Organization in Lipid Membranes Containing Cholesterol

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(Received 13 June 2002; published 9 December 2002)

A fundamental attribute of raft formation in cell membranes is lateral separation of lipids into coexisting liquid phases. Using fluorescence microscopy, we observe spontaneous lateral separation in free-floating giant unilamellar vesicles. We record coexisting liquid domains over a range of composition and temperature significantly wider than previously reported. Furthermore, we establish correlations between miscibility in bilayers and in monolayers. For example, the same lipid mixtures that produce liquid domains in bilayer membranes produce two upper miscibility critical points in the phase diagrams of monolayers.

DOI: 10.1103/PhysRevLett.89.268101 PACS numbers: 87.16.Dg, 64.70.Ja, 64.75.+g

Mammalian cells are surrounded by an outer wall or "plasma membrane" of proteins and lipids arranged in opposing leaflets of a bilayer. There is growing evidence that this membrane is not uniform, but instead laterally organizes into regions called "rafts." These are liquid domains in which cholesterol, saturated long-chained lipids, and particular proteins are concentrated [1]. Rafts are currently of great interest to cell biologists, immunologists, and physical scientists alike [2]. As an example, rafts have been implicated in important cell functions such as endocytosis, adhesion, signaling, apoptosis, protein organization, and lipid regulation [1,3,4] (and references therein). Furthermore, pathogens may recruit specific lipids to a site to aid infection of a cell [5].

A simpler system in which to study physical properties of lipids is in a bilayer membrane of a vesicle. Experimental support for both rafts in cells and coexisting liquid domains in vesicles has been gathered by a variety of methods including detergent insolubility [6], single-molecule tracking [7], fluorescence quenching [8], fluorescence energy transfer [9], and aggregation of fluorescently labeled proteins [4]. Recently, direct visualization of micron-scale liquid domains was accomplished in bilayers [10,11]. For bilayers of a particular mixture of phospholipids, sphingomyelin, and cholesterol, a miscibility transition was found below the lipid melting temperature [11]. To date, few lipid mixtures have been studied by this method and the miscibility transition temperature has not been systematically explored. We are interested in how strictly lipid composition must be regulated for liquid domains to form in vesicles. Therefore, we have investigated vesicle membranes containing several different phospholipids and a wide range of cholesterol compositions. By systematically varying the temperature of our vesicles, we have assembled extensive miscibility phase diagrams based on fluorescence microscopy.

Coexisting liquid phases also exist in lipid monolayers at an air-water interface, as observed by a variety of researchers (e.g., [12]). There has been a persistent question of how experimental results in monolayer systems can be applied to bilayers. By studying the same lipid mixtures in monolayers as in our vesicles, we are able to compare the phase behavior of both systems.

Giant unilamellar vesicles.—Vesicles were made with ternary mixtures of a saturated phosphatidylcholine lipid [either di(14:0)PC, di(15:0)PC, di(16:0)PC, or di(18:0)PC], an unsaturated phospholipid [di(18:1)PC], and cholesterol. These mixtures were chosen to mimic raft-forming compositions in cell membranes [10,11]. Individual vesicles within a population may vary slightly in composition, estimated as ± 2 mol % cholesterol. This uncertainty results in a range of transition temperatures [13]. Phospholipids (Avanti Polar Lipids, Birmingham, AL), cholesterol, and dihydrocholesterol (Sigma, St. Louis, MO) were stored at $-20\,^{\circ}$C and used without further purification. A minimal amount (0.8 mol %) of Texas Red di(16:0)-phosphatidylethanolamine (Molecular Probes, Eugene, OR) was used as a dye for contrast.

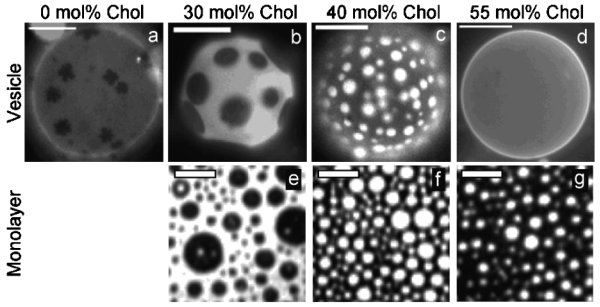

FIG. 1. Fluorescence micrographs of vesicles and monolayers of 1:1 di(18:1)PC/di(16:0)PC plus varying amounts of cholesterol. Scale bars are 20 μm. Vesicles are $<5\,^{\circ}$C below their phase transition temperatures and exhibit either solid-liquid phase coexistence (a), liquid-liquid phase coexistence (b),(c), or no visible phase separation (d). Vesicle domains are not necessarily at equilibrium sizes and coalesce with time. Monolayers are at $(27 \pm 0.5)\,^{\circ}$C and below their phase transition surface pressures and exhibit liquid-liquid phase coexistence (e)–(g). Monolayer domain sizes do not significantly change on the time scale of experiments (minutes).

