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Phase Separation and Fractal Domain Formation in Phospholipid/ Diacetylene-Supported Lipid Bilayers

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Phase separation in lipid bilayers is a phenomenon dependent on many environmental parameters such as pH, temperature, ionic strength, and pressure. Its importance in biological systems is reflected by the fact that it has been implicated in the spatial reorganization of plasma membranes, which leads to signaling and stimulation. Here, we present the study of phase separation, domain formation, and domain morphology of supported lipid bilayers composed of mixtures of diacetylene lipids and phospholipids. We have used high-resolution fluorescence and atomic force microscopy to characterize the phase separation between these lipids, and have found that at temperatures below 40 °C diacetylene molecules form fractal-like domains. These molecules aggregate in tetralayer stacks with an average monolayer thickness of 3 nm. Boundary and area fractal dimensions were calculated to quantify the domain growth and morphology. A transition from dendritic to dense branching growth was observed as the relative diacetylene concentration was increased. The ability to tailor the growth pattern by changing the relative amount of diacetylene molecules makes this a useful model system for the study of nonequilibrium growth phenomena. In addition, we have explored the possibility of promoting diacetylene domain nucleation through the use of nanostructured surfaces. We found that nanoscale perturbations acted as nucleation sites and modified the growth pattern of diacetylene domains. Phase separation induced by nanometer-scale perturbations could prove useful in selectively positioning lipid patches with specific compositions.

Introduction

Since the first description of supported lipid bilayer (SLB) formation on solid substrates,[1−3] model membrane systems based on such structures have gained much attention. It has been shown that, with the appropriate choice of lipid components and substrates, SLBs can be formed by vesicle rupture and fusion,[4,5] and can result in fully mobile bilayers which can serve as models for more complex multicomponent systems, such as cell membranes. SLBs offer several advantages over lipid monolayers formed at air−water interfaces or whole vesicles. They can be easily imaged through fluorescence and atomic force microscopy (AFM) under a wide range of environmental conditions. In this way, the effect of pH, ionic strength,[6,7] temperature,[8] substrate,[9] and lipid headgroup charge[7] have been studied extensively and have yielded a better understanding of lipid interactions with their environment. Other advantages of SLBs over vesicles are the ability to decouple effects induced by the inherent curvature of vesicles and the possibility to probe membrane and membrane-embedded protein properties across the bilayer.

Phase separation has been studied for a multitude of systems composed of lipids or amphiphilic molecules. In the past, most of these systems have been studied in Langmuir films with the aid of fluorescence or Brewster angle microscopy (BAM). In this way, the effect of temperature and compression rates on domain growth and morphology has been studied in myristic acid films,[10−12] a number of alkyl-based non-ionic surfactants with various headgroup moieties,[13−15] and diacetylene/phospholipid mixtures.[16,17] Such studies have provided a wealth of information on the molecular packing of lipids as a function of pressure and have allowed mapping the transitions from fluid to condensed phases along surface pressure−area isotherms.

SLBs have recently been introduced as viable systems in the study of phase separation with the added advantage of allowing the use of surface-sensitive techniques. These techniques allow the acquisition of parameters that are not easily accessed through Langmuir film studies. Thus, the study of phase separation of lipid bilayers on solid supports can provide information that complements that obtained through pressure−area and BAM. Phase segregation of lipid molecules is typically studied in SLBs with the aid of fluorescent probes that have different partition coefficients for the individual phases. Another useful tool in the study of phase segregation that does not require the use of additional lipid probes is AFM, where height differences between lipid phases are observed as a result of changes in lipid tilt and packing.[18] By using SLBs, it has been found that the segregation of lipid components in membranes can be triggered by changes in cholesterol concentration,[18] thermal history,[19] temperature,[20]

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and ionic concentration,[19] or by a rearrangement of lipid components as vesicles fuse down on the substrate.[21]

Model systems that can phase-separate are important because they can provide information about lipid interactions that can then be used to gain understanding of more complicated lipid systems.[22] The biological relevance of phase separation is underscored by the fact that lipid microdomains or lipid "rafts" have been observed as insoluble fractions from cell membranes,[23−25] and have been implicated in receptor clustering and membrane signaling events.[26−28] In addition, studies have shown that plasma membrane preparations derived from prokaryotic[29] and eukaryotic cells[30] show domains with ordering at multiple length scales as well as fractal behavior. Thus, the study of phase separation in SLBs, domain formation, and morphology through fractal analysis can have implications in the understanding of lipid segregation and microdomain formation.

For diacetylene molecules, previous studies have been performed on Langmuir films where it has been shown that diacetylenes phase-segregate from phospholipids and aggregate with characteristic morphologies at given surface pressures and temperatures.[16,17] It has also been shown that at high surface pressures the diacetylenes stack in multilayers.[16,17] However, the domain morphology is difficult to assess with high resolution in Langmuir monolayers. In contrast, SLBs can be imaged with high resolution through optical and atomic force microscopy. Furthermore, SLBs allow the use of nanostructured substrates to modify the phase-separation behavior.

In this article, we present a study on phase separation between diacetylene lipids and phospholipids in SLBs. We find that diacetylene domain formation happens spontaneously at temperatures below 40 °C in lipid films at the air−water interface or in SLBs, but not in giant unilamellar vesicles (GUVs). In addition, the use of AFM revealed that the diacetylene domains that form as a result of phase separation are composed of tetralayer stacks. Domains that formed as diacetylene molecules phase-separated from phospholipids showed fractal-like behavior with characteristic morphologies for each of the studied diacetylene−phospholipid ratios. A combination of fluorescence microscopy and fractal dimension analysis has been used for the characterization of domain formation, growth, and morphology. We have also used nanostructured substrates to selectively nucleate fractal domain formation. Throughout this manuscript, we use the terminology introduced by Ben-Jacob and Garik[31] to describe the domain morphology observed. The significance of the fractal dimension analysis is discussed in the theoretical framework developed for the description of diffusion-limited aggregation and fractal aggregate evolution.[31−33]

Materials and Methods

Materials and Chemicals. Diacetylene lipid BisDiynePC (1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine) and phospholipid DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) were purchased suspended in chloroform from Avanti Lipids and used without further purification. Fluorescent phospholipid probe DHPE-LR (1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine Lissamine rhodamine-B) was purchased from Molecular Probes and resuspended in chloroform. Single side polished 4 in (100) silicon wafers were purchased from Silicon Quest International. Thermal oxide was grown in a furnace by standard recipe to a nominal thickness of 200 nm as a substrate for the formation of fluid SLBs.

Lipid Vesicle Preparation. Small unilamellar vesicles (SUVs) were prepared by point probe sonication as previously described.[34] Appropriate amounts of component lipids were mixed in 2 mL of chloroform to obtain mixtures of BisDiynePC/DOPC/DHPE-LR at a final vesicle concentration of 1 mM in phosphate buffer saline (PBS). Lipid mixtures prepared were 10:90:1, 30:70:1, 50:50:1, 70:30:1, and 90:10:1 BisDiynePC/DOPC/DHPE-LR. Subsequently mixtures will be referred to by their diacetylene-phospholipid ratio, and the presence of fluorescent probe will be implied in all cases. Lipids were allowed to mix in chloroform for 1 h at room temperature. The solvent was evaporated in a glass vial under dry nitrogen to form uniform lipid films. The lipid films were then rehydrated in PBS at 55 °C for 1 h. This yielded a mixture of uni- and multilamellar vesicles with a wide size range. SUVs were obtained by point probe sonicating the solution with an input power of 5 W for 30 min. The final vesicle solution was filtered through a polycarbonate membrane with a pore size of 450 nm to remove residual particulates. Vesicles were stored at 1 mM concentration at 4 °C for later use.

