Accepted Manuscript

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PII: S0005-2736(07)00256-8
DOI: doi: 10.1016/j.bbamem.2007.07.009
Reference: BBAMEM 79465

To appear in: BBA - Biomembranes

Received date: 18 April 2007
Revised date: 11 July 2007
Accepted date: 13 July 2007

Please cite this article as: Jiang Zhao, Jing Wu, Huilin Shao, Fanrong Kong, Nieraj Jain, Geoffrey Hunt, Gerald Feigenson, Phase Studies of Model Biomembranes: Macroscopic Coexistence of Lα+Lβ, with Light-Induced Coexistence of Lα+Lo Phases, BBA - Biomembranes (2007), doi: 10.1016/j.bbamem.2007.07.009

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Phase Studies of Model Biomembranes:

Macroscopic Coexistence of L\(\alpha\) + L\(\beta\), with Light-Induced Coexistence of L\(\alpha\) + Lo Phases

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Footnotes

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Abbreviations: DPPC, 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine; DSPC, 1,2-Distearoyl-sn-Glycero-3-Phosphocholine; DOPC, 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine; POPC, 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine; SM, sphingomyelin; NPG, n-propyl gallate; TLC, thin-layer chromatography; GUV, giant unilamellar vesicle; RSE, rapid solvent exchange; FRET, fluorescence resonance energy transfer; (16:0,Bodipy-PC), 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine; C20:0-DiI, 1,1'-dieicosanyl-3,3',3''-tetramethylindocarbocyanine perchlorate; TR-DHPE, Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt.
Abstract

Phase diagrams of 3-component lipid bilayer mixtures containing cholesterol reveal major differences among the different types of lipids. Here we report that mixtures of cholesterol together with POPC and a high-melting temperature PC or sphingomyelin show different phase behavior from similar mixtures that contain DOPC or di-phytanoyl-PC instead of POPC. In particular, only one region of macroscopic phase coexistence occurs with POPC, a region of coexisting liquid disordered and solid phases, \{L_\alpha + L_\beta\}. Fluorescence microscopy imaging is useful for these studies, but is subject to artifactual light-induced domain formation, as reported by Ayuyan & Cohen [9]. This artifact can be attenuated by decreased illumination and low dye concentration. The use of the free radical scavenger n-propyl gallate can reduce the artifact, but this molecule enters the bilayer and itself perturbs the phase behavior. We suggest that the light-induced domain separation artifact might actually arise from pre-existing lipid clusters that are induced to coalesce, and therefore indicates highly nonrandom mixing of the lipid components.

Keywords: photo-induced; lipid peroxidation; liquid-disordered phase; liquid-ordered phase; antioxidant; phase boundary shift
1. Introduction

Knowing the phase of a material under study, including any possible coexistence of multiple phases, can be a prerequisite for understanding both the material properties (viscosity, elasticity, bending modulus), as well as any preferential locations (partitioning) of the components. In the case of lipid bilayers used as models for cell membranes, 3-component mixtures can show several different phases, and several different types of phase coexistence regions. Nonrandom arrangements of the bilayer components, as well as the underlying molecular-level interactions, can be studied by solving the phase diagrams of the bilayer mixtures [1]. A general question of interest is whether composition is either (a) relatively uniform over a distance scale corresponding to at least the time for an enzyme to turnover, or equivalently, for molecules to find each other over a period of milliseconds. This distance scale is ~ 50 – 100 nm, a size of bilayer known from small vesicle studies to be sufficient to have genuine phase separation [2]; or else (b) changing discretely over this distance scale, with components partitioning between compositionally-distinct domains.

Phase separations observed for certain lipid compositions, and nonrandom mixing in general, including any clustering, might provide important insights into natural membranes. A first-order phase transition in a biological membrane would involve an abrupt change in lipid and protein localization, whereas changes in nonrandom mixing would involve more gradual alteration of protein and lipid clustering. But simplifications are needed to understand biological membranes by study of chemically well-defined model bilayer mixtures; the large number of components of cell membranes makes it difficult to know whether any chosen mixture is “typical”, and whether the observed
mixing behaviors can be generalized. We seek to capture essential features of the complex system of the cell membrane by study of judiciously chosen chemically simple bilayer mixtures.

One strategy for model membrane studies is to find out whether some of the components of cell membranes can be grouped together based on similarity of mixing behavior. In this regard, studies to date have shown that high melting temperature phospholipids including DPPC, DSPC, and several sphingomyelins, seem to exhibit similar behaviors in 3-component bilayer mixtures that contain cholesterol [3]. Other studies have shown that the phospholipids DOPC and diphytanoyl-PC, which have low melting points, with acyl chains that pack poorly in ordered phases, also seem to show characteristic phase behaviors in 3-component mixtures that contain cholesterol [4].

