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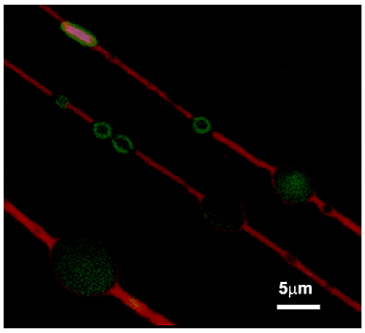

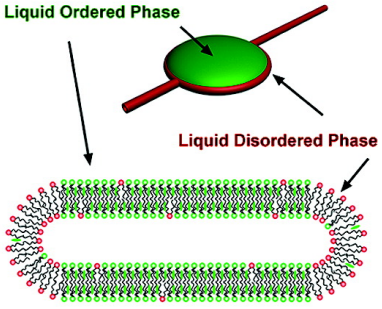

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Lipid Bilayer Discs and Banded Tubules: Photoinduced Lipid Sorting in Ternary Mixtures

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Abstract: The self-assembly of biological amphiphiles has proved a fascinating topic in recent years, the hollow cylindrical lipid tubule morphology being of particular interest due to its potential applications in "soft" microtechnologies. Lateral coexistence of liquid-ordered (l_o) and liquid-disordered (l_d) phases, which may resemble raft formation in cell membranes, was investigated in lipid tubules, prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, egg-sphingomyelin, and cholesterol. Fluorescence microscopy shows that the appearance of micrometer-scale l_o domains in the lipid tubule is not an intrinsic phase behavior of the system but a consequence of photoinduced lipid peroxidation. Most interestingly, new photoinduced bilayer structures: lipid discs, essentially stable flattened liposomes, were observed for the first time in a model membrane system. This investigation not only aids in our understanding of lipid sorting phenomena in cell membranes but also demonstrates how control of this process may provide a route to the generation of new, functional structures.

Introduction

Lipids are the main components of biological membranes, and as amphiphilic molecules, they display fascinating and diverse lyotropic phase behavior as a function of their concentration in aqueous solution. Recent studies of the cell membrane and its increasingly apparent complexity have initiated a rapidly expanding field following the realization that the bilayer of the membrane is typically not a homogeneous fluid-like structure, as described by Singer and Nicholson in their fluid mosaic model,[1] but that domains of differing lipid compositions to the surrounding bilayer may dynamically arise and, through their differing order and chemical environment, assist in protein sorting,[2] transport,[3] and activation.[4] The thermotropic phase transition in membrane lipids has been studied extensively.[5,6] Pure lipid membranes show a sharp phase transition at the melting temperature (T_m). Above T_m, the "fluid" membrane (L_α or L_d phase) exhibits a high lateral mobility of molecules in the plane, and a high degree of disorder in the conformational states of the alkyl chains. Below T_m, the "gel" state is characterized by quasi-long-range order in the plane and a high degree of conformational order among the alkyl chains. By mixing a high T_m and low T_m lipid with cholesterol it is possible to induce the formation of a new liquid ordered (l_o) state, where the alkyl chains are ordered but there exists lateral mobility of the molecules.[7] First described as "detergent resistant membranes" (DRMs),[8] these lipid domains or "rafts" are postulated to take the form of lateral patches of differing composition to the surrounding areas. The membrane may be described as phase-separated bilayers, with areas of differing functionality. Such phase separation has been observed in model membrane systems such as giant unilamellar vesicles (GUVs)[9−16] and supported lipid bilayers (SLB)[17−19] by a variety of techniques including fluorescence microscopy, atomic force microscopy, and nuclear magnetic resonance.