GUVs were prepared according to the protocol described by Angelova and collaborators.[35,36] Chloroform solutions containing the lipid and dye were uniformly spread onto the conductive sides of ITO (indium tin oxide)-coated slides (4−8 ohm, Delta Technologies) held at 65 °C. The lipid-coated slides were then placed in a vacuum for at least 3 h to evaporate the excess solvent. Two coated slides were then arranged to create a capacitor; the coated conductive sides face each other and are separated by a nitrile O-ring. The space between the slides was filled with 65−70 °C 100 mM sucrose solution (prepared with Milli-Q water). The capacitors were held at 65 °C in an aluminum chamber in a dry bath. Vesicles were electroswelled in the dry bath by applying an alternating sinusoidal electric field (V_{pp} = 2 V, 5 Hz) across the capacitor for 2 h. Small vesicles spontaneously form upon the addition of water to the slides, but the application of an alternating field is thought to cause them to collide and coalesce with each other to form giant unilamellar vesicles (25−60 μm diameter). The vesicles were finally "quench-cooled" to room temperature in 3 h.

Supported Lipid Bilayer Formation and Imaging. SLBs of BisDiynePC/DOPC/DHPE-LR mixtures were formed from SUVs by vesicle fusion onto hydrophilic substrates. Silicon oxide substrates were preconditioned with oxygen plasma in a Harrick Extended Plasma Cleaner for 5 min. This rendered the surface hydrophilic for SLB formation. SUVs at 1 mM concentration were applied to the substrate and incubated for 30 min at 55 °C for bilayer formation. The substrates were then rinsed three times with warm (at 55 °C) PBS to remove excess vesicles and delay phase-separation of lipids. SLBs were then allowed to cool to 25 °C (at a cooling rate of 2 °C/min), and phase-separation was monitored via fluorescence microscopy. Epifluorescence was observed using an AX70 Olympus upright microscope with excitation and emission filters for Rhodamine Red dye (Omega Optical), and 20×/0.5 NA, 40×/0.8 NA, 60×/0.9

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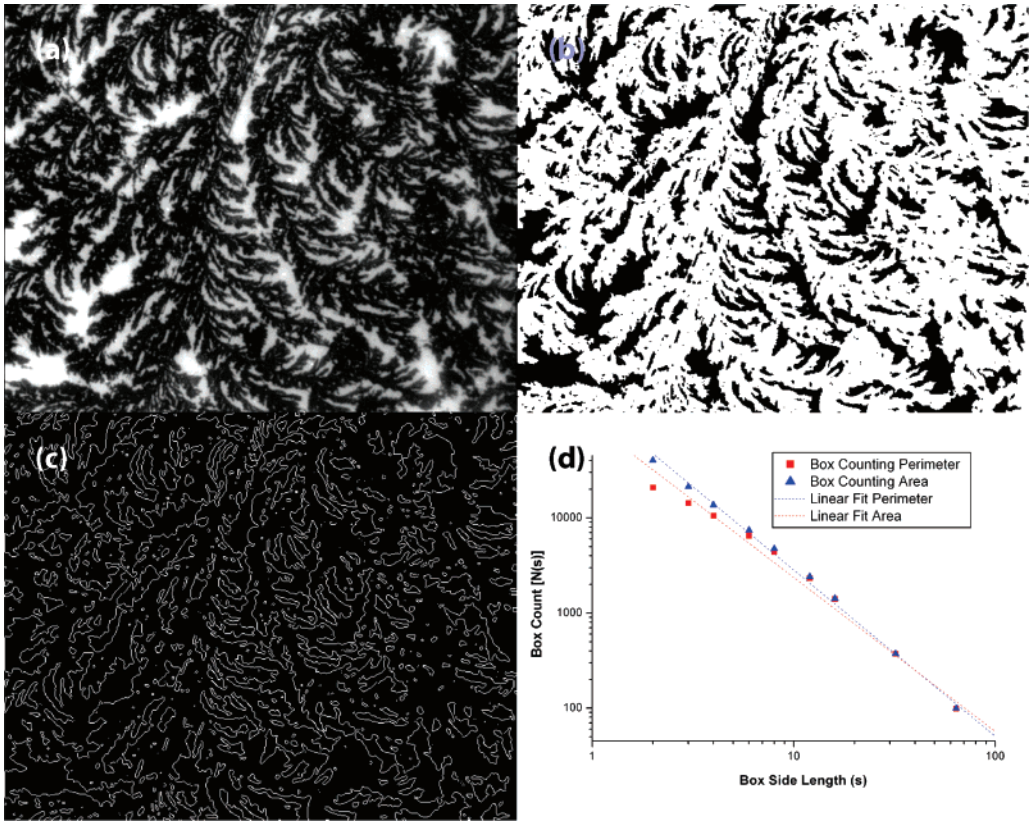

Figure 1. The area and boundary fractal dimensions were calculated via the box-counting method. (a) Original fluorescence microscopy image of fractal diacetylene domains formed on a glass substrate from a 70:30 BisDiynePC/DOPC mixture. Binary images used to calculate the area (b) and boundary fractal (c) dimensions. (d) Linear regression of the box count vs box side length. Fractal dimension corresponds to the absolute value of the slope of the linear fit.

NA, and 100×/1.0 NA physiological objectives from Olympus. Images were acquired with a CoolSnap ES camera (Photometrics) and recorded with *IPLab* software (Scanalytics). Movies of the phase separation in SLBs were recorded using *StreamPix* software (Norpix).

Fractal Dimension Analysis. The fractal dimension for BisDiynePC domains was calculated using the box-counting method.[37] The box-counting method calculates the fractal dimension by placing the image on a grid with box side size s and counting the number of boxes N required to regenerate the whole area or boundary comprising the domain in the original image. By varying the box size, an estimate of the fractal dimension D reflecting the self-similarity or scale invariance of the domain can be obtained from the equation[37]

$$\ln(N(s)) = B - D\ln(s) \quad (1)$$

where B is the linear regression offset. A program for the fractal dimension analysis was written in *Matlab* (MathWorks) where the raw image was imported, inverted, and transformed into a binary object where the BisDiynePC domains were represented by ones and the liquid disordered phase was represented by zeros (Figure 1). The binary 520 × 696 pixel image was then placed on grids of box sizes 2, 3, 4, 6, 8, 12, 16, 32, 64, and 128 pixels. The number of boxes containing ones was then counted, and the fractal dimension was estimated from a linear fit to eq 1. The program was tested against fractals of known dimensionality, e.g., Sierpinski triangle ($D = 1.585$), and compared to results obtained with the *Fractop*[38] program to validate the results obtained from the implemented method.