In this report, we focus mainly on POPC as representative of yet another group of phospholipids showing characteristic behaviors in 3-component mixtures. Lipids which we have studied in this group are POPC, SOPC, and DLPC. All have melting points not far from 0 °C. POPC and SOPC are commonly found in animal cell membranes. In the case of DLPC in bilayer mixtures with DPPC and cholesterol, we previously found one compositional region having coexisting macroscopic domains of \{L\alpha + L\beta\}, together with some other regions clearly having only one phase, and yet other compositional regions with extremely nonrandom mixing, but without macroscopic phase separation [5]. We have observed similar behavior in 3-component mixtures of cholesterol with DPPC/POPC, DSPC/DLPC, DPPC/SOPC and DSPC/POPC. However, during the studies with DSPC/POPC, we found that sample illumination with a fluorescence microscope could cause macroscopic domains to separate in GUVs that initially were uniform. Because such domains had not formed during the slow cooling of the GUV samples nor
subsequent incubation at 23 °C for up to several days, and could be minimized by reducing illumination of the sample on the microscope stage, these light-induced domains can be classified as “artifacts”, even without a complete analysis of the nature of the artifact.

Regarding these observations, several reports have appeared in the last few years that seem to show POPC behaving similarly to DOPC and diphytanoyl-PC in that 3-component mixtures containing POPC and cholesterol were reported to exhibit macroscopic phase separations of coexisting liquid disordered plus liquid-ordered \( \{L_\alpha + L_\alpha\} \) domains [3,6-8]. In agreement with Ayuyan & Cohen [9], here we report that light-induced domains readily form under common experimental conditions as artifacts that have misinformed studies of phase behavior in these bilayer mixtures. The light-induced artifacts appeared in three different lipid compositions for each of the three different types of SM/POPC/chol mixtures with egg-, palmitoyl- and stearoyl-sphingomyelin respectively, and three more compositions of palmitoyl-sphingomyelin/POPC/chol mixtures. In short, none of these mixtures exhibit macroscopic \( \{L_\alpha + L_\alpha\} \) phase separation as described in the published reports. Instead, the spurious separation of the immiscible, micron-sized round domains is caused by intense illumination during examination with a fluorescent microscope. Importantly, light-induced domains form more rapidly with higher concentration of fluorescent dyes in the mixture. Similar light-induced phase separations also occur in 1-phase regions of DSPC/DOPC/chol and other mixtures containing unsaturated phospholipids, namely DSPC/POPC/chol. In favorable cases, minimal illumination can enable observation prior to appearance of the artifacts. As previously noted by Ayuyan & Cohen [9], the free radical scavenger n-propyl gallate can yield GUVs that show no phase separations. However, these mixtures that contain
NPG are misleading in their own way because the NPG seems to enter the lipid bilayers, becomes a significant bilayer component, and alters the phase behavior, as we show below.

2. Materials and methods

2.1. Materials

Phospholipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (POPG), 1,2-distearoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (DSPG), egg sphingomyelin (egg-SM), palmitoyl sphingomyelin (palm-SM) and stearoyl sphingomyelin (stearoyl-SM) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL) and cholesterol from Nu Chek Prep (Elysian, MN). The fluorescent dyes 1,1’-dieicosanyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (C20:0-DiI), 1-hexadecanoyl-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-sn-glycero-3-phosphocholine (16:0,Bodipy-PC), and Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (TR-DHPE) were obtained from Invitrogen (Eugene, OR). Naphthopyrene was obtained from Sigma-Aldrich (St. Louis, MO). Purity greater than 99.9% was confirmed by thin-layer chromatography of 50μg of each of the lipids on washed, activated Adsorbosil TLC plates (Alltech, Deerfield, IL), developed with chloroform/methanol/water (65/25/4) for all phospholipids, chloroform/methanol (9/1) for C20:0-DiI and C18:2-DiO, and with petroleum
ether/diethyl ether/chloroform (7/3/3) for cholesterol analysis. N-propyl gallate (NPG) was from Sigma-Aldrich (St. Louis, MO). All solvents used were HPLC grade. Phospholipid stock concentrations were quantified by phosphate assay [10], fluorescent dye stocks by absorption spectroscopy using an HP 8452A spectrophotometer (Hewlett-Packard, Palo Alto, CA). Extinction coefficients used are 143,000 M⁻¹cm⁻¹ at 549 nm for C20:0-DiI; 91,800 M⁻¹cm⁻¹ at 504 nm for Bodipy-PC, 107,000 M⁻¹cm⁻¹ at 583 nm for TR-DHPE (all from Invitrogen, Eugene, OR), 23,700 M⁻¹cm⁻¹ at 460 nm for naphthopyrene (from Sigma-Aldrich, St. Louis, MO). Cholesterol stocks were prepared analytically.