Several recent studies have utilized GUVs to determine the phase diagram of ternary lipid mixtures[10,11] and relate their results to the living cell[16,20] as the specific mixtures in which phase separation occurs may be biologically relevant. Coexistence of the l_o and l_d phases in GUVs can be visualized by careful

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use of fluorescent labels, and micrometer size domains are easily observed.[9−16] Such a phenomenon has also been discussed theoretically,[21−23] linking domain size, shape, and elastic properties. There is some controversy, however: such large domains are not observed in biological cells, and other factors such as photooxidation or contamination can significantly affect the results.[24]

Hollow cylindrical lipid tubules have been of particular interest due to their potential relevance to intercellular transporting channels[25] and applicability to controlled-release systems,[26,27] chemical microreactors,[28] and nanotemplates.[29,30] Lipid tubules were first investigated by Hochmuth and Evans, formed by aspirating a portion of the cell membrane into a small micropepitte.[31] Apart from the self-assembled lipid tubules from diacetylenic lipid derivatives,[32−34] lipid tubules were generally considered to form from deformation of lipid membranes undergoing a certain force,[35] such as capillary extrusion,[28,36] molecular motors,[37,38] hydrodynamic flow,[39] and electro-osmotic flow.[40] Lipid tubules have a similar detectable scale in one dimension compared to GUVs, and their unique shape (diameter $\sim$1 μm or less, length $\sim$1 mm) and membrane properties make them a suitable candidate to investigate phase behavior in ternary lipid systems in a more restricted geometry.

In this paper, we describe a simple method to prepare lipid tubules using hydrodynamic flow and investigate photo-oxidation effects on domain formation in lipid tubules formed from ternary mixtures of a high T_m, a low T_m lipid and cholesterol. The phase diagram for this particular ternary mixture has been described previously in GUV studies.[10,11] By labeling lipids with two fluorescence probes, which partition into different regions in the "phase-separated" membranes, interesting structures, such as banded lipid tubules and membrane discs, are observed as a consequence of photoinduced lipid peroxidation. This is the first time such membrane disc structures have been reported in a model membrane system. This observation not only aids in our understanding of lipid sorting phenomena in cell membranes (suggesting a mechanism for bilayer disc formation in retinal rod-cells), but may provide a fascinating route to the generation of new, functional structures by controlling lipid morphologies through photo-effects.

Methods and Materials

Chemicals. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), (2S,3R,4E)-2-acylaminooctadec-4-ene-3-hydroxy-1-phosphocholine (egg sphingomyelin) (eSM), cholesterol, 1-oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (18:1−12:0 NBD-PC), 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 purchased from Avanti Polar Lipids (Alabaster, AL). Perylene was purchased from Fluka (Sigma-Aldrich, St. Louis, MO). All of the materials were used without further purification. Lipids were dissolved in chloroform (HPLC grade) and stored under −20 °C with a total lipid concentration of 0.5 mM. All the lipid mixtures were prepared with equal molar ratios of DOPC and eSM, but with a differing mol % of cholesterol from 0 to 50%. Fluorescent probes, NBD-PC and Rh-DPPE/perylene (or Rh-DPPE/NBD-DPPE) were added to DOPC and ternary lipid mixtures of DOPC/eSM/cholesterol, respectively, to visualize lipid tubules in an amount of 0.2 mol %.

Lipid Tubule Formation. Lipid tubules are prepared using flow-assisted rehydration. Briefly, a lipid solution is carefully dip-coated on a clean glass slide surface as small isolated droplets. After 2 h under vacuum, the dry lipid film is re-hydrated with a drop of Milli-Q water (18.2 mΩ cm^{-1}) or 100 mM sucrose solution at a temperature above its T_m, and then covered with a piece of cover glass. Following hydration, the formation of lipid tubules can be directly observed with fluorescence microscopy. To keep the lipid tubules from drying out, the edges of the cover glass are sealed with vacuum grease or wax. For the ternary lipid mixtures, water or sucrose solution used to rehydrate the lipids was prewarmed to 50 °C, and the lipid rehydration and sealing was carried out inside an incubator at 50 °C.

