039/b000000x/

sorting, function in the cell (recently reviewed by Baumgart et al.⁶). In addition, these relationships can be used to engineer and understand soft matter systems that use geometry to control energy, dynamics and mechanistic processes.

Recently, we introduced an example of this kind of an engineered system. Pixelation patterns of coexisting fluid lipid phases in multibilayers were formed by imposing patterned curvature using an underlying substrate, a square lattice array of hemispherical features, “bumps”, formed by standard e-beam lithography of a poly(methyl methacrylate) (PMMA) layer on silica.⁷ 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.⁸ We showed that the high-bending modulus L_o phase formed the fundamental units (or “pixels”) of the pattern by being confined to and centred on the flat region between the unit cell of 4 bumps and that the “domain pixels” connect up to form linear and rectangular 2 dimensional shapes surrounded by the L_d phase. We quantitatively showed that this initial domain pattern shape is driven by a lowered bending energy, where the L_o phase avoids the high curvature of the bumps, that is larger than the increase in line energy associated with a geometry of connected domain pixels. The lattice spacing of the e-beam patterned substrates was set between 600 nm and 75 nm, forming domain patterns with “line-widths” that ranged from the micrometer-scale to the submicroscopic. In comparison, other methods that have been used to form curved substrates for phase-separated lipid bilayers, such as standard photolithography, polymerization of PDMS on a template, and wrinkling of PDMS,^{1,2,3,4} have not formed such well-defined domain patterns because the patterns are confined to the micrometer-scale, where there is a weaker influence of line tension, or because they do not have the capability to form a highly repetitive pattern.

We show here that the L_o phase exists transiently, in the form of a lipid domain pixelation pattern, on the curvature-patterned sections of the substrate. The well-defined nature of the domain pixelation patterns provided us an ideal opportunity to study the influence of geometry on the dynamics and mechanisms of the transition of these metastable patterns toward a lower energy state. Four different underlying square lattice arrays were used that varied in lattice spacing and bump radius. Fluorescence microscopy was used for qualitative mechanistic observations as well as quantitative measurements that allowed us to describe the L_o phase area fraction vs. time by a kinetic rate equation for each underlying lattice. We will show that the kinetic rate equations and visual observations suggest two distinctly different mechanisms for this transition to zero domain area fraction. In addition, we note that lipid tubules spontaneously radiate from the high-curvature corners of the first two e-beam patterns. Finally, we investigated the extent to which we could regenerate each domain pattern by cooling. We then compare the results to known isothermal phase diagrams at the same temperatures^{8,9} and the work of Simonsen and colleagues¹⁰ who studied the influence of fixed nucleation sites on domain shapes.

Experimental

Materials

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol and 1-palmitoyl-2-(6-((7-nitro-2-3,1-benzoxadiazol-4-yl)amino) hexanoyl)-sn-glycero-3-phosphocholine (NBD-PC) were purchased in chloroform (10 mg/ml) from Avanti Polar Lipids, Inc. Milli-Q water (18 M Ω cm) was used for all the steps involving water. The hexane (ACS reagent 98.5%) and the Methanol (HPLC grade) needed for the lipid spin coating solvent, were purchased from Sigma-Aldrich Inc. and Fisher Scientific, respectively. The silicon wafers used for the production of the 1cm $\times$ 1cm substrates were purchased from Silicon Valley Group (San Jose CA) and the

Poly(methyl methacrylate) (PMMA) 950k 2% dissolved in Anisole from MicroChem (Newton MA).

