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Light Induced Liquid Crystalline Phases in the Lipid Bilayer

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The cell membrane is one the most well known examples of liquid crystalline behavior in living systems and in recent years more detailed attention has been paid to the different phases which may occur in biologically relevant lipid bilayers. In this paper we examine a ternary lipid mixture formed from DOPC, sphingomyelin, and cholesterol. In such mixtures fluorescence illumination has been shown to induce the formation of large, more ordered domains or 'rafts'. This mechanism is investigated using fluorescence microscopy and we find the formation of various "soft" structures including the newly discovered "disc instability" is induced by illumination of lipid tubules.

Keywords: biological-membranes; cholesterol; fluorescence microscopy; giant vesicles; model membranes; phase-separation; phase-transitions; rafts; tubules

INTRODUCTION

Lipid molecules form the primary constituent of the cell membrane. They are amphiphillic and therefore exhibit lyotropic phase behavior in an aqueous environment. Most relevant to the cell membrane is the lamellar phase, where lipids assemble into a sheet-like bilayer structure. Such lyotropic phases are sensitive to changes in solvent

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concentration however further rich phase behavior can be observed by varying the temperature of lipids in the lyotropic phase. Various thermotropic phases may be observed and in particular, in-plane ordering in the lamellar phase is modulated as a function of temperature. Such in-plane phase behavior may be relevant to biological function and in recent years it has been observed that such phenomena could be responsible for lipid "domains" or "rafts", areas of the membrane with increased in-plane order and differing composition to the surrounding regions. These domains have been linked to such mechanisms as protein sorting [1], transport [2], and activation [3]. A considerable amount of work on the thermotropic phases of membrane lipids has been carried out previously [4,5] and it is well known that we can observe a phase transition at the so-called melting temperature (T_m). Above this temperature, the membrane is described as being in the "L_α", "l_d" (liquid disordered) or "liquid crystalline" phase. Lipids in this phase display a high in-plane mobility and short-range order. Below T_m, the membrane forms the "gel" phase, or "$L_{\beta'}$". This phase exhibits long-range in-plane order and a high degree of conformational order among the alkyl chains.

Some very interesting phase behavior occurs if we mix different lipids with cholesterol, a method which provides an interesting model for the cell membrane. By mixing a high T_m and low T_m lipid with cholesterol (i.e., one lipid in the pure state will be in the liquid crystalline phase at room temperature and one will be in the gel phase in the pure state at room temperature) phase separation occurs and a new l_o or "liquid ordered" phase is formed. In this phase the membrane retains the fluid-like properties of the l_d phase, but with more highly ordered chain packing, reducing the rate of in-plane diffusion.

Such phase separation has been observed in several lipid systems now by various authors and several techniques are commonly employed to characterize the phase behavior. Most common is the use of fluorescence microscopy on giant uni-lamellar vesicles (GUVs) [6–12], and atomic force microscopy (AFM) on supported lipid bilayers (SLB) [13–15].

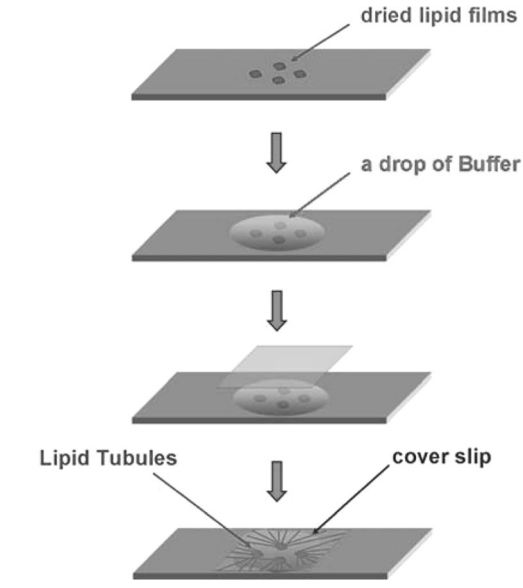
Coexistence of the l_o and l_d phases in a phase separated GUV can be observed by labeling the lipid mixture with small amounts (typically <0.5 mol%) of fluorescent lipids. In such experiments micrometer size domains are typically observed [6–12]. Such domains are very large however compared to the size of a cell so it is clear that our simplified models for the membrane do not accurately represent the cellular system. In addition recent experiments have linked photo-induced lipid peroxidation to this phase separation phenomenon [16,17]. In this paper we discuss the effects of peroxidation-induced phase separation