2.2. Formation of giant unilamellar vesicles (GUVs)

2.2.1. Electroswelling

Most of the GUV samples were prepared by the method of “electroswelling”[11], as described in the accompanying paper [12].

For some experiments, NPG was added to the GUV suspension after vesicle harvest by mixing with an isoosmotic NPG-containing sucrose solution. NPG was dissolved in sucrose solutions, assisted by 10-15 min sonication (Bransonic ultrasonic cleaner, Branson Ultrasonics Corp, Danbury, CT) followed by heating to 65 °C with occasional vortexing until a clear solution was achieved. Final NPG concentrations in stock solutions were 4 or 30 mM. After cooling to 23 °C, a GUV suspension was mixed with the 4 or 30 mM NPG + sucrose solution, followed by gentle stirring. The solution was then held for at least 20 min to allow for mixing before microscopy observations. We found that a 30mM NPG, 70 mM sucrose solution would remain clear at room temperature for at least 4 days before needle shaped crystals formed.
2.2.2. Gentle hydration

In order to prepare samples by a different method for the purpose of comparison with the electroswelling method, GUVs were formed by “gentle hydration”, which requires negatively charged lipid species in the mixture [13]. Details are described in the accompanying paper [12].

2.3. Confocal fluorescence microscopy of GUVs

GUVs were examined under the same condition as described [12], unless specified. TR-DHPE was illuminated at 543 nm and emission collected from 570 - 700 nm. Direct observations of GUVs were also made through the eyepiece using illumination from the 50W Hg arc lamp light with excitation filters set at red 510 – 560 nm or green 460 - 500 nm excitation. For some samples, the duration of illumination was important. Approximately 5 - 10 s of illumination was the shortest time needed to record an image. However, observations through the eyepiece without recording an image could be done with shorter illumination times of ~ 2 - 5 s.

For microscope observations at 10 °C, sample temperature was controlled by use of a drilled home-built aluminum block connected to a circulating water bath, model 910 (Fisher Scientific, Pittsburgh, PA). Temperature of the aluminum block was measured with a surface thermometer model 310C (Pacific Transducer Corp, Los Angeles, CA).

2.4. FRET and Single-Dye Fluorescence (These experiments are described in the accompanying paper [12]).
3. RESULTS

3.1. Equilibrium phase behavior

For bilayer mixtures of DPPC/POPC/chol, DSPC/DLPC/chol, DSPC/SOPC/chol and DSPC/POPC/chol, GUVs reveal a region of 2-phase coexistence, with macroscopic domains of L\(\alpha\) together with L\(\beta(\beta')\) phases. Fig. 1 shows compositions examined for these four mixtures. The dyes (16:0,Bodipy)-PC and C20:0-DiI show complementary partitioning between the L\(\alpha\) and L\(\beta(\beta')\) domains, with the domains that are bright with red-orange fluorescence from C20:0-DiI having straight sides and often appearing branched, corresponding to the L\(\beta(\text{or } \beta')\) phase. For these four lipid mixtures, at cholesterol concentrations greater than a particular value of \(\chi_{\text{CHOL}} 0.15 – 0.2\), the visibly separated domains of the GUVs disappear abruptly and fluorescence of dyes appears uniform at all higher \(\chi_{\text{CHOL}}\).

However, the phase behavior outside of this region of \{L\(\alpha\) + L\(\beta(\beta')\}\} is not simple. FRET and single-dye measurements reveal similarly complex behavior to that previously observed for DPPC/DLPC/chol [5]. This behavior could correspond to the formation of compositionally distinct clusters characteristic of highly nonideal mixing, or else to actual phase separation.

3.2. Light-induced domains form close to phase boundaries
During the course of these phase boundary studies, we found that GUVs with compositions within a 1-phase region would show light-induced domain formation upon illumination with the fluorescence microscope. Furthermore, formation of these artifacts was correlated to vicinity of a phase boundary, or perhaps equivalently, the degree of nonrandom mixing. For mixtures of DSPC/POPC/chol, in the compositional region that we chose to examine, GUVs having compositions farther from the upper boundary of \{L\alpha + L\beta\} required longer illumination times for light-induced domain separation. For example, along the fixed line of \(\chi_{DSPC} = 0.3\), GUVs of \(\chi_{CHOL} = 0.2\) required 2 - 3 min for light-induced domains to appear, whereas only 10 - 20 s were needed for GUVs of \(\chi_{CHOL} = 0.16\). (16:0,Bodipy)-PC exhibited preferential partitioning between the light-induced domains favoring the L\alpha phase. C20:0-DiI initially exhibited barely discernible brightness difference between domains, with more pronounced brightness differences appearing upon longer illumination times. The partitioning behavior of the two dyes (16:0,Bodipy)-PC and C20:0-DiI was complementary between the light-induced domains (Fig. 2A), indistinguishable from that of non-induced domains.