Preparation of the Substrates

Poly(methyl methacrylate) PMMA (100 nm thickness) coated substrates were produced in the Northern California Nanotechnology Center, University of California, Davis. A stock solution of PMMA 950k 2% dissolved in Anisole was spin coated onto pre-cleaned 4 inch silicon wafers in a Silicon Valley Group (SVG, San Jose CA) 8100 spin coater with automated handling and proximity bake; 2 nm uniformity was recorded at 5 points around the wafer using a NanoSpec 210 film thickness measurement tool and Veeco Dektak 3030 Profilometer. PMMA was coated at 3,000 rpm for 45 s and then proximity baked 1cm above a hotplate at 190 °C for 5 mins. Then individual 1cm² sections were subjected to electron beam writing in an FEI 430 NanoSEM (FEI, Hillsboro, OR) equipped with a Nabity Pattern Generating System (NPGS v.9.0) and high speed beam blanker. Since PMMA is a positive electron beam resist, the bumps, which were patterned in a squared lattice arrangement, were formed by the removal of the PMMA from the silicon wafer. The patterned chips were immersed in 1:3 methyl isobutyl ketone:isopropyl alcohol (MIBK:IPA) developer solution for 90 seconds.¹¹ The final inspection of the samples was performed in a FEI 430 NanoSEM. Scanning electron microscope (SEM) images were used to determine the projected radius and lattice spacing of the patterned bumps. Atomic force microscopy (AFM) (Veeco Metrology, Inc., Santa Barbara, CA) using contact mode imaging was used to confirm that the bumps were of hemispherical shape.

Preparation of the supported lipid multibilayers

The lipid mixture used for this study was (6:4, mol: mol) DPPC: DOPC and 20 mol % cholesterol. In order to use fluorescence microscopy, NBD-PC (2 mol %) was added to the mixture. The lipids were mixed in a 1 mL conical vial and dried with nitrogen. The sample was combined with a spin coating solvent, 97:3 (v/v) hexane/ methanol, in order to completely dissolve the lipids. The volume of the solvent was the amount necessary to have a 5mM solution of lipids. 30 µl of this solution was dispersed onto the 1cm x 1cm substrate which was placed on the spin coater (Chemat Technology, Inc., Northridge, CA) set for 3000 rpm for 40 seconds. After spin coating, each sample was placed under mild vacuum (while in the dark) for 24 hr in order to evaporate the solvents. The lipid multilayer of each sample was hydrated (while in the dark) by immersing each sample in a 70°C buffer bath (10mM Tris-HCl, 150 mM NaCl and 2mM CaCl₂-H₂O at pH 7.4) for 30 minutes. Once each sample reached room temperature (22°C ± 1°C) it was imaged with a 60× water immersion lens on a Nikon TE400 fluorescence microscope, using a FITC filter set (Chroma Technology, Bellows Falls, VT). Further sample cooling was achieved using a Bionomic Controller, 20/20 Technology, Inc., Wilmington, NC. The software used for image analysis was ImageJ/ Micromanager (National Institutes of Health).

Results

L_o domain pattern on square lattice curvature array

Electron beam lithography of 100 nm thickness PMMA coated silicon wafers was used to form four arrays of hemispherical PMMA features, “bumps”, projecting from a flat silica surface in a square lattice pattern. Two of the array lattices varied in the spacing between the bumps (400 nm and 200 nm) while maintaining the same projected bump radius of 100 nm. The other two lattices varied in their projected bump radii (65 nm and 60 nm) while maintaining the same spacing between the bumps of 375 nm. More accurate measurements of these dimensions, from SEM images, are contained in Table 1. The SEM image of each pattern can be found in the supporting information (Fig. S1). Note that each array pattern is

imprinted multiple times into a PMMA-coated silicon wafer (Fig. S1a) which allows statistical analysis. Lipid multibilayers were formed on each patterned silicon wafer by spin coating followed by hydration of a (6:4 mol:mol) DPPC:DOPC and 20 mol% cholesterol mixture containing an additional 2 mol % of a fluorescent dye, NBD-PC. This mixture forms a liquid-ordered (L_o) phase coexisting with a liquid-disordered (L_d) phase^{8,12} below its miscibility temperature (T_m), approximately 32°C.¹² NBD-PC partitions into the L_d phase such that the L_d and L_o phases can be identified their bright and dark appearances respectively.