on a typical ternary lipid mixture and how such an effect can be used to create interesting new lipid-based structures. "Soft" fluid state microfluidics have been explored in recent years, where networks of vesicles and tubules can be formed by micropipette aspiration to create complex functional structures [18] and networks of ultra-small scale micro-reactors.

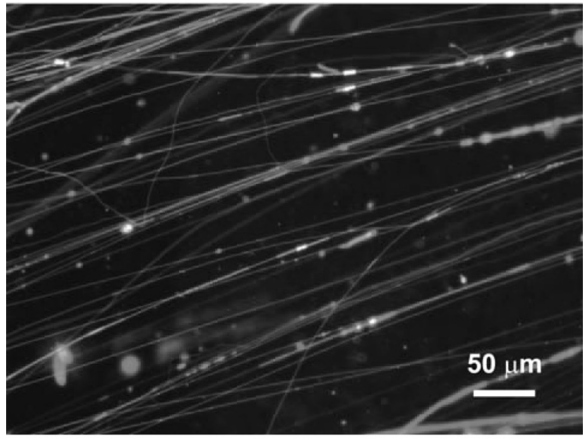
METHODS

Ternary lipid mixtures were prepared consisting of 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC, T_m:−20°C), (2S,3 R,4E)-2-acyl-aminooctadec-4-ene-3-hydroxy-1-Phosphocholine (Egg sphingomyelin) (eSM, T_m:40°C), and Cholesterol. The labeled lipids, 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (Ammonium Salt) (NBD-DPPE), and 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine-N-(Lissamine Rhodamine B Sulfonyl) (Ammonium Salt) (Rh-DPPE) were added in small amounts to the mixture (<0.5 mol%), where necessary for fluorescence microscopy. All materials were purchased from Avanti Polar Lipids (Alabaster, Al) and used without further purification. The lipids were dissolved in chloroform (HPLC grade) and stored under −20°C with a total lipid concentration of 0.5 mM. All mixtures were prepared with equal molar ratios of DOPC and eSM, but with a differing mol% of cholesterol.

Although lipids may form many different structures in solution, such as unilamellar vesicles, lipid tubules, multilamellar structures or the more exotic "Pearling instability" [19], one geometry which is particularly interesting is the lipid tubule. If we are interested in constructing a "soft" fluid-fluid microfluidic system, then "vesicle-like" chambers can be connected by tubular channels [20]. Lipid tubules structures are essentially very long, narrow unilamellar vesicles and can be formed by flow-assisted rehydration. A lipid solution with a total lipid concentration of 0.5 mM is carefully dip-coated on a clean glass slide surface as small isolated droplets. After 2 hours under vacuum, the dry lipid film is re-hydrated with a drop of Milli-Q water or 100 mM sucrose solution at a temperature above T_m, and then covered with a piece of cover glass. Following hydration, the formation of lipid tubules can be directly observed with a fluorescence microscope provided a fluorescent probe is incorporated. To keep the lipid tubules from drying out, the edges of the cover glass are sealed with vacuum grease or wax. Fluorescent probes, Rh-DPPE for DOPC and Rh-DPPE/NBD-DPPE for ternary lipid mixture of DOPC/eSM/cholesterol, were added to lipid solutions to visualize lipid tubules at 0.2 mol% before drying. This method is summarized in Figure 1a



(a)



(b)

FIGURE 1 (a) Schematic showing the process for lipid tubule formation and (b) Fluorescence microscope image of lipid tubules formed by this method from DOPC labeled with Rh-DPPE.

and fluorescence microscopy images of lipid tubules labeled with Rh-DPPE can be seen in Figure 1b. Tubules prepared using this method are typically oriented parallel to each other and remain

anchored to the lipid film, providing a nice array of tubules for observation.