Other lipid mixtures also exhibited light-induced domain separations for compositions close to a phase boundary. As shown in Fig. 2B, round domains appear in time upon illumination of GUV samples of DSPC/DOPC/chol of \(\chi_{CHOL} = 0.40\), which is in the 1-phase Lo region but very close to the boundary of \{L\alpha + Lo\} coexistence [12]. Here, (16:0,Bodipy)-PC and C20:0-DiI show complementary dye partitioning between the light-induced domains, again accompanied by a gradually enhanced C20:0-DiI partitioning during continuous light-induced domain formation. Domains usually coalesced during observation, as shown in Fig. 2B. These light-dependent separated
domains have the appearance and show the dye-partitioning behaviors indistinguishable from GUVs of composition $\chi_{\text{CHOL}} = 0.38$, just inside the boundary of $\{L_\alpha + L_0\}$ coexistence, except for their time-dependent formation. Moreover, after the initial induction of separated domains, the fraction of the surface occupied by each type of domain does not seem to change much in time. GUVs of DSPC/DOPC/chol having compositions $\chi_{\text{CHOL}} > 0.42$, farther from the boundary, required much longer time or more intense illumination to show light-induced domain separations.

3.3. SM-containing mixtures show light-induced domains

Investigating further, we prepared samples of POPC and cholesterol together with a series of high-melting temperature lipids, egg-SM, palm-SM, and stearoyl-SM. Upon illumination with the fluorescence microscope, GUVs initially showing uniform fluorescence of (16:0,Bodipy)-PC or C20:0-DiI or naphthopyrene would exhibit tiny domains depleted of both dyes that moved rapidly over the vesicle surface, then fused together, in some cases into a stable honeycomb structure, in other cases into larger separated domains, as shown in Fig. 2C. Instead of attempting to characterize these behaviors over all of composition space, we followed Veatch & Keller [3] to examine in detail three defined compositions for each mixture: SM/POPC/chol = 0.33/0.34/0.33, 0.25/0.5/0.25, and 0.4/0.4/0.2. GUVs of the first two compositions initially show uniform fluorescence of (16:0,Bodipy)-PC or C20:0-DiI, with the uniformity persisting long enough that a vesicle could be selected and an image could be scanned and saved. However, in almost all cases the 0.4/0.4/0.2 mixtures exhibited the light-induced domain
separation so quickly that even when working as fast as possible, the first images saved by the light detection system showed domain separation. Only in a few cases with egg-SM at this composition were GUVs initially observed to be uniform over the entire surface; within seconds, visible circular domains appeared on these GUVs. Very fast light-induced domain separation was also noticed at palm-SM/POPC/chol = 0.5/0.25/0.25. The development in time of domain structure is shown in Fig. 3.

For palm-SM/POPC/chol we examined two additional compositions that are within or close to the likely region of coexisting liquid and gel \{L_{\alpha} + L_{\beta}(\beta')\} [14]. For palm-SM/POPC/chol = 0.5/0.5/0, no light-induced domains appeared even after a long period of intense illumination, as shown in Fig. 4. For palm-SM/POPC/chol = 0.47/0.47/0.06, within or very close to the proposed phase boundary of this region, light-induced domains formed that first showed linear features, including branched domains like those seen in Fig. 1, which then evolved over a time of ~1 min into completely round domains.

As for the dye partitioning behavior between the light-induced domains in SM/POPC/chol, for all compositions having $\chi_{\text{CHOL}} > 0.1$ and for all SM species that we examined, (16:0,Bodipy)-PC, C20:0-DiI and TR-DHPE partitioned favorably into the same phase, whereas napthopyrene partitioned in a complementary manner to these dyes (data not shown). This is the type of dye partition behavior that we have previously observed for brain-SM/DOPC/chol mixtures in the compositional region of \{L_{\alpha} + L_{\text{O}}\} coexistence [15]. Thus, the light-induced domains have at least some of the characteristics of "genuine" phase separation of \{L_{\alpha} + L_{\text{O}}\}, including that in SM-containing mixtures both C20:0-DiI and (16:0,Bodipy)-PC partition into the L_{\alpha} phase, whereas in DSPC-containing mixtures, the dyes partition in a complementary manner. In
contrast, for palm-SM/POPC/chol with $\chi_{\text{CHOL}} = 0.06$, Fig. 4 shows that the light-induced domains have the initial appearance of \{L$\alpha$ + L$\beta$\}.