Phase-separated DPPC:DOPC:cholesterol multibilayers, deposited onto these curvature arrays and cooled to 22 ± 1 °C, formed pixilation patterns of interconnected and isolated liquid-ordered phase domain pixels, as shown in previous work. Fig. 1a and b show an AFM image and SEM image, respectively, of a square lattice array of 100 nm projected radius bumps with 200 nm spacing between the bumps. Fig. 1c shows a fluorescence microscopy image of a typical pixelated L_o domain pattern (dark phase) observed for a multibilayer supported by this same curvature array pattern. Only large rounded domains exist on the non-patterned regions of the substrate as shown in Fig. 1d. Typically we spin coat 2 to 3 bilayers and the distal 1 or 2 bilayers display microscopically visible L_o phase, giving potentially two shades of grey for overlapped and non-overlapped domains as previously reported by Simonsen et al.¹³ The linear and/or networked appearance of these domain patterns occurs because of the mechanically-driven partitioning of the L_o phase to the grid of silica between the bumps⁷ shown by the green dashed lines between Fig. 1 b and c and illustrated in Fig. 1e. In this work, we investigate the 2 and 3-dimensional evolution in time of these patterns.

L_o domain pattern disappearance

Qualitatively, the L_o domain patterns on the four bump lattices have the same evolution in time at 22 ± 1 °C. The domain patterns initially show an increase in their interconnectedness and roundedness from their initial states as shown in Fig. 2 (left panels). This effect is produced because isolated L_o domain pixels and narrow projections are disappearing from the pattern (arrows, Fig. 2 insets) first. This initially stage is followed by more uniform disappearance of the patterned L_o domains as shown in Fig. 2 (right panels). Quantitatively, the kinetics of this process and accompanying remodelling of the membrane was distinctly different for each of the four underlying array lattices. We did not notice any visible change in domain area fraction of the multibilayers on flat PMMA that surrounded the bump arrays demonstrating that the L_o phase clearance is caused by the high curvature imposed on the multibilayers by the arrays.

The rate at which the patterned L_o domains disappeared varied dramatically between the first two patterns which differed in the spacing of the underlying bump lattice while maintaining the same 100 nm projected bump radius. With an underlying 400 nm lattice spacing (Fig. 2a), an initial domain area fraction of 0.48 ± 0.01 decreases to 0.27 ± 0.04 in a period of 42 hours. The individual domains appear to fade away slowly during this process as highlighted by the circled region in Fig. 2a. However, when the spacing between the underlying bumps is narrowed to 200 nm the pattern disappears much faster as demonstrated in Fig 2b, especially from the bottom right corner where more domains are a lighter shade of grey, thus in only one bilayer. The initial area fraction of 0.43 ± 0.01 decreases to 0.018 ± 0.004 in 18 hrs. With both of these underlying lattices, we typically observed a vesicle or two (bright spots) at time = zero, however they did not grow significantly during the disappearance of the pattern.

To introduce higher curvature into the arrays, the projected radius of the bumps was reduced to 65 nm and 60 nm and the bump spacing was very similar, 375 nm, to the first lattice array

so that a direct comparison could be made with the 400 nm lattice spacing used for the 100 nm projected radius bumps. In comparison, sections of the pattern disappear all at once (Fig. 3 a and b) rather than “fading” to the background intensity (Fig. 2 a). The initial domain area fraction of 0.506 ± 0.002 and 0.486 ± 0.011 decreased to 0.112 ± 0.002 and 0.106 ± 0.003 in a period of 25 and 28 hours for the 60 nm and 65 nm bump radii respectively. At first inspection, these rates appear to be in between the two rates observed for the domain patterns formed on the arrays of 100 nm bumps. However, kinetic analysis will reveal that they follow different rate equations.