Fluorescence microscopy imaging was carried out on a Leica DM LP (Leica Microsystems Inc., Bannockburn, USA) upright microscope. All microscopy measurements were carried at a room temperature (~22°C). By labeling lipids with two fluorescence probes, which partition preferentially into different regions in the "phase-separated" membrane, we are able to observe contrast between the two different phases and some interesting structures are observed.

Supported Lipid Bilayers (SLBs) for AFM were prepared using the well known vesicle fusion method. Briefly, small unilamellar vesicles (SUVs) solutions (0.5 mM) were prepared by tip-sonicating a multilamellar vesicle solution for 10 mins, with a water bath (60°C) and Nitrogen protection. After centrifuging (to remove titanium debris from the tip sonicator), about 2 ml of SUV solution was transferred into a Petri-dish, in which a freshly cleaved mica substrate was glued on the bottom, and incubated at 55°C for 30 min. The sample was rinsed with Deionized water thoroughly, taking care to keep the sample surface under water at all times. Finally the Petri-dish was transferred to the AFM stage and imaged with a multimode AFM (Dimension 3100, Digital Instruments, Santa Barbara, CA) using fluid contact mode.

RESULTS

Figure 2a shows an AFM image of a single lipid bilayer on mica formed from a lipid mixture consisting of DOPC/eSM/Cholesterol at a molar ratio of 1:1:1. Small lipid domains can be seen to form, the l_o phase being slightly thicker than the l_d phase. A height difference of 0.58 nm is measured (Fig. 2b) by plotting the height line profile across this image. Figure 2c shows a schematic of a single bilayer in cross-section in which the lipid sorting which leads to domain formation can be seen. Cholesterol molecules preferentially partition with the saturated sphingomyelin chains, away from the more disordered packing of the unsaturated DOPC molecules. It is important to note here that the size of the membrane domains observed using AFM is on the nanometer length-scale, i.e., too small to resolve optically. When this mixture is used to prepare vesicles in solution, domains on the surface are often not optically observed initially, as would be expected: they are smaller than the resolution of the microscope.

Macroscopic domains can be formed in giant vesicles by a photo-oxidation process and this effect has been discussed recently in the literature [21,17]. In this case, domains tens of microns in