Free-floating giant unilamellar vesicles of 20–50 μm diameter were prepared in > 18 MΩ cm water as by Angelova *et al.* [14] with modifications to increase yield and compositional uniformity and to detach the vesicles from the substrate on which they were grown. Vesicles were viewed using a Nikon Eclipse fluorescence microscope with a Photometrics Cool Snap FX camera.

Fluorescence micrographs of vesicles are shown in the top row of Fig. 1. By changing composition and temperature, three distinct phase morphologies are observed in the thin shell of the bilayer membrane: solid-liquid coexistence, coexistence of two liquid phases, and one uniform liquid phase. These three phases appear the same independent of whether the vesicles contain di(14:0)PC, di(15:0)PC, di(16:0)PC, or di(18:0)PC. We observe that solid domains are noncircular, rotate as rigid bodies, occur at low cholesterol composition, and exclude the dye. When two solid domains collide on the surface of our vesicles, they do not deform upon contact. The melting temperatures of solid phases are recorded in Fig. 2. The coexistence of solid and liquid phases in vesicles, the disruption of solid by higher cholesterol fractions, and the correlation of higher melting temperatures with longer acyl chains observed here are consistent with previous results [15].

As cholesterol concentration is increased in the vesicles beyond $\sim$10 mol %, the solid phase is replaced by liquid, which is more applicable to rafts in biological membranes. As in the solid phase, liquid domains are large (micron scale), stable, and self-assemble without the aid of proteins. Coexisting liquid phases are found from approximately 10 mol % to near 50 mol % cholesterol. The range of lipid compositions over which micron-sized liquid domains are recorded here is significantly wider than previously known [10,11]. We characterize liquid phases by their circular domains. When two liquid domains collide on the surface of our vesicles, they coalesce by continuously deforming into a larger circular domain. Given that our vesicles are expected to have the same composition in both leaflets of the bilayer and that we do not observe overlapping domains, we conclude that domains in each bilayer leaflet are in registration, as reported previously [10,11]. In our experiments, two phases are observed, bright and dark (although it is always possible that an additional phase is present that the probe does not distinguish). Liquid domains that are cholesterol