Microscopy. Laser scanning confocal fluorescence images of lipid tubules are acquired with an inverted Nikon TE2000-E2 Eclipse C1si (Nikon Instruments Inc., Melville, NY, USA) Confocal Laser Scanning Microscope (CLSM) equipped with a Nikon CFI Plan Apochromat TIRF 60× oil immersion objective (NA 1.49, 0.12 mm WD). A 404 nm modulated diode laser and a 561 nm diode pumped solid-state laser are simultaneously used to excite perylene and Rh-DPPE, respectively (filters: 450/35, 515/30, 605/75), using a 65.1 μm pinhole. Conventional fluorescence microscopy imaging was carried out on a Leica DM LP (Leica Microsystems Inc., Bannockburn, USA) upright microscope with a 120 W mercury lamp and a 63× water immersion objective. Under this objective, samples were exposed to a 0.2−0.4 mW illumination depending on the wavelength used. A Linkam LTS350 temperature stage was used to control the temperature, and unless stressed, microscopy measurements were carried at a room temperature ($\sim$22 °C).

Results and Discussion

Single Component Lipid Tubules. Successful preparation of lipid tubules is shown in Figure 1. Using fluorescence microscopy, the dynamic growth of lipid tubules can be directly visualized. Typically, the tubules are well aligned with each other, forming parallel to the flow direction. The diameter of each tubule is about 1 μm or less, with a length of up to several millimeters. Formation of such a spectacular array of tubules can be attributed to the hydrodynamic flow. No tubule formation was observed in a closed chamber, and the formation of new tubules was stopped once we sealed the edges of the cover glass

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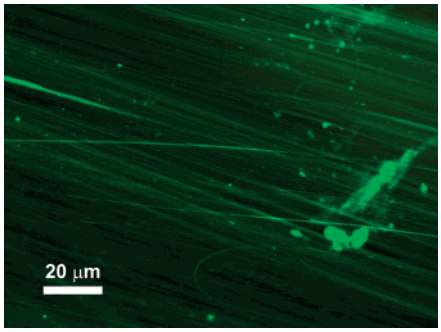

Figure 1. Fluorescence microscope image of highly aligned, flow generated DOPC lipid tubules labeled with 18:1−12:0 NBD-PC.

with vacuum grease or wax. These structures are relatively stable (last for up to 24 h), thus providing the possibility to obtain more detailed structural information.

Photoinduced Banded Patterns in Ternary Lipid Tubules. The coexistence of two liquid phases (l_o and l_d) has been directly observed on the surface of giant unilamellar vesicles (GUVs) by several individual groups using fluorescence microscopy.[10,13−15,20] Lipid tubules have a similar detectable scale in one dimension; their typically high aspect ratio makes them a suitable candidate to investigate the phase behavior in ternary lipid systems in a more restricted geometry. DOPC, eSM, and cholesterol were selected to generate the tubules, and all the samples were prepared with equal molar ratios of DOPC and eSM, but with a differing mol % of cholesterol.

Figure 2 shows lipid tubules comprised of 1:1:1 mol % DOPC/eSM/cholesterol. Although the same mixture has been previously reported to form equal proportions of coexisting l_o and l_d domains in GUVs,[10] we initially observed continuous, uniform lipid tubules (Figure 2a), indicating that spontaneous micrometer-scale phase separation does not occur in the tubular geometry prepared by this method. However, lateral reorganization of lipids and phase separation was observed after ~5 s of illumination (Figure 2b). This observation was made based on the partitioning properties of the fluorescent probe Rh-DPPE, which preferentially stays in the l_d domains. Further illumination of the same region gave rise to more dark domains, which subsequently organized to form a "banded" pattern (Figure 2c and d).

The dark patches are fairly uniform in size ($2 \pm 0.5\ \mu$m) and do not coalesce into longer patches even after exposure to illumination for an extended period of time. This is in contrast to the GUV system, in which the l_o and l_d phases often coalesce to form two distinct phase domains.[9−12] The size of the patches may be modulated by lipid composition, temperature, and illumination intensity. Due to the complexity of the system and the multiple physical forces in play, this will be systematically investigated in the future. It is not surprising that the bands do not coalesce on the tubule once formed as the cylindrical geometry restricts their motion, compared with circular "islands" on a spherical vesicle surface. To fuse with each other, neighboring domains along the lipid tubule would have to meet, but they are blocked from doing so by the other phase in-between, whereas on the GUV surface, molecules in the l_d phase can more easily diffuse two dimensionally to allow l_o domains to meet. This geometrical difference results in trapping the banded pattern in a quasi-equilibrium state.