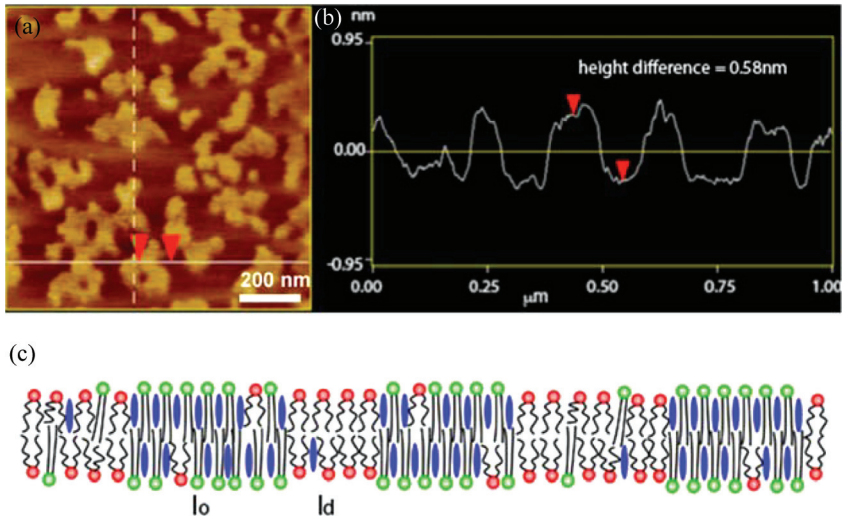


FIGURE 2 Atomic force microscope image 1 μm square (a) and profile plot (b) of a single phase-separated bilayer on mica revealing the topological differences between the l_o (thicker – pale domains) and l_d (thinner) phases. (c) Schematic of the membrane molecular arrangement demonstrating the membrane organization. (Red lipids = DOPC, green lipids = eSM, blue molecules – cholesterol).

diameter are observed. The mechanism by which this photo-induced phase separation can be initiated originates in the labeled lipid molecules. Excitation of the fluorophore (e.g., Rh-DPPE in our case) in the presence of oxygen may result in the production of free radicals in the solution. These radicals are highly reactive and are able to peroxidize the double bonds of an unsaturated lipid in the mixture, in this case the DOPC. Peroxidation of this double bond also results in a *cis* to *trans* conversion [22] [Porter, N. A. Caldwell, S. E. Mills, K. A. Lipids, 1995, 30, 277]. The mechanism by which this leads to macroscopic phase separation is still not fully understood although it clearly increases the disorder in the DOPC dominated domains, possibly making it a less favorable environment for the more ordered sphingomyelin/cholesterol packing and promoting phase separation [23].

Lipid tubules were formed from the ternary lipid mixture DOPC/eSM/Cholesterol at a molar ratio of 1:1:1 with ~0.2 mol% Rh-DPPC added to investigate. Initially optical observations show no evidence of phase separation however after ~30s clear domains are observed to appear in the tubules. Dark patches of the l_o phase form as the labeled lipid preferentially partitions into the more fluid l_d

phase and away from the growing l_o domains. As in the case of GUVs, this peroxidation-induced phase separation only occurs when the ternary lipid mixture contains an intermediate amount of cholesterol ($10 \sim 50\%$). Outside of this range, phase separation cannot be induced even after long illumination times. Such a result is in good agreement with the phase diagram for GUVs of the same composition [8] indicating that the phase behavior in lipid tubules is very similar to that of the GUVs. In addition, by using the antioxidant n-propyl gallate (NPG (5 mM)), shown to retard the peroxidation in GUV samples, [21] we did not observe micrometer-scale domains at any ratio.

A time sequence of domain coalescence can be seen in Figure 3 on the surface of a particularly thick tubular structure. Over ~20s after illumination the formation of macroscopic domains is observed. These domains merge to form large micron-scale areas in the membrane. It should be noted however that the tubule seen in Figure 3 is much thicker than those typically observed and is shown merely to demonstrate the domain growth over time. The tubules shown in Figure 1b are more typical with a diameter of $<1\,\mu$m.

On the formation of domains in the lipid tubule two clear domain organizations can be observed, a "band" structure, where domains form an alternating pattern along the tubule and the "disc instability".

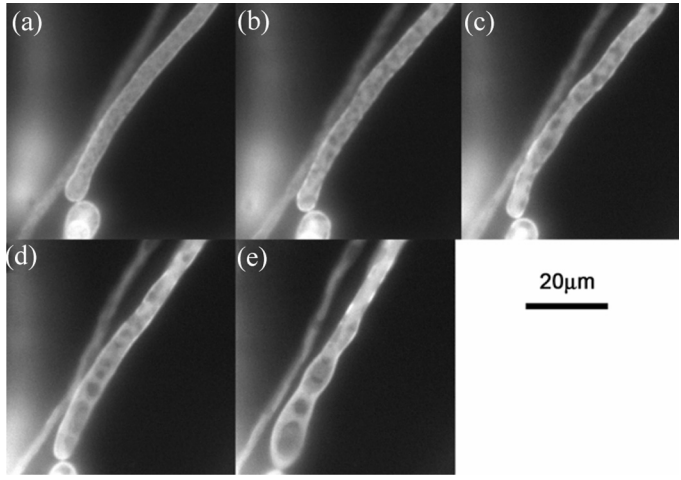


FIGURE 3 Fluorescence microscopy time sequence demonstrating the formation of large liquid ordered domains in a particularly thick lipid tubule formed from DOPC:eSM:cholesterol at a molar ratio of 1:1:1. Images are taken sequentially over ~20s using fluorescence microscopy.