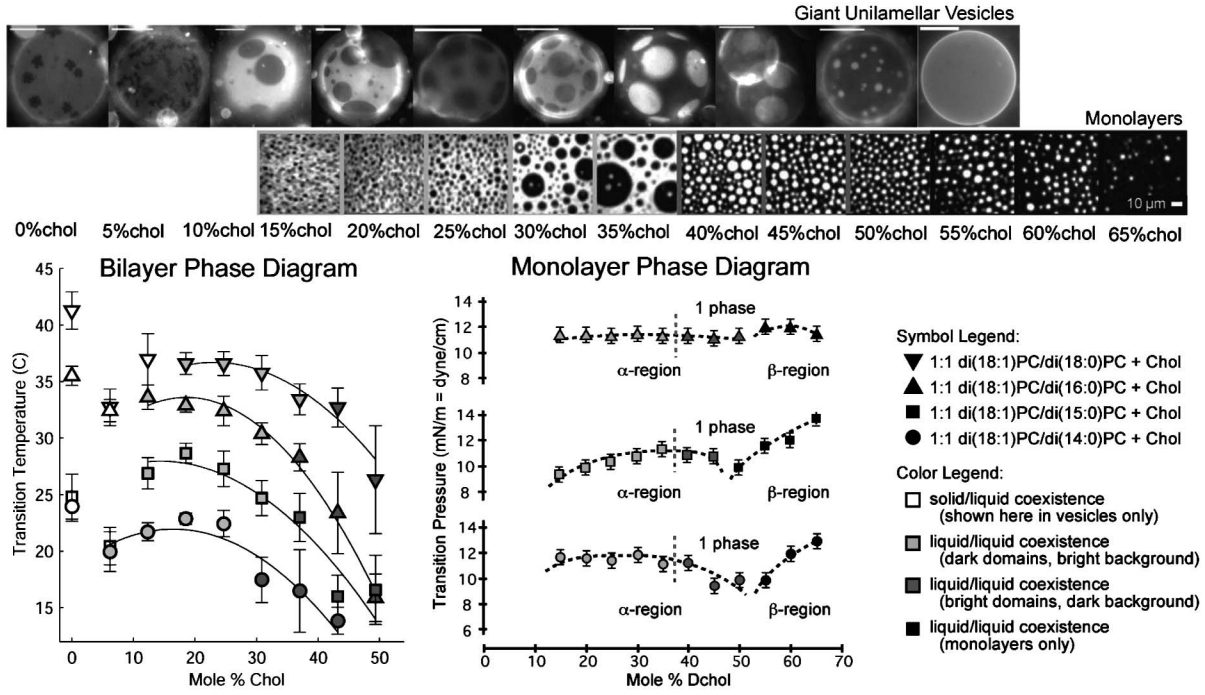

FIG. 2. Top: A series of fluorescence micrographs of vesicles and monolayers of 1:1 di(18:1)PC/di(16:0)PC with varying cholesterol. Scale bars are 20 μm for vesicles and 10 μm for monolayers. From left to right, the phases are solid-liquid coexistence, coexisting liquid phases, and either one uniform liquid phase (in vesicles) or coexisting liquid phases (in monolayers). Bottom left: Miscibility transition temperatures for vesicles. Points at low cholesterol represent melting of the solid phase. Filled points mark miscibility transition temperatures for two liquid phases. Temperatures from (10–50) °C are experimentally accessible. Error bars represent standard deviations over multiple measurements. Bottom right: Miscibility transition surface pressures for monolayers. The change of contrast is marked by a vertical gray dashed line. For all domains ≥ 10 μm, striping of domains was seen near the transition. Striping may occur in smaller domains beyond our resolution. In all cases, curves are drawn to guide the eye and are not explicit fits of the data.

rich exclude the probe so that they appear dark. At low cholesterol concentrations, the area fraction of dark domains is small, so dark domains appear on a bright background [Fig. 1(b)]. As cholesterol concentration is increased, the contrast reverses to bright domains on a dark background [Fig. 1(c)]. Reversals occur at 30–40 mol % cholesterol. At 50–55 mol % cholesterol, domains are no longer observed and the vesicles appear uniform.

This third phase, a uniform liquid, appears both at high cholesterol and at high temperature in vesicles. At moderate cholesterol compositions below 50 mol %, liquid domains spontaneously form from this uniform liquid as temperature decreases through the miscibility transition ($\sim 0.05\,^{\circ}\mathrm{C}/\mathrm{sec}$) [10]. Transition temperatures are shown in Fig. 2, down to an experimental cutoff of 10 °C. As acyl chain lengths of the saturated lipid increase, we observe that miscibility transition temperatures increase. Hence, liquid domains containing lipids with longer chains are stable to higher temperatures, proving a correlation between chain melting temperature and miscibility temperature in bilayers [11].

We expect that a large family of similar ternary mixtures containing saturated phospholipids, unsaturated phospholipids, and cholesterol would also yield coexisting liquid domains in vesicles. Noting that miscibility transition temperatures in Fig. 2 range from 13–38 °C, we speculate that some biological lipid mixtures may spontaneously separate into cholesterol-rich and cholesterol-poor domains near physiological temperatures.

Comparison with monolayers.—Lateral separation of liquid domains of phospholipid and cholesterol is not limited to bilayers in vesicles, but has also been previously observed in monolayers at the air-water interface of a Langmuir trough (e.g., [12,16]). Studying monolayers containing unsaturated lipids presents a challenge of oxidation. This was minimized by substituting dihydrocholesterol (Dchol) [17] and a lower dye concentration (0.6 mol %), by opening a new ampule of di(18:1)PC for each set of experiments, by using lipids promptly, by reducing air exposure, and by concluding trials within 3 min. As in vesicle experiments, 18 $\mathrm{M}\Omega\,\mathrm{cm}$ water was used in the trough subphase. To confirm our results, two trials were reproduced at Stanford University using a Langmuir trough flushed with argon. Transition pressures of monolayers containing 1/1 di(18:1)PC/di(14:0)PC + 35% Dchol were 11.2 mN/m in our experiments without argon and 11–12 mN/m in the Stanford experiments with argon. Similarly, transition pressures of monolayers containing 1/1 di(18:1)PC/di(16:0)PC + 35% Dchol were 11.4 mN/m without argon and 9.5–10 mN/m with argon. We estimate our experimental errors as ± 0.5 mN/m and systematic errors due to oxidation as $< +2.5$ mN/m.

An obvious question is how the phase behavior of bilayer and monolayer systems compare. Figure 2 presents transition temperatures vs mol % cholesterol in bilayers as well as transition surface pressures vs mol % cholesterol in monolayers of the same lipid mixtures. To date, every mixture that produces liquid domains in bilayers exhibits two upper miscibility critical points in the corresponding monolayer phase diagram. The reverse is not always true (the results are unpublished). Domains are large and stable and self-assemble without the aid of proteins. As in the case of vesicles, monolayer phases look the same independent of whether the vesicles contain di(14:0)PC, di(15:0)PC, or di(16:0)PC.