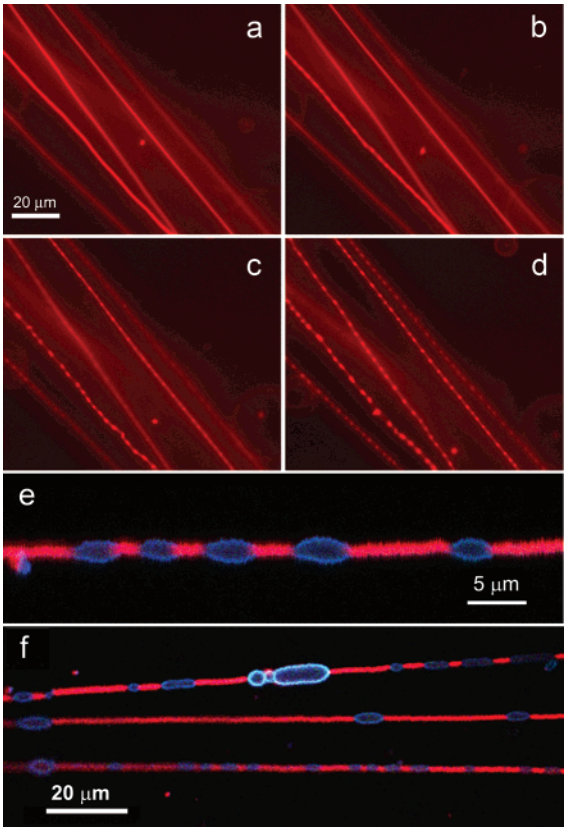

Figure 2. Photooxidation-induced lipid sorting in Rh-DPPE labeled DOPC/eSM/cholesterol (1:1:1) lipid tubules with increasing illumination time (a) 0 s, (b) 10 s, (c) 20 s, and (d) 30 s; (e, f) Confocal images of phase separated lipid tubules labeled with perylene (blue) and Rh-DPPE (red).

The process of photoinduced phase separation takes about 30 s. The possibility that the phase separation was induced thermally by illumination was investigated but was found not to be a factor. The phase separation process was found to be irreversible as the banded pattern was never observed to relax back to the uniform state, even if kept away from illumination for more than 1 h. Increasing the temperature to 45 °C resulted in a miscibility of the two fluid phases, which is in good agreement with the reported miscibility transition temperature (T_{mix}) in a similar GUV system.[10] The banded pattern was then recovered after cooling the sample back down to room temperature; however, due to drifting of the tubules at high temperatures and the continual illumination of the sample as the temperature is varied, it was difficult to compare the same region before and after heating. Another important observation is that this phase separation only occurs in the small region illuminated by the microscope. Whenever the focus is moved to a fresh region, similar lipid sorting and phase separation can be observed again, initiated from uniform lipid tubules.

The mechanism by which this photoinduced phase separation can be initiated was described in detail in a recent paper and originates in the labeled lipid molecules.[24] Excitation of the fluorophore (e.g., Rh-DPPE in our case) initiates a chain reaction whereby unsaturated lipids photooxidize at the double bonds to create lipid peroxides.[41] This process may directly contribute to the formation of micrometer-scale domains in model membranes.[24] In our ternary lipid tubule system, we have found that,

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in the same way, generation of lipid domains is initiated by microscope illumination in the tubule geometry as well as in more vesicle-like structures. Moreover, peroxidation-induced phase separation only occurs when the ternary lipid mixture contains an intermediate amount of cholesterol (10∼50%). Outside of this range, phase separation cannot be induced even after long illumination times. This result is in good agreement with the phase diagram for GUVs of the same composition,[11] 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,[24] we did not observe micrometer-scale domains at any ratio.