Atomic Force Microscopy. Atomic force microscopy images were obtained using a Dimension 3000 AFM (Veeco Instruments) in contact mode in fluid. DNP−S cantilevers with a nominal spring constant of 0.58 N/m were used for contact mode imaging at scanning rates of 1−2 Hz. Images obtained were processed and analyzed with *WSxM* program (Nanotech Electronica).[39]

Structured Substrate Fabrication. Structured substrates containing 50-nm-wide gold posts were fabricated with e-beam lithography using 2% PMMA positive resist, gold evaporation, and lift-off as previously described.[40]

Results and Discussion

Phase Separation of Diacetylene/Phospholipid Mixtures. SLBs from BisDiynePC/DOPC/DHPE-LR 1 mM mixtures formed by vesicle fusion on silicon oxide substrates at 55 °C within 30 min of incubation. SLB formation occurred as a result of the adhesion of vesicles on the substrate until a critical surface coverage was achieved and vesicles started fusing, forming spreading bilayer patches. The bilayer appeared uniform and fluid at high temperatures, as confirmed by qualitative FRAP. However, the bilayer was observed to phase-separate as the lipid bilayer cooled down to temperatures below 40 °C, which corresponds to the main transition temperature of BisDiynePC. Phase separation of the lipid molecules was observed by monitoring the fluorescent lipid probe DHPE-LR. As the SLB separated, DHPE-LR partitioned to the liquid disordered phase

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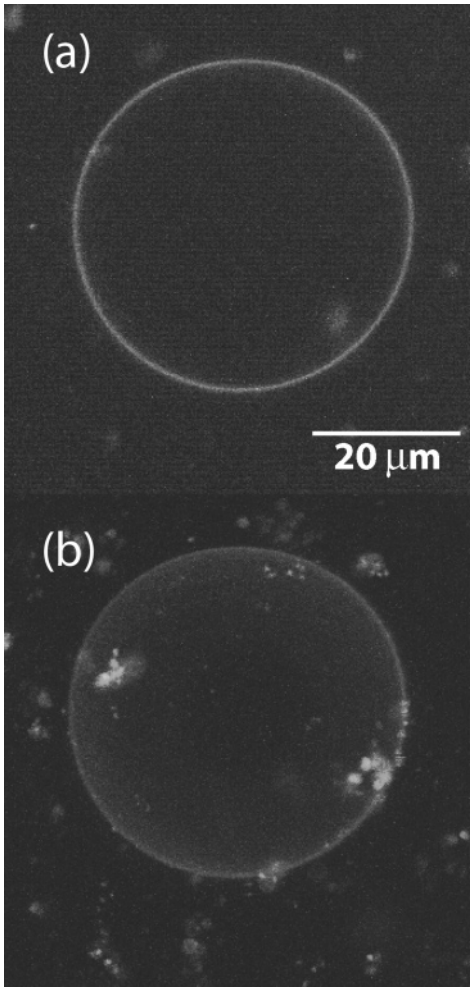

Figure 2. Confocal imaging of a giant unilamellar vesicle of 70:30 BisDiynePC/DOPC labeled with naphtopyrene show no domain separation between the two lipid components. (a) Equatorial plane image. (b) Compounded view of cross-sectional images of an entire vesicle.

(corresponding to DOPC), leaving the BisDiynePC domains as dark regions on the SLB. The observations suggest that BisDiynePC molecules become more ordered at low temperatures, forming a gel phase which precludes the incorporation of fluorescent probes that partition to the liquid disordered or liquid ordered phases such as DHPE-LR or naphtopyrene. The phase separation was reversible, as the diacetylene domains dissolved and the SLB homogenized when it was heated above 40 °C. Additionally, the diacetylene domains could be polymerized by flushing the chamber where the SLB is formed with argon and exposing the bilayer to 254 nm UV light. This confirmed that the dark domains in the bilayer corresponded to diacetylene molecues. Once the domains were polymerized, they remained stable upon heating above the transition temperature.

Phospholipid−diacetylene phase separation occurred in SLBs or in monolayers formed at the air−water interface, as has been previously observed with Brewster angle microscopy.[16,17] However, no phase separation was observed on intact GUVs labeled with naphtopyrene (Figure 2). GUVs appeared smooth and homogeneous at temperatures well below the transition temperature of BisDiynePC (down to 0 °C) while in suspension, but phase-separated as they ruptured and formed SLB patches on the underlying substrate. Such behavior suggests that phase separation between diacetylene lipids and phospholipids occurs only if there are nucleation sites provided by defects at the interface where the mono- or bilayer is formed.

The effect of surface defects was confirmed by comparing the phase-separation behavior on smooth silicon oxide surfaces formed from crystalline silicon wafers versus amorphous borosilicate glass. The borosilicate surfaces, with higher surface roughness, promoted the formation of a multitude of submicrometer BisDiynePC domains, and reduced the number of large, micrometer-scale domains. On the other hand, the smooth, thermally grown oxide surface limited the number of submicrometer-scale domains and promoted the formation of larger aggregates. The possibility of introducing well-defined surface defects and selectively nucleating the formation of BisDiynePC domains was explored with the aid of nanostructured surfaces as described below.

Atomic Force Microscopy of Diacetylene Domains. Contact-mode AFM in fluid was used to characterize the domains resulting from phase separation between BisDiynePC and DOPC lipids. Domains composed of BisDiynePC were 6.4 ± 0.2 nm higher (31 measurements in 3 different samples, Figure 3) than the surrounding DOPC bilayer which has a typical thickness of 5.5 nm in high ionic strength environments.[41] This result shows that diacetylene domains are not bilayers but tetralayer stacks, with monolayer thickness *ca.* 3 nm. Trilayer stacks have also been inferred from molecular area measurements performed in Langmuir films.[17] Our measurements confirm previous reports of diacetylene molecule stacking[16] and add a value for the average monolayer thickness. We also observe that molecular stacking occurred in BisDiynePC/DOPC at atmospheric pressure, while previous reports of molecular stacking in DMPC/diacetylene mixtures happened only in high surface pressure conditions.[16,17] It is possible however that, at higher surface pressures, the diacetylene molecules could aggregate as higher multilayer stacks. The stacking of diacetylene domains stems from the crystalline-like packing of individual BisDiynePC molecules, which is energetically favorable over a disordered packing when the diacetylene molecules are mixed with phospholipid in a liquid disordered phase. Thus, the difference between our system and those studied by Jelinek and collaborators in the pressure value at which stacking is observed could stem from the phospholipid used in the mixture and its inherent properties in acyl chain packing. Further experiments that elucidate the impact of acyl chain length and main transition temperature are required to confirm this hypothesis.

Morphology of Diacetylene Domains. The morphology of diacetylene domains produced by phase separation from DOPC was highly dependent on the relative BisDiynePC/DOPC concentration. Concentrations studied (in molar percentage) were 10:90 (not shown), 30:70, 50:50, 70:30, and 90:10 (Figure 4). The diacetylene domain morphology showed a crossover from dendritic growth at low BisDiynePC concentrations to dense branching formations at higher concentrations. Similar domain morphology has been observed in other systems composed of lipids and amphiphilic molecules.[10−13,16,17,19,42,43] These systems have been described in detail for their behavior as a function of surface pressure and temperature. However, little attention has been put on domain formation, morphology, and evolution, and its dependence on relative concentration of the segregating species. This has a potential impact on the understanding of nonequilibrium growth phenomena.