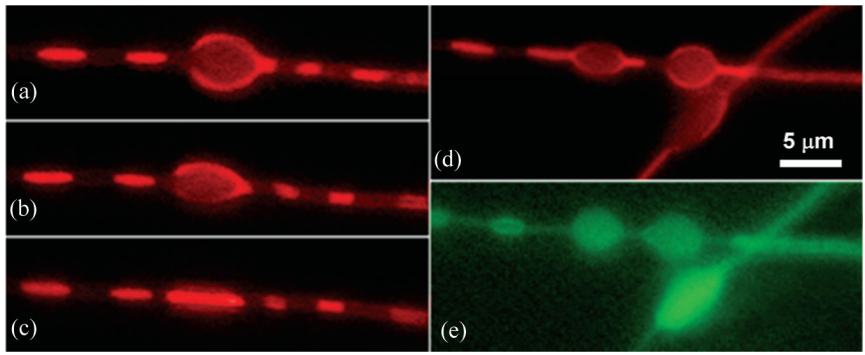


FIGURE 4 (a–c) A single lipid disc formed in a lipid tubule between l_d (bright) and l_o (dark) bands. Images are taken sequentially over a time period of ~10s, revealing the flat nature of the disc as it rotates and (d–e) Double labelling of tubules exhibiting the two structures showing (d) the l_d phase (fluoresence emmission from Rh-DPPE) and (e) the l_o phase (fluorescence emission from NBD-DPPE).

Lipid bilayers discs are formed along the length of the tubule, their flat surfaces consisting of islands of the l_o phase [17]. The coexistence of these two structures in a single tubule can be seen in Figures 4 and 5.

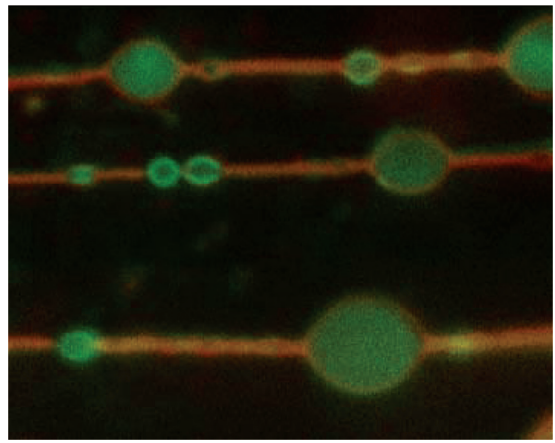


FIGURE 5 Fluorescence microscopy image displaying coexistence of the "band" structure and the "disc" structure in phase-separated lipid tubules. The l_o phase is labeled in green (NBD-DPPE) and the l_d phase in red (Rh-DPPE).

CONCLUSIONS

In this paper we describe some of the interesting macroscopic membrane structures which can be formed as a result of in-plane phase separation in the lipid bilayer in ternary lipid mixtures. The lower curvature of the l_o phase over the l_d phase (compositionally different sub-phases of the classic L_α liquidcrystalline phase) can lead to the formation of non-spherical geometries such as a bilayer disc structure and banded tubules. As changes in morphology can be photo-induced it should now be possible to create custom structures based on inducing localized changes in curvature for "soft" membrane based nanofluidic devices using this technique. Most interestingly such changes in structure can be effected remotely without mechanical interference to the delicate system.

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