The sectional morphology of a phase-separated lipid tubule can be more clearly visualized with CLSM by labeling the lipid mixture with perylene and Rh-DPPE, which partition into the l_o and l_d phases respectively (Figure 2e and f). No difference in the phase behavior of this system was observed on the addition of perylene. The l_o domains (blue) look like hollow elongated vesicles connected by cylindrical l_d domains (red) at both ends. A close examination reveals that the l_o phase bulges outward. This feature is characteristic of l_o/l_d phase-separated tubules and is never reversed. The swollen shape of the l_o domains might be attributed to the rigidity of their ordered packing, which is partial to a lower curvature than the l_d phase.[42] Such an observation is notable as it indicates the significance of the differing bending rigidities of the phases. Narrow tubules prepared by this hydration flow method have a diameter of <1 μm; however, the l_o phase is clearly not stable in this geometry and on forming a large domain attempts to minimize its curvature by increasing the diameter of the region. Narrow tubules ($d \approx 1$ μm) composed purely of l_o phase have not been observed.

Phase Separation in "Pearled" Lipid Tubules. This discovery provides an interesting mechanism for the generation of new structures. In addition to the predominant structure of straight lipid tubules, we have observed photoinduced phase separation effects in some other lipid structures, including chains of spherical vesicles (a structure known as the "Pearling Instability"[43]). Although the straight tubules are usually fixed on both ends by fusion onto glass surface or other tubules, pearling was occasionally observed in tubules having at least one end freely fluctuating. Formation of the "pearling" structure may be induced by fluctuation and relaxation of the tubules as a result of competition between the surface tension energy and bending energy.[44] Similar to the basic tubule system, in this necklace of vesicles, no micrometer-scale phase separation was observed on initial illumination (Figure 3a and c). After several seconds, however, dark patches (l_o phase) appear, which preferentially partition into the lower curvature regions rather than staying in the narrow tubular "necks". (Figure 3b and d).

Phase Separation Induced Lipid Discs. The most exciting structure observed in this system is the bilayer disc. As shown in Figure 4a, lipid discs are composed of an outer ring in the l_d phase (Rh-DPPE stays in the l_d phase), with a dark circle (l_o phase) inside and are connected by tubular segments. Discs were observed to rotate freely and move laterally along the tubules

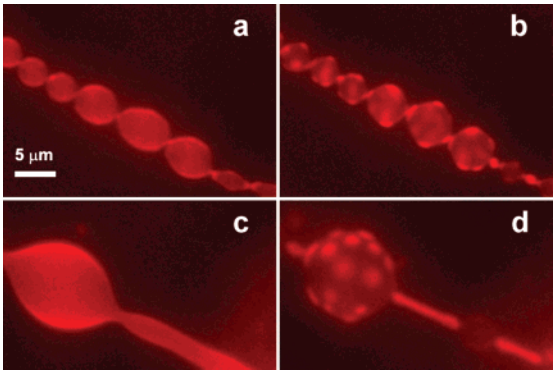

Figure 3. Lipid-Peroxidation-induced macroscopic phase separation in a Rh-DPPE labeled DOPC/eSM/ Cholesterol (1:1:1) lipid tubule exhibiting the "pearling instability", at $t \approx 0$ s (a and c) and $t = 30$ s (b and d).

(see Web-enhanced objects). The discs often coexist in the same sample with other structures, such as vesicles and tubules. The mechanism for the disc formation is illustrated in Figure 4b. Tiny patches of the l_o phase appear in the tubule, which subsequently form larger patches. Instead of stabilizing in the banded pattern however, a chain of bilayer discs forms along the tubule. This presumption is supported by the observation of the intermediate state during the disc formation, as shown in Figure 4c.

A close-up of a single disc at different rotational angles, labeled with Rh-DPPE and NBD-DPPE is shown in Figure 5. The l_o phase (green) and the l_d phase (red) can be clearly distinguished. In particular a flat, disc-like morphology can be verified by rotating the disc by 90° (Figure 5a and c). We propose a model for the structure of the lipid disc, in which the two flat faces are composed of eSM and cholesterol enriched l_o phase, with a rim of DOPC enriched l_d phase, as shown in Figure 5d.