3.4. High dye concentration promotes light-induced domains

TR-DHPE was used by Keller and co-workers at concentrations from 0.2 to 2 mol%, mostly 0.8 mol% [3]. We examined palm-SM/POPC/chol = 0.33/0.34/0.33 using TR-DHPE at both 0.15 and 0.8 mol%. At the lower dye concentration, GUVs appeared uniform at the earliest observation times, as shown in Fig. 2D. However, light-induced domains started to appear after illuminating with the Hg lamp for ~ 10 – 20 s. Domains appeared initially as tiny irregular specks, which then grew into large irregular patches that eventually became more rounded in less than a minute. At the higher concentration of TR-DHPE of 0.8 mol%, light-induced domain formation was much faster, with most GUVs displaying micron-size rounded plus irregular domains at the earliest observation through the eyepiece, with only rare GUVs caught via the eyepiece in a seconds-long transition from uniform to separated domains, shown in Fig. 2E.

3.5. Temperature is not the key variable

For both palm-SM- and stearoyl-SM-containing mixtures of SM/POPC/chol at 0.33/0.34/0.33 and 0.25/0.5/0.25, similar light-induced domains were observed at 10 °C as at 23 °C. For all samples examined, the timescale for appearance of light-induced
phase separation was similar for these mixtures at 10 °C and 23 °C, using either 0.15 mol% TR-DHPE or (16:0,Bodipy)-PC and C20:0-DiI at 0.1 mol% each (data not shown).

3.6. N-propyl gallate enters the bilayer phases

Ayuyan and Cohen [9] recently reported that the free radical scavenger NPG added to GUV preparations inhibits formation of light-induced domains. In agreement, we observed this inhibition for stearoyl-SM/POPC/chole with (16:0,Bodipy)-PC and C20:0-DiI at 0.1 mol% each: With 2mM NPG added to the aqueous phase, the appearance of light-induced domains required several-fold longer at the composition 0.33/0.34/0.33, and ~ 10-fold longer at 0.25/0.5/0.25. Similarly, for palm-SM/POPC/chol at 0.33/0.34/0.33 containing 0.15 mol% TR-DHPE, vesicles remained uniform under continuous Hg lamp illumination for > 3-5 min, 10-fold longer than without NPG. With the higher dye concentration of 0.8 mol% TR-DHPE, vesicles remained uniform for a much shorter time of ~ 2 - 5 s before the appearance of light-induced domains.

For the composition 0.4/0.4/0.2, the presence of 2 or 5 mM NPG did not change the observation that most GUVs showed separated domains with our quickest observation through the eyepiece. When the NPG concentration was increased to 10 mM, a mixture of uniform and phase-separated GUVs was seen at the earliest observation, and after 3 min of continuous Hg lamp or laser illumination all vesicles showed micron-sized domains. At the highest concentration that we examined, 20mM NPG, GUVs of 0.4/0.4/0.2 were uniform upon initial observation. Light-induced phase separation occurred in most of these GUVs only after ~10 min of continuous illumination (Fig. 5).
We wanted to explore whether NPG enters the bilayer and perturbs the phase behavior. Because we had previously determined an accurate compositional phase diagram for DSPC/DOPC/chol [12], we could use that study to examine an important property of NPG: does this molecule change the equilibrium phase behavior of a lipid mixture? For convenience, we chose an especially distinct boundary of the coexistence region of \{L_\alpha + L_0\}. For samples containing a fixed concentration of \(\chi_{\text{CHOL}} = 0.2\), the boundary occurs at \(\chi_{\text{DSPC}} = 0.09\). With 2 mM NPG, this boundary shifts to the higher DSPC concentration, \(\chi_{\text{DSPC}} = 0.11\). With 10 or 20 mM NPG, all GUVs with \(\chi_{\text{DSPC}} = 0.06 - 0.16\) were uniform, indicating that the phase separation which occurs for all \(\chi_{\text{DSPC}} > 0.09\) at \(\chi_{\text{CHOL}} = 0.2\), had been abolished in this composition range (Fig. 6).

4. Discussion

4.1. Two categories of phase diagrams

The phase diagrams of Fig. 1 imply a different type of phase behavior for all of these 3-component bilayer mixtures that contain cholesterol and either POPC or SOPC or DLPC, together with a higher-melting temperature phospholipid, as compared with 3-component mixtures that contain cholesterol and either DOPC or di-phytanoyl-PC together with a high-melting temperature phospholipid. Although more work is required to characterize more completely the behaviors of the POPC-, SOPC-, and DLPC-containing mixtures, a key point is that lipids can be sensibly grouped into categories that
exhibit similar phase behaviors in complex mixtures containing cholesterol. Recognition of these different lipid groups according to their phase behaviors has been hindered by artifacts induced by intense illumination during fluorescence microscopy.