At least one giant vesicle emerged and grew from each phase-separated multibilayer supported by an array of the 65 nm projected radius bumps as shown in Fig. 3a (between red arrows). Each giant vesicle appears to be of one homogeneous phase with such a low level of fluorescence that the underlying domain pattern can be easily observed. In addition, in a case where only one giant vesicle formed (see Fig. 3a), we were able to estimate that the approximate surface area of the vesicle of $919 \mu\text{m}^2$ (assuming a flattened vesicle) corresponded approximately to the decrease in area of the L_o domain pattern on the array ($1,110 \mu\text{m}^2$) over the same time, 27.5 hours. For this pattern, disappearance of micrometer-scale sections of the domain pattern could occur over the time frame of seconds with accompanying changes in size of the giant vesicle(s) as demonstrated in Fig. S2. These rapid changes in the domain pattern had not taken place for the 100 nm projected radii patterns. The connection from each vesicle to the actively vanishing portion of the domain pattern appeared to take place through lipid tubule(s) as demonstrated in Fig. S2. In the case of the 60 nm bump array, giant vesicles were not being formed. Instead, vesicles of 0.5 to several μm -scale appear to be forming and some are actively shedding from the multibilayer on the patterned array (as shown by red arrows in Fig. 3b). As these vesicles do not grow to giant vesicle size, it cannot be determined if their membranes are uniform in appearance and thus of a single phase.

Kinetics of L_o pattern disappearance

The decreasing area fraction, A_f , of patterned domains with time was fit with equation 1

$$\frac{dA_f}{dt} = -kA_f^n \quad (1)$$

where k and n are constants. To determine the best fit, the method of least squares was applied in order to minimize the sum of residual errors between the experimental A_f vs. t data and integral form of equation (1). Note that in the case of the 400 nm lattice with the 100 nm projected radius bumps, the sum of residual errors was significantly less by using the $n = 1$ integral form of the equation rather than allowing n to vary. The A_f value for each t was an average from at least four identical lattice patterns. The best fits to the A_f vs. time data are shown in Fig. 4 (a and b). Values for n and k are given in Table 1. The value of n was 1 or approximately 1 for the domain patterns on the two arrays containing 100 nm projected radius bumps. However, the value of k for the 200 nm spacing was approximately 15 times higher in comparison to the 400 nm spacing. For the other two domain patterns with smaller projected radii of the bumps the value of n was approximately 2 indicating that a different mechanism was controlling the disappearance of the patterned L_o phase in comparison to the $n \sim 1$ cases.

Tubules patterned at corners of arrays

For the multibilayers supported by the 100 nm projected radius bump arrays, we observed a lipid tubule extending from each corner of each array into the surrounding flat unpatterned

multibilayer as shown in Fig. 5a. The tubules were readily visible extending from the 400 nm lattice pattern and these were as much as tens of micrometers in length and persisted for hours as shown in Fig. 5b ($t=0$) and 5c ($t=20$ hours). They extended symmetrically from the corners and sometimes formed small vesicles at the end or around the middle of the tubules as shown in Fig. S3a. In contrast, the tubules extending from the 200 nm pattern are only a couple of micrometers in length and are barely resolvable with the fluorescence microscope as shown in Fig. S3b. The formation of tubules at the corners was independent of the presence of domains since we also observed them when pure DOPC multibilayers were formed on a bump array (Fig. S3c). In one case, we observed tubule connections between neighbouring lattices at these corner sites as shown in Fig. 5d. These tubules may have been fed by excess lipid that appeared to initially be accumulated at the inside edges of the arrays (Fig. 5b) and was cleared over time (Fig. 5c).

Interestingly, there was no formation of tubules in the corners of the 65 and 60 nm bump lattice patterns. As presented earlier, giant vesicles and smaller vesicles were formed from the interior of those arrays.