In the tubule based system presented in this paper, other morphologies are observed, but the disc structure and banded pattern predominate. The occurrence of a band or a disc in a particular location appears to depend on the spatial configuration of l_d patches as they form and future observations are planned to capture the mechanism of disc formation in real time. The aspect ratio of the discs can vary from apparently very flat (<1 μm), to oblate spheroids, biconcave discs and even circular vesicles with an l_d "waist", although flat discs are very common in this system, in which the observed thickness appears to match that of the host tubule. In vesicles with coexisting fluid domains, a line-tension σ, at the phase boundaries, has been proposed as a mechanism to control membrane deformation and budding.[21] It has been shown that this line tension drives vesicles to "limit shapes" by attempting to minimize the domain boundary.[13] This effect is clearly observed for the banded pattern as domain boundaries are minimized (Figure 2f). For the disc instability, however, this appears not be the case and large domain boundaries form. This illustrates the delicate balance between membrane curvature and line-tension, where we see the l_o phase minimizing its curvature at the expense of increasing domain boundaries. The disc structure is however unstable and occasionally discs are observed to expel the l_o phase by budding. This phenomenon can be seen clearly in Figure 6 where two different lipid discs are observed to "bud" in this way. It should be noticed that, as can be seen from the intensity profile

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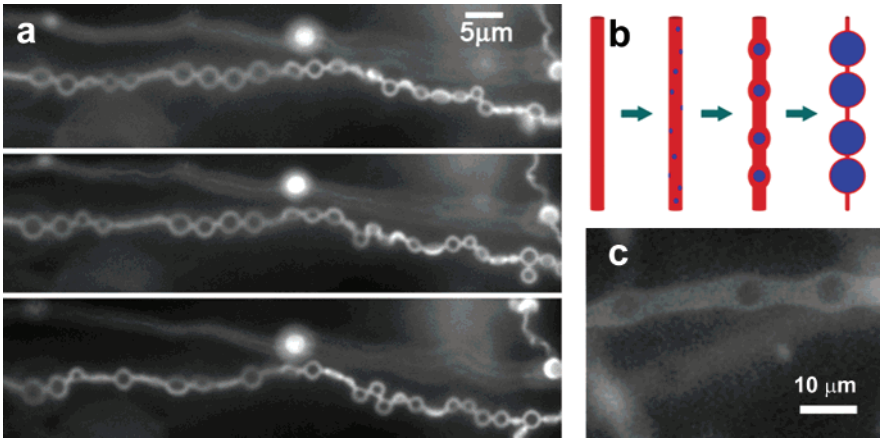

Figure 4. (a) Lipid tubule of Rh-DPPE labeled DOPC/eSM/cholesterol (1:1:1) exhibiting the "disc instability" at three different times; (b) cartoon illustrating the mechanism of disc formation; (c) fluorescence image of a fat lipid tubule with dark patches.

Ⓦ Videos (Ⓦ 1 and Ⓦ 2) showing rotation of lipid discs in AVI format are available.

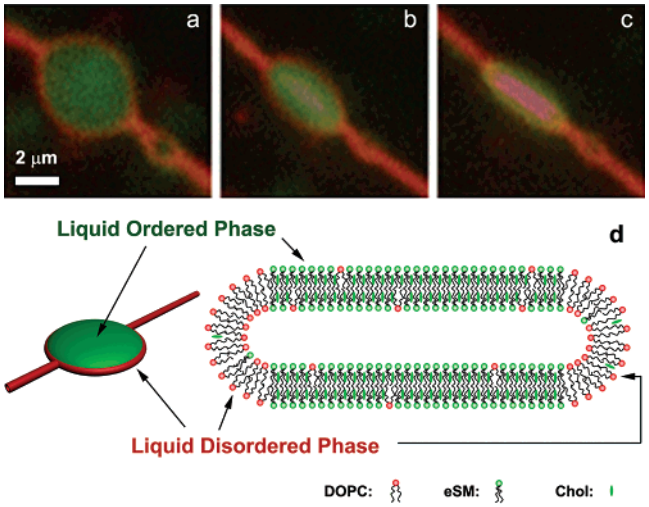

Figure 5. (a−c) Fluorescence microscopy images of a single DOPC/eSM/Cholesterol (1:1:1) lipid at varying angles of rotation, labeled with NBD-DPPE (green − the l_o phase) and Rh-DPPE (red − the l_d phase); (d) 3D schematic (left) and cross-sectional view (right) of a lipid disc.