The growth of two-dimensional fractal aggregates has been described in the past in the frame of diffusion-limited aggregation

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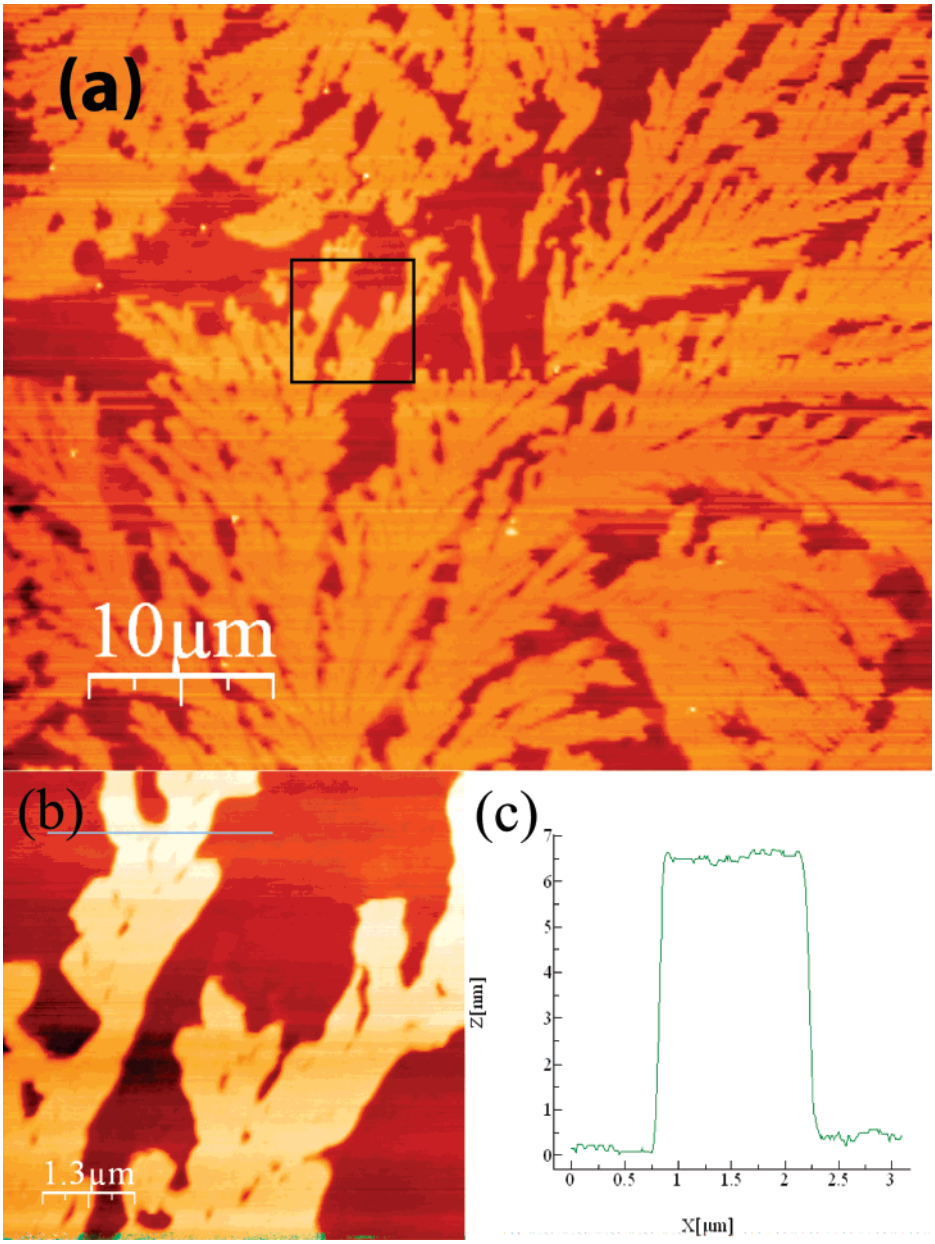

Figure 3. Atomic force microscopy characterization of diacetylene domains formed as the BisDiynePC separate from the DOPC molecules in a supported lipid bilayer from a 70:30 BisDiynePC/DOPC mixture. (a) Representative image of a BisDiynePC domain. (b) Magnified image corresponding to the black square in (a). (c) The height profile of a diacetylene domain, corresponding to the blue line in (b), shows that these domains are 6.5 nm taller than the surrounding DOPC bilayer.

(DLA).[32] In the simplest model of DLA, an aggregating species forms a growing cluster from an initial seed (which in this case can be represented by an impurity). The cluster grows by the individual collisions of the aggregating molecules that have a certain probability of adhering to the boundary of the growing cluster.[16] In our case, the fractal-like morphology exhibited by the diacetylene domains can be interpreted as a result of the interfacial instability of the growing boundary, where nonaggregating particles (DOPC and DHPE-LR molecules) limit the growth of the cluster. Therefore, growth is controlled by two characteristic processes, one relating to the diffusion of nonaggregating molecules away from the growing interface and the other pertaining to the adhesion of the aggregating species to the cluster. Evidence of the first process can be seen as an increase in fluorescence of the fluid bilayer near the interface of the growing aggregate (*cf.* Figure 5). This is the result of a higher density of DHPE-LR molecules that are excluded from the BisDiynePC domains and slowly diffuse away from the interface.

Dendritic pattern formation has been ascribed to growth dominated by the rate at which aggregating molecules incorporate to the cluster,[44] thus producing aggregates that reflect the inherent symmetry of the individual aggregating molecules at the microscopic scale.[31] This type of pattern was observed at low diacetylene concentrations. On the other hand, dense branching morphology is dominated by the diffusion rate of nonaggregating molecules[44] and yields anisotropic aggregates that reflect the macroscopic nature of diffusion fields,[31] and is typical of saturating concentrations of the aggregating molecules. Consistent with this description, we observed the crossover from dendritic growth

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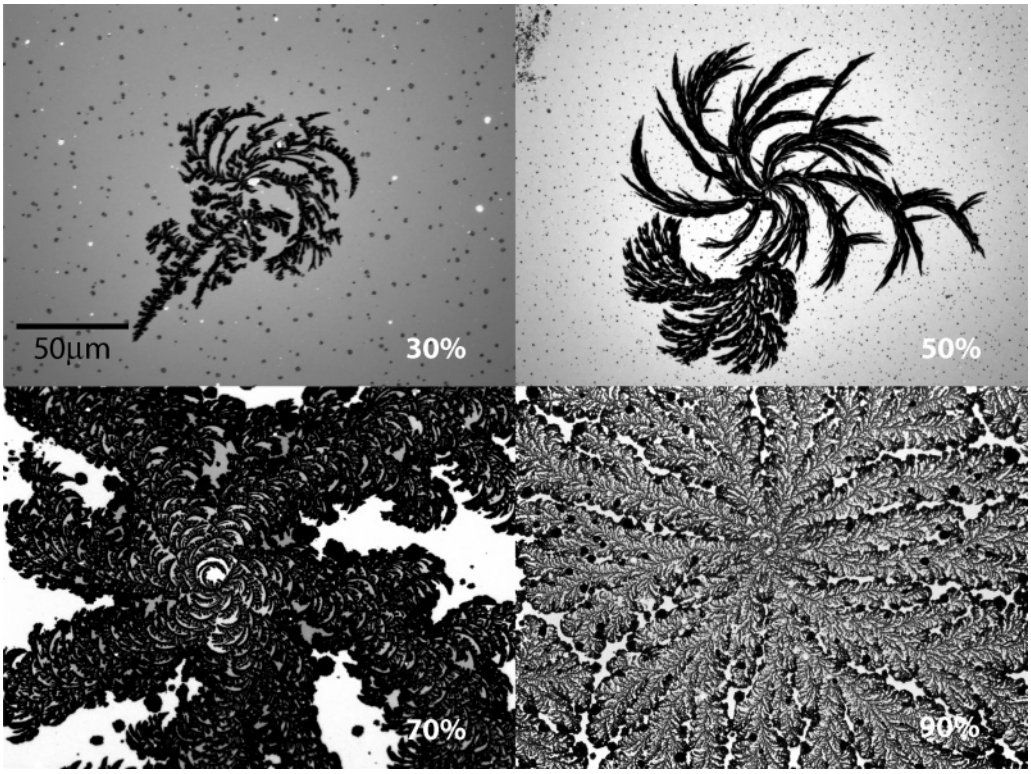