Regenerating domain patterns by cooling

The presence of L_o domains reduced drastically over time in the multibilayer atop the bump array as it is clearly shown in Figs 2, 3, and 4. After the domain area fraction was near zero, we dropped the temperature below $22 \pm 1^\circ\text{C}$ at a rate of 3.3°C/hr to investigate to what extent we could regenerate the domain patterns for all 4 underlying lattices. On the order of ten domains had nucleated and grown to $1\text{-}\mu\text{m}$ -scale patterns by the time 20°C was reached for all four underlying patterns as seen in Fig. 6a (21.2°C) and 6b (19.4°C) that shows regeneration of the patterns from Fig. 2b and 3b respectively. These newly nucleated domain positions generally corresponded to the original locations of the L_o phase at $22 \pm 1^\circ\text{C}$ (see Fig. S4a for an overlay). During growth, the L_o phase pattern always appeared as pixels clearly confined to a grid pattern and clearly arranged in clusters forming rectangular patterns as shown in Fig. 6. We measured the L_o domain area fraction with temperature for the case where the underlying array contained 60 nm radii bumps of 400 nm lattice spacing as shown in Fig. 7, choosing an array where there was nearly 0% L_o phase visible before the temperature drop. The area fraction increased nearly linearly from 0.026 at $22 \pm 1^\circ\text{C}$ to 0.22 at 10°C . The average area fractions at $\sim 10^\circ\text{C}$ of the domain patterns on three of the lattices reached approximately the same value, 0.2 . However, recovery of domain area fraction on the 100 nm projected radius and 200 nm spacing lattice was significantly less, ~ 0.1 . These difference probably reflect differences in the rates of domain disappearance, which will serve as a competing process. The final patterns, after cooling was complete, overlapped substantially with the original patterns but were not exact duplicates as shown in the overlay of Fig. S4b.

Discussion

In our previous work we quantitatively showed that the initial L_o phase pattern shape is driven by a decrease in bending energy that is larger than the increase in line energy.⁷ We showed that in these patterns, domains are pixelated by being confined to and centred on the flat region between the unit cell of 4 bumps and they connect up to form linear and rectangular 2 dimensional shapes. We show here that these domain pixelation patterns represent a metastable state that is only present transiently after cooling the multibilayer composition through the L_o - L_d miscibility transition. The L_o phase spontaneously disappears in the multilayers supported by the bump array over a matter of several to tens of hours. The likely reason for this metastability is the substantial line energy that is created in these patterns.

We can estimate the energetic driving force of the L_o domain pattern metastability by multiplying the total domain perimeter, P_{array} , ($\sim 1050 \mu\text{m}$) of a pattern such as in Fig 2b (zero timepoint) by the approximate line tension, σ , of $\sim 2.5 \text{ pN}$ obtained from the literature¹⁴ to obtain a line energy, σP_{array} , of $\sim 2.6 \times 10^{-15} \text{ J}$. In comparison, a circular domain of the same area ($\sim 730 \mu\text{m}^2$) has a perimeter, P_{circle} , of $\sim 96 \mu\text{m}$ and thus a line energy, σP_{circle} , of $\sim 2.4 \times 10^{-16} \text{ J}$. A Boltzmann distribution (equation 2),

$$A_{f,array} = A_{f,bulk} \exp[-(\sigma P_{array} - \sigma P_{circle})/kT] \quad (2)$$

applied to these values and the bulk area fraction of L_o domains in the areas outside of the arrays ($A_{f,bulk}$) of 0.6, predicts that the equilibrium area fraction of domains in the supported multibilayer on the array ($A_{f,array}$) is basically zero.

Our data suggests that different mechanisms are possible for spontaneous clearance of the domains from the curvature patterned regions, i.e. to reach the thermodynamic equilibrium state. For the arrays with the 100 nm projected radius bumps we did not observe growth or expulsion of vesicles as a possible mechanism. Instead, a nearly linear decrease in A_f with time and measured exponential rate constant near $n=1$ in equation 1 point to a diffusion-limited process such as Ostwald ripening.^{15,16} In the supporting material we derive equation 3, showing that there exists an $n=1$ dependence, for the diffusion-controlled dissolution of the pattern by an Ostwald ripening-type mechanism, assuming the domain pattern is elongated in geometry.

$$\frac{dA_f}{dt} \propto -\frac{DC_b v A_f}{r^3} \quad (3)$$

Where D is the diffusion coefficient of domain species in the L_d phase surrounding the domains, C_b is the bulk mole fraction of the L_o species (essentially DPPC and cholesterol) in the L_d phase, v is the capillary length which is directly proportional to the line tension (σ), and r is the radius of a domain pixel.