In the monolayer phase diagram, the two distinct regions of coexisting liquids are called α and β [18]. The α region in monolayers has similarities with the liquid coexistence region in bilayers. Specifically, liquid domains that are cholesterol rich appear dark such that at low cholesterol concentrations dark domains appear on a bright background [Fig. 1(e)]. Contrast reverses as cholesterol fraction increases to 35–40 mol % (Fig. 2). A transition from the α to the β region occurs at 50–55 mol % cholesterol, the same concentration as the termination of the liquid phases in vesicles. In both the α and the β regions, two phases (bright and dark) are observed, although it is possible that an additional phase is not distinguished by the fluorescent probe [19], as in vesicles. Monolayer domains in the β region appear similar to those in the α region, but are typically smaller [18]. No domains are observed in vesicles corresponding to the β region in monolayers (Fig. 3). This may be related to limited solubility of cholesterol in bilayers [20].

In monolayers, many simple mixtures of phospholipids and cholesterol produce coexisting liquid phases, although most have a single miscibility critical point, rather than two as seen here [16]. The appearance of two upper critical points in monolayer phase diagrams has previously been modeled by the formation of a chemically distinct "condensed complex" such that C (cholesterol) and P (phospholipid) undergo the reaction $q\mathrm{C} + p\mathrm{P} \rightarrow \mathrm{C}_q\mathrm{P}_p$ [12,16,21]. Within this model, the α region we observe is a coexistence between phospholipid-rich

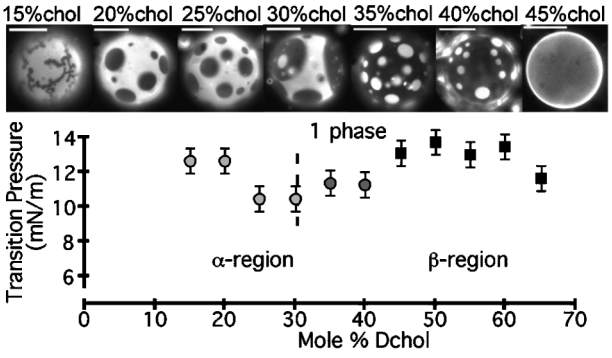

FIG. 3. Top: Vesicles of 2:1 egg phosphatidylcholine/brain sphingomyelin with varying cholesterol concentrations and imaged $<5\,^{\circ}\mathrm{C}$ below their phase transition temperatures. All scale bars are 20 μm. Bottom: Monolayer phase diagram for the same lipid compositions at 26 °C.

and complex-rich phases, and the β region is between complex-rich and cholesterol-rich liquid phases. Complexes can contain more than one phospholipid species [16,22]. Although our monolayer results are consistent with the formation of condensed complexes, and our monolayer and bilayer phase diagrams are similar, we have not provided direct evidence that the bilayer phase behavior we observe is a consequence of condensed complexes. Related results have led to a superlattice model that shares similarities with the condensed complexes model [20,21,23].

The similarities above between monolayer and bilayer phase behavior are not limited to simple ternary model mixtures. We chose to study 2:1 egg phosphatidylcholine/brain sphingomyelin mixed with cholesterol, a popular lipid mixture also thought to approximate rafts in cells [10,11,13]. As before, we investigated a wide range of cholesterol compositions. We observed liquid phases in vesicles over the range of 20–40 mol % cholesterol, with the same domain morphology and contrast described above (Fig. 3). At 45% cholesterol, the vesicles are in one uniform liquid phase. As expected, the corresponding monolayers have two upper miscibility critical points with a transition from the α to the β region between 40 and 45 mol % cholesterol (Fig. 3).

In summary, we have seen that the formation of liquid domains occurs spontaneously in free-floating unilamellar vesicles containing a wide range of ternary mixtures of cholesterol, saturated phospholipid, and unsaturated phospholipid. This work also establishes a correlation between monolayer and bilayer phase behavior, both of which are similarly dependent on cholesterol composition. The liquid domains we observe are analogous to liquid-ordered and detergent-resistant phases in bilayers [1,24]. In cells, it has been shown that many proteins cluster in rafts with lipids [4]. Since a protein's activity can change with lipid environment [25], it is an interesting possibility that the lateral separation of lipids recorded here may play a role in the regulation of cholesterol in membranes [26].

We thank Harden McConnell, Arun Radhakrishnan, Rob Carlson, and Ray Goldstein for comments on our manuscript. We are particularly grateful to Tamara Okonogi for confirming two monolayer pressures. This work was funded by a NSF CAREER Award, a Research Corporation Innovation Award, the UW Royalty Research Fund, and the Petroleum Research Fund. S. L. V. received support from the UW Center for Nanotechnology.

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