Figure 4. The morphology of BisDiynePC domains formed as the diacetylene molecules phase-separate from DOPC is heavily dependent on the relative diacetylene−phospholipid concentration. The percentages of diacetylene present in the mixture used to form the bilayer are shown on each image. Dendritic growth is observed for mixtures with diacetylene molar percentage of <50%, while dense branching morphology is observed for percentages of >50%. Competing morphologies are observed for diacetylene content of 50%.

to dense branching growth in the morphology of BisDyinePC domains as the diacetylene concentration was increased. Similar transition from dendritic growth to dense branching has been observed in fatty acid Langmuir monolayers as a function of compression rate.[45] At diacetylene molar percentages of <50%, we found that domain growth was predominantly dendritic, with the main dendrite typically curving as the cluster grew. Smaller branches typically grew to the sides of the main dendrite, but remained much smaller and did not seem to be paired. Growth at molar percentages of >50% showed dense branching morphology, with growth quickly extending in thin branches (typically smaller than a couple of micrometers) that did not seem to follow particular orientations. When the concentration of BisDyinePC was 50%, mixed growth was observed with some domains resembling dendritic growth and others resembling feathered patterns (as shown in Figure 4). Feathered patterns have been previously described in cationic lipid bilayers.[19] The competition between the two growth morphologies was also reflected in the fractal dimension calculated from aggregates as discussed below. The onset of diacetylene domain formation and the speed of domain growth also depended strongly on the relative concentrations, being slower as the percentage of BisDiynePC was decreased. The decrease in the growth rate of fractal domains is also consistent with DLA models, as fewer collisions and therefore fewer aggregation events would occur at the growing interface when the concentration of diacetylene molecules is decreased.

Fractal Dimension Analysis of Diacetylene Domains. Since its inception,[46] fractal analysis has been used to study a multitude of physical systems such as metal surface fractures,[47] myelin membrane domain formation,[30] colloid aggregation,[48] and multicomponent polymer membranes,[49] as well as in the description of domain formation in single-component or multicomponent lipid mono- and bilayers.[42−44] The fractal dimension *D* allows the characterization of self-similarity and order of an object at different length scales, as well as quantification of the domain morphology. Therefore, it represents an objective, quantitative measure of the characteristics of two-dimensional domains formed as a result of phase segregation. It can also be used as a metric to describe the growth process controlling domain formation and evolution. We used fractal dimension analysis through the box-counting method (as described in Figure 1) to further characterize the domains formed during the phase separation of BisDiynePC and DOPC at different molar ratios and at different magnifications.

To gain understanding of the information obtained from the fractal dimension analysis, phase separation was recorded in real time as the SLB cooled below 40 °C. It was observed that, once a domain nucleated, its growth was directional, forming fractal-like patterns (Figure 5). As discussed above, the formation of dendritic or dense branching morphologies arises from the nonequilibrium conditions at the growing interface and is consistent with DLA of BisDiynePC molecules. A progression of the growth of a diacetylene domain is shown in Figure 5. The graph in this figure presents two estimates of the fractal dimension based either on the boundary or the area of the fractal domain.

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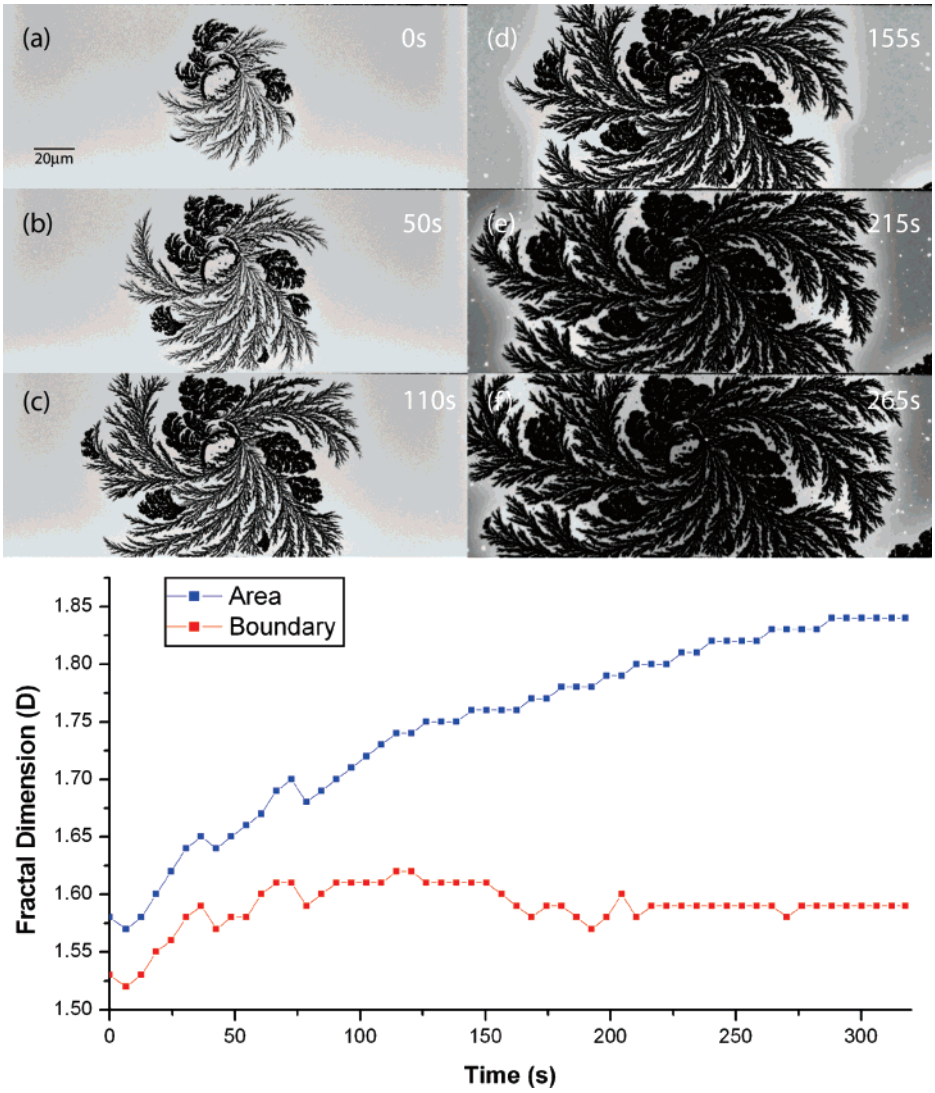

Figure 5. The morphology of a growing domain in a 90:10 BisDiynePC/DOPC mixture can be described by the computation of the boundary and area fractal dimensions. Panels (a−f) show the growth of a single domain. The BisDiynePC domain looks dark because the fluorescently labeled lipid partitions to the disordered phase. The bilayer surrounding the domain is brighter due to an increased concentration of fluorescently labeled lipids. Time stamps are shown on each of the panels. The bottom graph shows the calculated boundary and area fractal dimensions for the lapse during which the growing domain was recorded.