4.2. Light-induced domain formation is dependent on nonrandom mixing

It is clear from the report by Aruyan and Cohen [9], and confirmed in this study, that intense illumination can induce domain separations in highly nonrandom bilayer mixtures that contain a fluorescent dye. Bilayers of DSPC/DOPC/chol provide a clear example: As the boundary of the 2-phase region \{L_\alpha + L_0\} is approached from the high \chi_{\text{CHOL}} direction, the more randomly mixed 1-phase mixtures, those farther from the phase boundary, do not show light-induced domains. But mixtures closer to the phase boundary, hence more nonrandomly mixed, show light-induced domains.

4.3. Different kinds of crosslinking promote domain separation

Interestingly, a number of published experiments show that for bilayer mixtures that are highly nonrandom, crosslinking of a membrane component can induce phase separation from a 1-phase mixture that had been merely nonrandomly mixed. In one example, crosslinking the ganglioside GM1 by cholera toxin B subunit induced phase separation in mixtures of brain-SM/SOPC/chol [15]. In another case, actin that is bound via PI-P3 which resides predominantly in the L_\alpha phase can be polymerized to induce macroscopic phase separations [16]. These observations imply that the POPC-containing mixtures that we studied are also close to phase separation that can be induced by light.
An implication for cells is that membrane components that are not initially separated into macroscopic, coexisting phases but are sufficiently nonrandomly mixed might readily undergo phase separations, for example by protein-mediated linking of membrane-bound components.

4.4. Lipid clusters might have incipient phase properties

SM mixtures containing POPC that seem to be within the coexistence region of liquid and gel \{L_\alpha + L_\beta(b')\} exhibit uniform GUVs in the fluorescence microscope. However, x-ray diffraction, differential scanning calorimetry, and ESR indicate coexisting phases at 23 °C [17-19]. Because the phase coexistence is not apparent with microscopy using various dyes, at least one dimension of the domains must be much smaller than ~ 300 nm. Fig. 4 shows that in such samples, light-induced domains of a solid phase might have formed. This observation might be significant: Within a single phase the compositionally distinct clusters of lipids that exist for highly nonrandomly mixed lipids might become much larger upon intense illumination, in effect by entropy reduction via aggregation, while retaining some essential features characteristic of their preexisting compositional distinctness. Perhaps if the coexisting clusters have compositions that would correspond to solid and to liquid phases were the scale much larger, then the visible light-induced domains have at least some of the characteristics of those compositions and phases. In different compositional regions, the coexisting cluster compositions correspond to the liquid-ordered phase and to the liquid-disordered phase, and then the visible light-induced domains have some of the characteristics of those compositions and phases.
4.5. Details of the mechanism of induced domains are lacking

The important work by Ayuyan and Cohen [9] points to lipid peroxides and their breakdown products as the origin of the light-induced domain artifacts. We agree. But as to the step-by-step mechanism of domain formation that starts from the free radicals, we emphasize that molecular details are not known. One possibility is a very general impurity-induced increase in the critical temperature of the lipid mixture [20]. Another possibility is that the actual molecular agent that induces macroscopic domains is polymerized or oligomerized lipid [21], just as for the case of oxygen and light-dependent curing of oil-based paints, but this has not yet been proven for bilayers. Starting from the excitation of a fluorophore and proceeding to the actual molecular event(s) of domain formation, events are complex and not well understood [9,22,23]. A direct experimental approach, for example, to isolate putative polymers and to estimate the concentration of any of the light-induced reaction products, is daunting, since the phenomenon occurs within the illuminated field of the fluorescence microscope. Efforts to detect light-induced domains in an intensely illuminated cuvet have failed because of the difficulty of achieving the needed light intensity (Heberle, experiments not shown).

4.6. Comparison with other published studies

In addition to published studies that involved light-induced domains, two groups that did not rely upon imaging of domains have reported that mixtures of SM/POPC/chol do indeed show coexistence of \{L_\alpha + Lo\} phases. One lab describes rich phase behavior of the mixture palm-SM/POPC/chol [24]. These investigators interpreted rather ordinary,
small variations in their data as evidence of phase boundaries. For a proper analysis, in addition to much more data, tielines would need to be determined rather than estimated from work by others on different mixtures. A more recent study from another group [25] of the mixture brain-SM/POPC/chol also reports rich phase behavior. In this case, the authors used averaged relaxation times of a complex multistep mechanism of protein-mediated dye efflux to infer phase boundaries and even fractions of coexisting phases, without the tieline information that was crucial for a proper analysis. A theme that runs through these and other studies is the failure to distinguish nonrandom lipid mixing within a single phase from coexistence of distinct phases.