The driving force of Ostwald ripening is line tension (σ) at the curved domain interface which enriches a curved interface in the L_o species in comparison to the bulk, setting up a concentration gradient ($C_i - C_b$). The concentration gradient drives a flux of domain lipids from the L_o phase to the bulk L_d phase. Therefore, domain pixels dissolve away (we noted a faded appearance), starting with those that are completely isolated and those that form narrow projections into the L_d phase. The multibilayer area outside of the array is basically an infinite sink for these lipids and therefore we cannot detect the growth of domains in the flat area. Additional evidence for Ostwald ripening is the magnitude of the ratio in the constant k between the 200 nm lattice spacing and 400 nm lattice spacing, k_{200}/k_{400} . Overall, k depends upon the domain pixel size as $1/r^3$ stemming from 3 different terms in the Ostwald ripening equation (see Supporting Material). Firstly, k is proportional to $C_i - C_b$ which is proportional to $1/r$ by the Gibbs-Thompson equation.¹⁷ Secondly, k includes a $1/r$ term that derives from simplifying the total domain perimeter (P_t), where diffusion takes place, as A_t/r where A_t is the total domain area. Thirdly, k includes a mass transfer coefficient that can be estimated as D/r where D is the diffusion coefficient and r is the diffusion length.¹⁸ Domain pixel radii can be estimated as 180 nm for the 200 nm lattice spacing and 320 nm for the 400 nm lattice spacing, thereby predicted a ratio $k_{200}/k_{400} \sim 6$, a reasonable estimate of the actual value (~ 15) considering the approximations made in deriving equation 3. This analysis shows that the sustained existence of the pattern is very sensitive to the unit cell size of the bumps, which sets the domain pixel radii. More recently

we have taken advantage of this phenomena by creating a gradient of lattice size that selectively partitions L_o phase to the larger lattice spacing.¹⁹

Vesiculation as a major mechanism for lowering the line energy of the system did not take place in the domain patterns on the 100 nm bumps with 200 nm and 400 nm lattice spacing. This makes sense when it is considered that a domain must bulge outward to initiate vesiculation. This bulging lowers the line energy by decreasing the perimeter, however the curvature energy increases. For a critical radius of curvature at the base of the bulge less than approximately $2(K_{L_o}+K_{L_d})/\sigma \sim 500$ nm as given by Lipowsky,¹⁶ the bulge will be unstable. Since the radii of the domain pixels are approximately 180 nm and 320 nm, they will be unstable to vesiculation. The higher curvature patterns (65 and 60 nm projected radii) appeared to be able to overcome this energetic barrier despite the fact that their domain pixels are ~ 300 nm in radius, i.e. less than the critical radius. The higher curvature may play a role. However a simpler explanation is that multiple domain pixels are cooperatively merging to form a bulging domain with a radius larger than 500 nm as a first step toward vesiculation or toward being incorporated into a growing vesicle or tubule. The second-order kinetics would be consistent with such a cooperative transition state. The smaller bumps yield a more “open” pattern with only about 5% of the area taken up by the bumps, in comparison to 10 and 20% for the two patterns that do not vesiculate. Perhaps the domain pixels are simply less “pinned” to their positions, and merge fairly readily.

In addition there is an energetic penalty for initially forming a vesicle of any size of approximately $8\pi K_{L_o}$.^{20,21} This energy can be balanced by the decrease in line energy $\sigma\Delta l$ that takes place when a L_o domain buds outward to form a vesicle. Therefore, a decrease in domain boundary of $\Delta l = 8\pi K_{L_o}/\sigma = 6\mu\text{m}$ is needed. This would represent a section of the domain pattern, e.g. 2 domain pixels wide and 4 domain pixels long, being converted into a vesicle of approximately 1 micron in diameter through budding. Once a vesicle of 1 micron is formed, the bending energy to incorporate more lipid becomes more favourable the larger the vesicle becomes. Therefore, it would be expected that vesicle would remain attached to the lipid multibilayer, continuing to take up L_o phase thus most efficiently lowering the energy of the system. This can be achieved by the formation of lipid tethers of tubule geometry that are observed here for the 65 nm bumps. The 60 nm bump pattern is the most open, with only 4.6% of the area occupied by bumps. We observed vesiculation, but it was more subtle since the vesicles did not grow to nearly the scale seen in the 65 nm pattern although they were more numerous. Nonetheless, the kinetics were very similar in both patterns indicating that the domain pixels are cooperatively interacting in the budding and growth of vesicles.