The images show the domain growing and progressively covering a larger portion of the frame. This progression in area coverage is represented by the fractal dimension calculated on the basis of the domain area. As expected, as the fractal domain grows, it progressively approaches a dimensionality of two, corresponding to full coverage of a two-dimensional plane. On the other hand, the boundary fractal dimension approaches a limit early on in the domain growth and remains roughly constant throughout the growth of the fractal. This number reflects the "jaggedness" of the domain boundary and has a characteristic value for each of the relative diacetylene concentrations. The type of growth that is observed can be characterized by the boundary fractal dimension, since dendritic growth has a boundary fractal dimension of 1.5,[44] while dense branching growth has typical boundary fractal dimensions of >1.7. A cluster grown by a process that is purely attributed to DLA would have a fractal dimension of exactly 1.7.[32] Therefore, we can characterize the resulting domains by two parameters, the boundary and area fractal dimensions, with the first describing the growth characteristics and the second describing the extent to which the domains can fill a two-dimensional space.

Figure 6 shows plots of the two fractal dimensions calculated for domains formed with the BisDyinePC mixtures studied. Fractal dimensions were calculated using at least five images of each mixture at each magnification used. Error bars on the plots represent the standard deviation of the calculated values between different images. All images were taken 24 h after the SLB was formed and the sample cooled to allow full domain formation. This provided a means to compare the extent to which the fractal domains covered the bilayer plane. In both calculated fractal dimensions, there is a generalized rise in the dimension value as the BisDyinePC percentage is increased, consistent with a crossover from dendritic to dense branching growth. However, it must be noted that samples containing 50% diacetylene content showed behavior that did not follow the general trend, with lower values on boundary and area fractal dimensions than expected from the general trend. This could be a direct result of the competing mechanisms at work in the formation of the fractal domains at this concentration. When these samples were imaged, domains exhibiting both dendritic and dense branching growth were observed. Yet, when each type of domain was analyzed independently, the resulting fractal dimensions were not different,

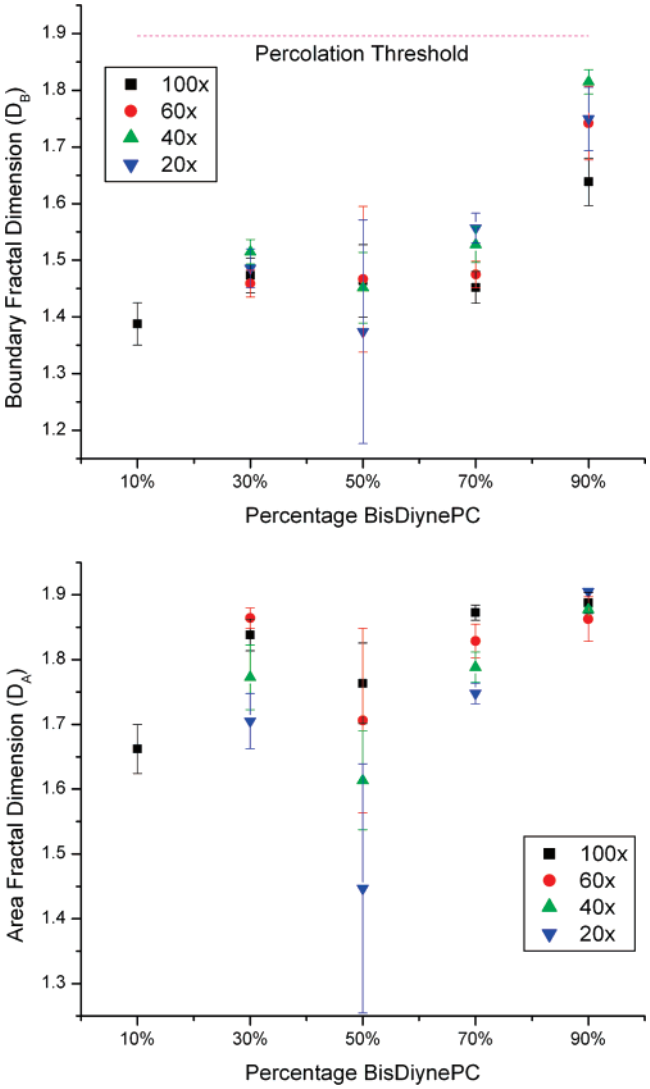

Figure 6. Boundary and area fractal dimensions show an upward trend as the concentration of BisDiynePC is increased. Domains formed at diacetylene content of 50% show an abnormal behavior which reflects the appearance of domains of different morphology. Top: Boundary fractal dimension for different BisDiynePC concentrations taken at various magnifications 24 h after cooling. Only mixtures with diacetylene content of 90% get close to reaching the threshold for a percolating network. Bottom: Area fractal dimension for different BisDiynePC concentrations at various magnifications. Note the same abnormal behavior for diacetylene content of 50%.

arguing that, although the morphology appears different, the underlying growth mechanisms are the same for both types of domains. This is an important strength of quantification based on the fractal dimensionality, which reflects the inherent characteristics of the growth mechanism that are not apparent visually. An important note is that, as the diacetylene concentration is increased, the boundary fractal dimension approaches that of a percolating network ($D_B = 1.896$),[37] meaning that the domain is fully connected across the plane. Only fractal domains with 90% BisDyinePC content came close to the percolation threshold (and in some images reached it), reflecting that the amount of the aggregating diacetylene is enough to form a continuous percolating network with DOPC fjords in liquid disordered phase. From the images taken, it is observed that in some cases the domains formed with this mixture seem to form a completely connected network in the SLB, which supports the conclusion drawn from the dimensional analysis.

The long-term evolution of diacetylene domains has also been described theoretically in terms of the fractal dimension for systems with conserved order parameter.[33] The evolution of fractal domains was followed for up to a week after their initial formation. According to the model proposed by Kalinin and co-workers, evolution of fractal aggregates following Ostwald ripening under conservative conditions can take two paths: coarsening or fragmentation.[33] Theoretically, the path taken by the fractal domain depends on the initial size and morphology of the seed cluster. In these samples, Ostwald ripening can describe the long-term evolution of the fractal aggregates, because the lipid constituents are mobile, allowing the relaxation of interfacial tension.[50] The samples studied for long-term evolution of fractal aggregates showed both coarsening and fragmentation.

Coarsening was the most common occurrence, as the metastable fractal shape relaxed, and by minimization of the excess interfacial energy, the dendritic features coarsened, forming discoidal shapes (Figure 7a,c). The fact that diacetylene domains did not completely relax to circular forms indicates that the line tension of the domain boundaries was not strong enough to overcome the unstable shape, and complete relaxation could take periods of time much longer than those studied. Longer time scales should reveal that the coarsened aggregates completely relax to circular domains, as can be seen in the much more mobile lipid monolayers formed at air−water interfaces (data not shown). The long relaxation time reflects the relative lack of mobility of the diacetylene molecules in the gel phase, which could be further reduced by the direct interaction between the lipids and the substrate.