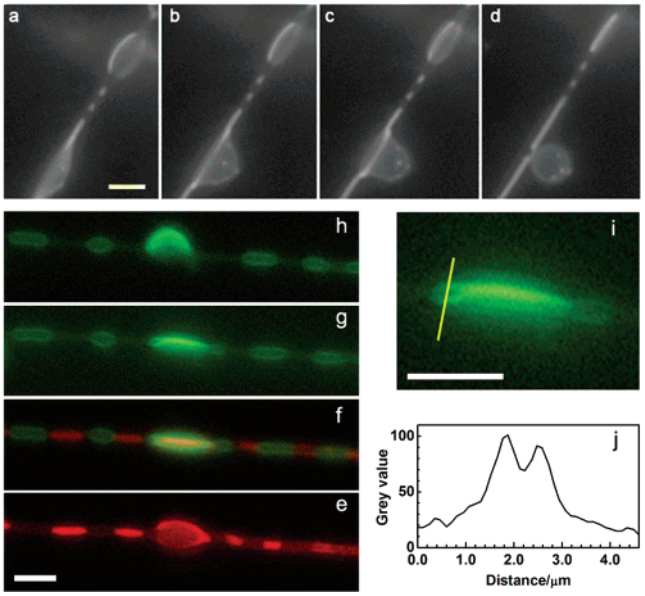

Figure 6. Fluorescence microscopy images of lipid discs exhibiting a "budding phenomenon". All samples consist of DOPC/eSM/cholesterol (1:1:1) and are labeled with Rh-DPPE (red) and NBD-DPPE (green). (a−d) a time series ($\Delta t \approx 1$ s) for a disc budding with only Rh-DPPE fluorescence shown; (e−h) a time series ($\Delta t \approx 1$ s) for a lipid disc with (e) Rh-DPPE only, (f) NBD-DPPE and Rh-DPPE, and (g, h) NBD-DPPE only; (i, j) a close up of the disc in (Figure 6g) with intensity profile as marked. Scale bars are 5 μm.

observed at one edge of an expelled disc (Figure 6j), the membrane disc is composed of double bilayers. Such an observation would not be possible in the case of lipid bicelles[45,46] as described by several authors and provides additional evidence for the structure as proposed in Figure 5d.

The structure of this "disc instability" may have some biological relevance, as it is strongly reminiscent of the bilayer discs found in the rod-outer segment (ROS) of the vertebrate retina. In the ROS, bilayer discs are generated in the cell body and move progressively up the rod, away from the cell body as they age, starting with a high cholesterol content, which gradually decreases. Detergent resistant membranes, or lipid rafts, have been isolated from the ROS,[47] and these domains are believed to be lipids in the cholesterol-assisted l_o phase. Other imaging work confirms the presence of ordered domains in the retinal discs.[48] It is significant that in high-cholesterol lipid tubules, the generation of membrane discs predominates. A similar bilayer disc stack is also observed in the Golgi apparatus.[49] Such discs are also connected by narrow neck regions, but occur in a stacked geometry, different to the longitudinal chain we observe. The observed discs appear to form by phase separation of the l_d phase into a ring structure, and this suggests a mechanism for the generation of the ROS discs. Although the retinal membrane is a much more complex system than this simplified model, it is possible that low T_m lipids in the l_d phase accumulate at the edges of the ROS disc to minimize the elastic energy of the membrane and stabilize the high edge curvature.

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Conclusions

Photoinduced micrometer-scale lipid sorting has been observed in ternary lipid mixtures in a tubule geometry, leading to the formation of lipid bilayer discs. It is clear that the micrometer-scale phase separation in this ternary lipid mixture is not an intrinsic phase behavior of the system, but may occur due to photoinduced lipid peroxidation. How this interesting mechanism leads to domain coalescence is not fully understood; however, it does allow the generation of fascinating structures and provides a mechanism for the formation of bilayer discs.

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