This work suggests that e-beam formed patterns can be used to control three dimensional remodelling of the lipid bilayer in specific locations. In previous studies, three dimensional membrane remodeling was performed by the application of external forces⁵, aqueous phase separation²², by lateral crowding of bound proteins on the membrane surface²³, and introduction of nanoparticles.^{24,25,26} Particularly interesting here is the presence of tubules in the corners of the arrays. Each corner consists of a 90 degree angle cut into a 100 nm PMMA layer and therefore they impose a high local curvature on the membrane. It appears that the corners alone are able to template tubule formation and growth from the multibilayers coating the arrays or from excess lipid bilayers structures found at the inside edges of the arrays.

Finally, the reappearance of domains upon cooling is consistent with the change in composition brought about by Ostwald ripening and vesiculation. If we assume that the majority of the phospholipids and cholesterol that were in the domains escaped the multibilayers in the array areas, then we can follow the tie line in the known phase

diagram^{8,9} crossing (6:4 DPPC:DOPC) 20 mol% cholesterol to the L_d phase composition of approximately (33:67 DPPC:DOPC) 14 mol% cholesterol at the temperature closest to ours (25 °C) on the known phase diagram. This L_d phase composition would be the starting composition before the temperature quench. Using this composition and applying the lever rule, the phase diagrams for 20 °C and 15 °C indicate that approximately 11 mol% and 25 mol% of the lipid multibilayer should be in the L_o phase respectively. At 10 °C, the 3-phase region crosses into this composition and 27 mol% of the membrane is L_o and $L_{\beta'}$ phase. We have plotted these points on Fig. 7 for comparison with our area fraction data at the same temperatures. Our area fractions are 50 – 80% of these values, explainable by the competing processes that deplete the L_o phase from this high curvature area.

The pattern formed by this slow temperature quench, appears primarily from a small number of fixed nucleation sites that overlap substantially with the original L_o phase locations. This result is not unexpected in comparison to experiments of domain nucleation on flat mica by Simonsen and colleagues who found that more than half of domains repeatedly nucleated at the same sites.¹⁰ In addition, as observed here, similar patterns should be generated upon heating and re-cooling for fixed nucleation sites because the geometry of the “capture zone” for the growing domain is fixed as well.¹⁰ Controlling the exact positions of these nucleations sites would be one mechanism to define the shape of the pattern, for example forming rows of domain pixels, for future sensor applications.

Conclusions

In conclusion, we have developed a soft matter system where the geometry of localized curvature imposed by an e-beam fabricated substrate is used to control energy, dynamics, and the mechanistic process. The geometry of the lattice patterns of “bumps” imposed upon a phase-separating (coexisting L_o - L_d phases) lipid multibilayer determines the L_o domain pixel size and the percentage of excluded area. We found that pixilation patterns formed of these domain pixels exist transiently in the curvature patterned areas of the substrate because of the large perimeter generated by pixilation of the L_o phase. The two geometric parameters of pixel radius and bump projected radius (which represents the excluded area) controlled the kinetics at which the L_o phase cleared the patterned areas and the mechanisms used. For the underlying 100 nm projected radius bumps with 200 and 400 nm lattice spacing, a mechanism of dissolution, similar to Ostwald ripening, was associated with first-order kinetics and a strong dependence of the area fraction vs. time rate on a decreasing domain pixel radius. However, for the 65 and 60 nm radius bumps we observed second-order kinetics associated with vesiculation of the L_o phase as a mechanism to eliminate L_o - L_d perimeter. We suggest that the kinetics are cooperative, i.e. second-order, because domains must merge in order to overcome the critical domain radius to form a stable domain bulge and that the more “open” structure of these patterns may promote vesiculation. Upon cooling, a domain pixel pattern is partially regenerated that overlaps significantly, but not exactly, with the original pattern as evidence of a lowered DPPC and cholesterol content and fixed nucleation sites. Interestingly, the corners of the patterned sections of the substrate appear to template the formation of lipid tubules which may have future applications, for example in nanofluidics. Overall, this work stands as a stepping stone toward a deeper understanding of biological membranes and using localized geometry to control the energy, dynamics, and mechanisms in future applications such as biomolecule arrays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