A less common occurrence in the samples was the fragmentation of the initial domain. The fragmentation was observed as collections of small discoidal domains which outline a fractal shape (Figure 7b,d). Similar fragmentation has been observed in monoglyceride Langmuir monolayers, as a result of the disparity between orientations of domain parts that come into contact.[43] These observations could provide experimental evidence of Ostwald ripening in the fractal domain morphology. However, the interactions with the solid support should be taken into account in a complete model, because they are bound to influence domain evolution.

Nucleation of Diacetylene Domains with Arrays of Nanogold Posts. We also explored the possibility of selectively nucleating BisDyinePC domains through the use of nanostructured surfaces. DLA models assume an initial seed cluster from which the aggregates grow. In the past, such nucleating sites have been ascribed to impurities in the system, which in the case of lipid mono- and bilayers would be represented by fluorescent molecules or surface defects.[42,44] The motivation behind selectively nucleating phase separation is that such a system could serve as a model that mimics processes that take place in cell membranes, where small perturbations lead to specific functionality such as signaling. Thus, we fabricated silicon oxide surfaces which contained 50-nm-wide, 50-nm-tall gold post arrays which had a spacing of 1, 2, 5, or 10 μm. As the SLB was formed on the oxide surface, these posts acted as impurities that would trigger the nucleation of diacetylene domains.

The mixture used in all gold post nucleation experiments was 30:70 BisDyinePC/DOPC. The SLB was formed at 50 °C followed by rinsing with PBS buffer at the same temperature and then cooling the SLB to 25 °C. Before cooling of the sample,

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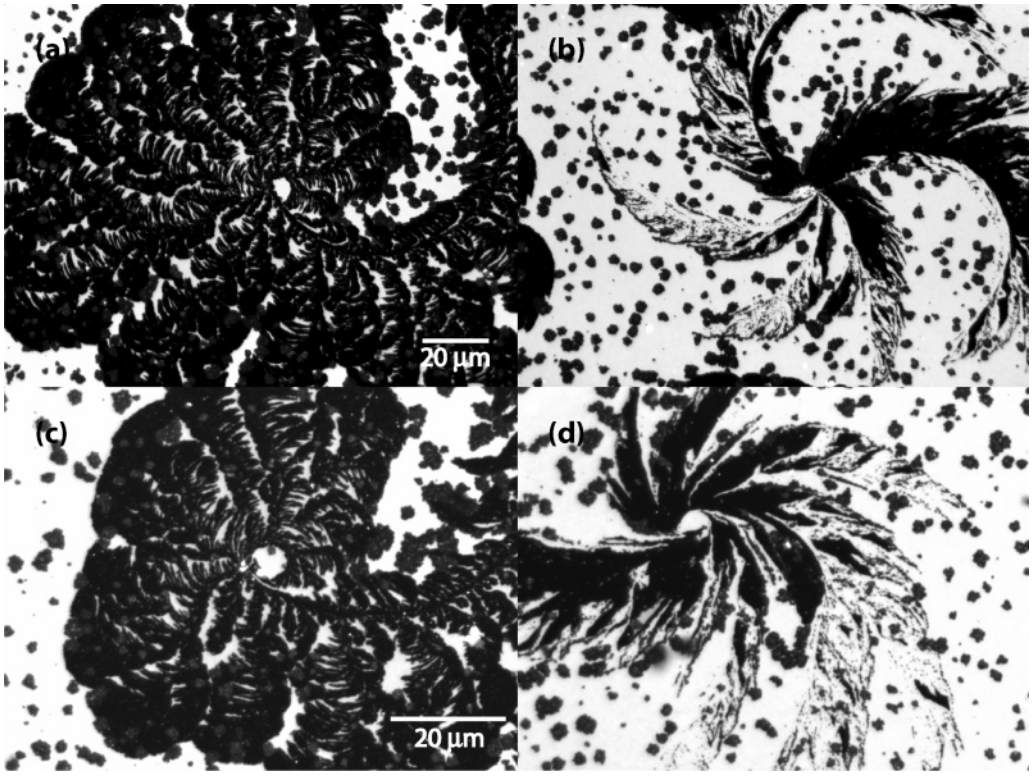

Figure 7. Fractal domain evolution over prolonged incubation times shows either coarsening or fragmentation. Images shown were taken from individual domains formed on a 50:50 BisDiynePC/DOPC bilayer two weeks after incubation and cooling to room temperature. Coarsening of fractal domains was a more common occurrence, shown in (a) and (c) for two different magnifications. Fragmentation was a rare occurrence, shown in (b) and (d) for two magnifications.

the SLB was mobile and looked homogeneously fluorescent, revealing no domain formation before cooling. As the lipid bilayer cooled, small discoidal diacetylene domains appeared preferentially around the gold pillars. Two hours after the initial formation of the SLB and after cooling, approximately 60% of the pillars showed nucleation in the form of small domains. The small domains that formed around the pillars had dimensions that were below 2 μm, with the smallest features observable being limited by optical diffraction. A few larger clusters, with fractal morphology, also formed after 2 h of incubation. At longer times (24 h), more than 90% of the pillars had promoted the nucleation of diacetylene domains. Although more fractal patterns had developed than at 2 h and had coarsened to become almost discoidal, some pillars retained only small domains, with dimensions between 2 and 4 μm. Thus, the gold pillars acted as nucleation sites for diacetylene molecules and effectively promoted the growth of diacetylene domains.

It was observed that the fractal domains formed within areas containing pillars and those outside the pillar regions had significantly different morphology. This can be seen from Figure 8a, where only half the plane in the image is covered by pillars. Fractal domains formed outside the pillar regions followed the characteristic dendritic growth observed previously for the 30:70 mixture (*cf.* Figure 4a). In contrast, fractal domains formed within the pillar regions were much more compact and had the appearance of dense branching growth, more akin to that observed with 90:10 mixtures.

Figure 8 shows fractal domains which formed in the 1, 2, and 5 μm spacing pillar arrays. Figure 8a shows a diacetylene domain which formed at the boundary of the pillar array and shows a clear difference in the domain growth outside and inside the pillar regions with 1 μm spacing. Calculation of the fractal dimension for each side of the domain yields an area fractal dimension of 1.45 for the domain outside the pillars and 1.75 for the domain inside the pillars. The boundary fractal dimension also showed an increase from 1.35 for the fraction of the domain outside the pillars to 1.54 for the portion inside the pillar region. This estimate supports the notion of a shift in the growth pattern from dendritic to dense branching. Figure 8b,c also shows that the branches of a domain forming within the pillar arrays grow preferentially in the directions following the rows and columns of pillars. From Figure 8, it can also be observed that, as the spacing between the pillars increases, the morphology more closely resembles that found outside the pillar arrays, as expected by the decrease of impurities present per unit area. Figure 9 shows the fractal dimension for domains formed in the different gold post arrays. It is observed that, as the spacing increases, the fractal dimension for the observed domains approaches that of domains grown in absence of the pillars.

Growth controlled by the pillar arrays is reminiscent of the patterns obtained with Hele-Shaw cells when periodic impurities and boundaries are introduced.[31] We propose that the possibility of selectively nucleating lipid domains is an efficient way of patterning lipid mixtures into predefined shapes, and even isolating phases after sample cooling. By creating an array of impurities that coarsely outlines the boundary of the area where the mobile lipids are to be confined, patches of fluid lipids can be isolated by the formation of a diacetylene barrier after the sample is cooled. In principle, this could be achieved by pillar arrays or even by steps etched into the substrate, as we have observed that etched features also serve as nucleation points for diacetylene domains (images not shown). These features could provide advantages in the design of novel biosensing interfaces where the polymerizable character of BisDiynePC can be exploited to permanently immobilize the diacetylene patterns.