4.7. Factors that influence light-induced phase separation

4.7.1. Composition

We observed domain separation to depend upon the type of components and the component concentrations. Essentially, the closer a 1-phase composition to a phase boundary, the faster the artifactual domains appeared. However, we have not yet established a quantitative link. This might be useful, if for example a measure of nonrandomness of mixing were tied to the time to induce phase separation.

4.7.2. Dye

Bodipy, DiO, DiI, Texas Red, and naphthopyrene could all give rise to light-induced domain separation. The dye we examined for concentration-dependence, TR-DHPE, showed ~ 50x faster formation of artifactual domains at the 5x higher dye concentration of 0.8 mol% compared with 0.15 mol%.
4.7.3. Temperature

Temperature was not an important factor. Samples under similar conditions at 23 and 10 °C showed the same time required to induce phase separation.

4.7.4 GUV preparation details

Ayuyan and Cohen [9] reported that free radicals that formed during electrolysis on ITO-coated slides contributed to the formation of artifactual domains. Because we also found light-induced domains to form readily in GUVs formed by the gentle hydration method [13], which does not generate free radicals, we conclude that any free radicals that form during sample preparation are not the dominant cause of the artifactual domains.

4.7.5. Free radical scavengers

It might well be that suitably anoxic conditions or use of a suitable free radical scavenger might be useful for eliminating the artifact of light-induced domains. However, we found that one such free radical scavenger, NPG, changes the phase behavior of mixtures of DSPC/DOPC/chol at concentrations that are required for effective scavenging of free radicals. This is almost certainly a result of NPG entering the bilayer and becoming a significant component. Molecules of similar structure to NPG are known to be localized in the lipid bilayer, for example, a series of ubiquinones [26].

5. Conclusions
One category of 3-component lipid bilayer mixture shows only one region of macroscopic phase separation at 23 °C, that of coexisting \( \{L\alpha + L\beta(\beta')\} \) phases. This type of mixture contains cholesterol together with a high melting temperature phospholipid such as DSPC or SM, and a lower melting lipid POPC, SOPC, or DLPC. No region of coexisting immiscible macroscopic liquid domains occurs in these mixtures.

For several mixtures containing POPC, as well as other mixtures that are close to a phase boundary of \( \{L\alpha + Lo\} \) coexistence, light can induce domain separation. This behavior is reminiscent of that previously observed when membrane components are crosslinked. In most cases, light-induced domains could be distinguished from genuine phase separation by sufficiently fast observation of uniform GUV appearance through the microscope eyepiece, or by noticing the progression of domains from tiny specks to large domains under intense illumination. Depending upon the composition, the light-induced coexisting domains behave like either \( \{L\alpha + Lo\} \) phases, or else like \( \{L\alpha + L\beta\} \) phases in terms of the domain shapes and the partitioning of dyes. These observations imply that some key features of highly nonrandom mixing without phase separation might be captured and imaged by means of light-induced domains.

Acknowledgement

This work was supported by grants from the National Science Foundation (MCB-0315330) and the American Chemical Society (PRF-38464-AC7) to G.W.F. The authors are grateful for help from Fred Heberle.
References


FIGURE CAPTIONS

Fig. 1. A group of 3-component bilayer mixtures containing cholesterol show a single region of 2-phase coexistence \( \{ L_\alpha + L_\beta(\beta') \} \) DPPC/POPC/chol (a), DSPC/DLPC/chol (b), DSPC/SOPC/chol (c) and DSPC/POPC/chol (d). “○” compositions showing uniform fluorescence on GUVs; “●” compositions showing 2-phase coexistence. “×” indicates the boundary locations revealed by FRET or single dye fluorescence. GUV images of DSPC/POPC/chol show regions where no phase separations are apparent (A) as well as the 2-phase coexistence region of \( \{ L_\alpha + L_\beta(\beta') \} \) (B). Fluorescent dyes are (16:0,Bodipy)-PC (green) and C20:0-DiI (red), both at 0.1 mole %, and observed at 23 °C. Each image is color-merged from the simultaneously collected fluorescence emission from both dyes using Leica Confocal software. Images constructed from confocal microscopy z-scans in 1-µm increments. Scale bars are 5µm.