(a) Atomic force microscopy image and (b) scanning electron microscopy image of the square lattice array of 100 nm projected radius bumps with 200 nm spacing between the bumps formed by e-beam lithography. (c) Fluorescence microscopy image of pixelation pattern of coexisting L_o (dark) and L_d (light) lipids deposited on the array by spin-coating followed by hydration. Green dashed lines serve as guides to show the positioning of the L_o phase on the flat grid between the bumps. (d) Fluorescence microscopy image of large rounded L_o phase domains coexisting with L_d phase on the non-patterned regions of the substrate (e) Side view sketch of the organization of the pixelated L_o and L_d phases in the distal bilayer (not drawn to scale).

Fig. 2. Evolution of the domain pattern over time on the 100 nm projected radius bumps array. (a) 400 nm lattice spacing. (b) 200 nm lattice spacing. White arrows in insets show the narrow projections and isolated domain pixels disappear first. White circle emphasis the slow fading of the patterned domains on the 400 nm lattice. Time zero images reprinted with permission from our previous publication,⁷ copyright 2012, American Chemical Society.

Fig. 3.

Time sequences of domain patterns supported by 375 nm lattice arrays with smaller bump projected radii. (a) Giant vesicle formation and growth (between red arrows) over 27.5 h with simultaneous reduction in the domain area fraction on the 65 nm projected radius array. (b) Formation and shedding (red arrows) of a small vesicles with simultaneous reduction in the domain area fraction on the 60 nm projected radius array.

Fig. 4.
(a and b) Decrease in the patterned domain area fraction with time on the 4 lattice arrays as noted with lines showing best fits to equation (1)

Fig. 5. Lipid tubules at corners of arrays. (a) Image showing that tubules radiate from each corner of the array (100 nm projected radius, 400 nm spacing) where the green box emphasises the location of a tubule. (b) Zoom of the tubule in (a) at time zero and (c) after 20 h. (d) Tubules connecting between arrays (400 nm spacing pattern). Scale bar 5μm

Fig. 6. Regenerating the domain patterns by cooling. The underlying arrays contain (a) 100 nm radius bumps with 200 nm lattice spacing. Note the clear recovery of the pixilation pattern and (b) 65 nm radius bumps and 400 nm lattice spacing. Note the high area fraction recovery (0.22) at 10 °C.

Fig. 7.

Black data points showing that cooling regenerates L_o phase in the multibilayer on the array and a comparison to area fractions from phase diagrams for DOPC/DPPC/cholesterol at the same temperatures.^{8, 9}

Table 1

Lattice dimensions from SEM images and kinetic rate constants from A_f vs. time

Lattice dimensions		Kinetic rate constants	
Projected Radius (nm)	Spacing (nm)	n	k (h^{-1})
101 $\pm$ 2	398 $\pm$ 3	1	0.014 $\pm$ 0.001
100 $\pm$ 2	199 $\pm$ 3	1.10 $\pm$ 0.02	0.219 $\pm$ 0.007
66 $\pm$ 1	372 $\pm$ 3	2.06 $\pm$ 0.11	0.29 $\pm$ 0.04
61 $\pm$ 1	380 $\pm$ 3	2.06 $\pm$ 0.18	0.28 $\pm$ 0.06