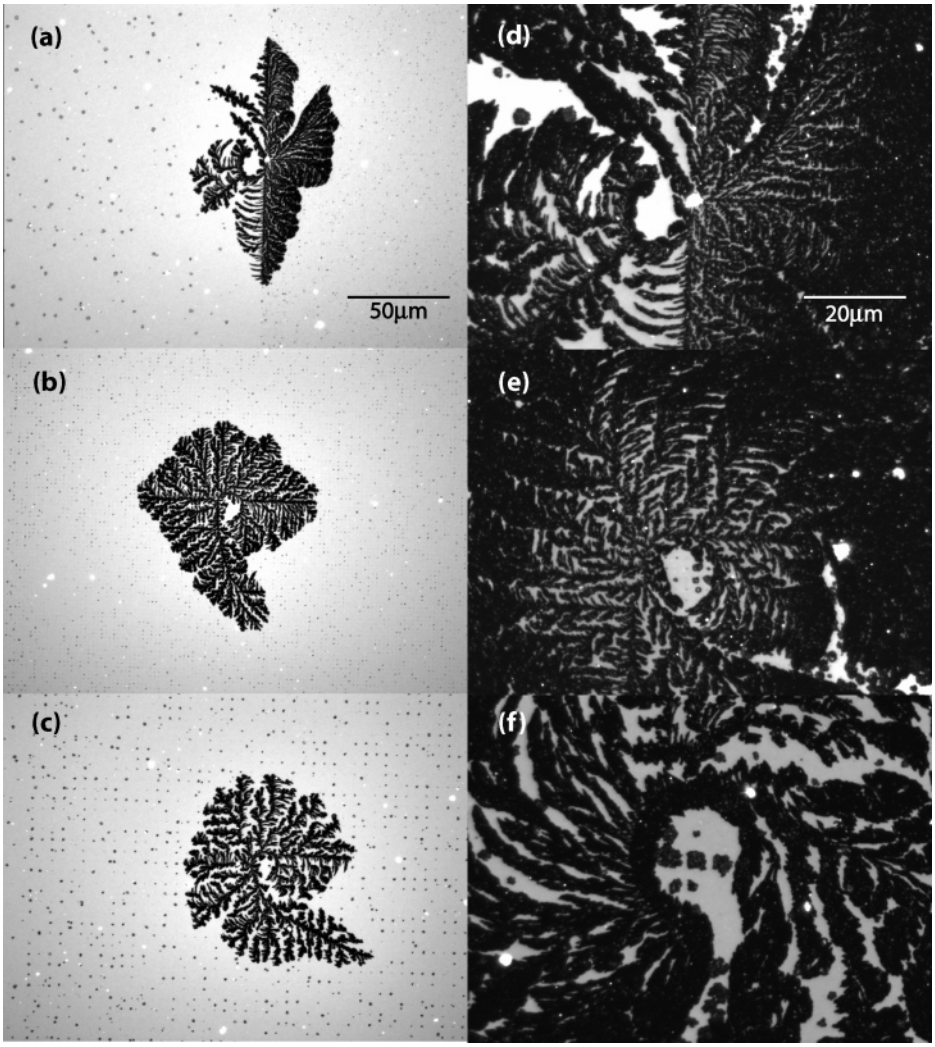

Figure 8. Growth patterns of diacetylene domains can be modified with nanostructured surfaces. Gold post arrays function as impurities which promote the nucleation of diacetylene domains. All images were taken from domains of 30:70 BisDiynePC/DOPC mixture. Panels (a−c) show domains grown for 2 h after cooling on gold post arrays with 1, 2, and 5 μm spacing (40× magnifications). Panels (d−f) show the central part of domains 24 h after cooling on gold post arrays with 1, 2, and 5 μm spacing (100× magnifications). A dramatic change in appearance is observed in panel (a) where the left half of the domain lies outside the pillar array and resembles images for domains grown in the absence of pillar arrays.

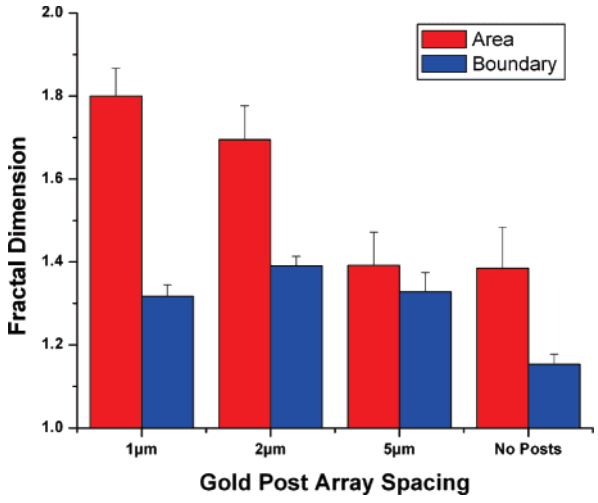

Figure 9. The boundary and area fractal dimensions are changed by the pillar arrays and reflect the change of the domain morphology from dendritic growth observed outside the pillar arrays to dense branching growth in the pillars. The fractal dimensions converge as the spacing of the pillars increases.

Conclusions

Phase separation of diacetylene lipids and phospholipids has been studied in SLBs. We have observed that phase separation and diacetylene domain formation happened in SLBs but not in intact GUVs, suggesting that the presence of surface impurities that act as nucleation sites is required for domain formation. Through AFM, we found that the domains formed by the diacetylene lipids were tetralayer stacks with an average monolayer thickness of 3 nm.

Domains resulting from phase separation had characteristic morphologies depending on the relative diacetylene−phospholipid concentration. Lower diacetylene concentrations yielded domains that grew as dendrites. Higher diacetylene concentrations shifted the growth behavior to dense branching morphologies. At diacetylene concentrations of 50%, the domains showed two different types of growth. The transition from dendritic to dense branching growth can be explained as the result of the interplay of two processes with different time scales. These processes are the rate of aggregation of diacetylene molecules and the rate of diffusion of the nonaggregating lipids away from the growing domain interface. When the growth is limited by the aggregation rate, the domains appear dendritic, while if the growth is controlled

by the diffusion rate of DOPC, the growth is skewed toward dense branching. Such domain morphology characterization was also reflected on the boundary and area fractal dimensions. Fractal analysis through the box-counting method allowed quantification of the diacetylene domain morphology. The fractal dimensions found for dendritic and dense branch domains agree with theoretical estimates developed in the frame of DLA. Evolution of fractal domains was also observed to undergo either coarsening or fragmentation over prolonged incubation.

We also assessed the possibility of selectively nucleating the diacetylene domains through the use of nanostructured substrates. Gold post arrays were found to effectively promote domain nucleation and dramatically affected the appearance of diacetylene domains, making them more compact and densely branched. These observations were also supported by the boundary and area fractal dimensions calculated. It was found that, as the array spacing was increased, the fractal dimensions for the formed domains converge to those of domains formed outside the pillar arrays. We suggest that the selective nucleation can be used for the construction of membrane morphologies and lipid distributions which can be manipulated and incorporated into biosensor design. We also propose that such nanometer-scale perturbations that promote phase separation could mimic protein aggregation and clustering in cell membranes, which promote the formation of lipid rafts and ultimately result in specific membrane functionality.

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