Fig. 2. Time-dependent development of light-induced domains on a GUV with composition DSPC/POPC/chol = 0.3/0.5/0.2 (A), DSPC/DOPC/chol = 0.36/0.24/0.4 (B), stearoyl-SM/POPC/chol = 0.4/0.4/0.2 (C), and palm-SM/POPC/chol = 0.33/0.34/0.33 (D, E) under continuous illumination, labeled with (16:0,Bodipy)-PC (green) and C20:0-DiI (red), both at 0.1 mole % for A, B and C; TR-DHPE at 0.15 and 0.8 mol% for D and E respectively, and observed at 23 °C. Time interval between images ~15 s in series A and D, ~30 s in B and C. With C, uniform GUVs were observed at the shortest times through the eyepiece, but this image could not be collected. Tiny dye-depleted domains appeared at the very start of any image collection. The great number of domains induced at the earliest times implies a large number of nucleation sites, with domains proceeding to fuse in time. The area fraction of the dark phase was measured to be ~ 20% for C1, ~ 50% for
C2, ~ 55% for C3, and ~ 65% for C4. With D, only rare GUVs were seen at the earliest times via the eyepiece to be uniform. Microscopy conditions as in Fig. 1. Scale bar 5µm.

**Fig. 3.** GUVs exhibit light-induced domain separation for three mixtures of SM/POPC/chol = 0.33/0.34/0.33 (A), 0.25/0.5/0.25 (B), and 0.4/0.4/0.2 (C) with egg-, palmitoyl- and stearoyl- sphingomyelin, and palmitoyl-SM/POPC/chol also at 0.5/0.25/0.25 (D). Mixtures 0.33/0.34/0.33 and 0.25/0.5/0.25 exhibited slower transition from uniform to light-induced domains compared with 0.4/0.4/0.2 and 0.5/0.25/0.25. In a few cases with egg-SM at 0.4/0.4/0.2, uniform GUVs were observed through the eyepiece with minimal illumination, followed by the visible domains appearing within seconds. Large open circles represent uniform GUVs seen at early time via the eyepiece but not imaged due to fast appearance of light-induced domains. The letter refers to the composition A, B, C, or D. The number refers to the earliest observation (1), or a time (2) some tens of sec later. Labeling and microscopy conditions as in Fig. 1. Temperature 23 °C, scale bar 5µm.

**Fig. 4.** In the \( \{L\alpha + L\beta(\beta')\} \) coexistence region, GUV imaging reveals no light-induced domains for palm-SM/POPC/chol = 0.5/0.5/0 (A), but light-induced solid domains appear initially at 0.47/0.47/0.06 (B). Time interval between figure images was ~20 s in series B. Labeling and microscopy conditions as in Fig. 1. Temperature 23 °C, scale bar 5µm.

**Fig. 5.** n-propyl gallate slows the appearance of light-induced domains. Uniform features of GUVs were captured at lipid compositions and high probe concentration where light-induced domains form rapidly without NPG. (A) palm-SM/POPC/chol = 0.4/0.4/0.2 with 10 mM NPG (A1) and 20 mM (A2). Dyes were (16:0,Bodipy)-PC (green) and C20:0-DiI (red) at 0.1 mole%; (B) palm-SM/POPC/chol = 0.33/0.34/0.33
with 10 mM NPG. GUVs were labeled with 0.8 mol% TR-DPPE; (C) palm-SM/POPC/chol = 0.4/0.4/0.2 with 20 mM NPG. GUVs were labeled with 0.8 mol% TR-DPPE. Microscopy conditions as in Fig. 1. Temperature 23 °C, scale bar 5µm.

**Fig. 6.** NPG becomes a bilayer component and changes the equilibrium phase boundary of DSPC/DOPC/chol. All samples had χ_{CHOL} = 0.2, with χ_{DSPC} changing in 0.02 stepwise increments from 0.06 ≤ χ_{DSPC} ≤ 0.16. Image shown is from sample with χ_{DSPC} = 0.1, the first point in the region of 2-phase coexistence of {Lα + Lo} of the series. With the addition of NPG, mixtures previously having phase separation (A) remain uniform upon illumination (B). B includes 20 mM NPG. Labeling and microscopy conditions as in Fig. 1. Temperature 23 °C, scale bar 5µm.
Figure 1

(a) and (b) show the phase diagrams of POPC, DPPC, and DLPC with cholesterol in different ratios. The diagrams illustrate the phase transition behavior of the lipid mixtures. (c) and (d) depict similar phase diagrams with an additional marker indicating a specific condition or phase, labeled 'Lα+Lβ' and 'uniform' respectively. The images at the bottom right provide visual confirmation of the phase behavior observed in the diagrams.
Figure 4
Figure 5
Figure 6

Jiang_Fig. 6