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Review

GUV preparation and imaging: Minimizing artifacts

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ABSTRACT

The components of biological membranes are present in a physical mixture. The nonrandom ways that the molecules of lipids and proteins mix together can strongly influence the association of proteins with each other, and the chemical reactions that occur in the membrane, or that are mediated by the membrane. A particular type of nonrandom mixing is the separation of compositionally distinct phases. Any such phase separation would result in preferential partition of some proteins and lipids between the coexisting phases, and thus would influence which proteins could be in contact, and whether a protein could find its target. Phase separation in a plasma membrane would also influence the binding of molecules from outside the cell to the membrane, including recognition proteins on viruses, bacteria, and other cells. The concept of these and other events associated with membrane phase separation are sometimes grouped together as the "raft model" of biological membranes. Several types of experiments are aimed at detecting and characterizing membrane phase separation. Visualizing phase separation has special value, both because the immiscibility is so decisively determined, and also because the type of phase can often be identified. The fluorescence microscope has proven uniquely useful for yielding images of separated phases, both in certain cell preparations, and especially in models of cell membranes. Here we discuss ways to prepare useful model membranes for image studies, and how to avoid some of the artifacts that can plague these studies.

Contents

Abbreviations: GUV, giant unilamellar vesicle; PG, phosphatidylglycerol; PS, phosphatidylserine; PC, phosphatidylcholine; DSPC, 1,2-Distearoyl-*sn*-Glycero-3-Phosphocholine; SM, sphingomyelin; POPC, 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine; EDTA, ethylenediaminetetraacetic acid; ITO, indium tin oxide; DOPC, 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine; L_d, liquid-disordered; L_o, liquid-ordered; SOPC, 1-Stearoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine; chol, cholesterol; TLC, Thin layer chromatography; C12:0-DiI, 1,1'-didodecanyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; C18:0-DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; C20:0-DiI, 1,1'-dieicosanyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; (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; TR-DHPE, Texas Red 1,2-dihexadecanoyl-sn-glycerol-3-phosphoethanolamine; PAH, polycyclic aromatic hydrocarbons; LR-DPPE, Lissamine Rhodamine 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine; NPG, n-propyl gallate; b-SM, brain sphingomyelin

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1. Introduction

Ever since the first clear pictures of coexisting gel + fluid domains in bilayer mixtures [1], fluorescence microscopy imaging of giant unilamellar vesicles (GUVs) has been an important tool for researchers who are interested in phase and mixing behavior of both model and real biological membranes. The unique usefulness of such images is twofold: (i) phase immiscibility is decisively ascertained; and (ii) whether the observed domains are liquid or solid is discerned. As with so many other types of image data, problems of observer bias should always be considered in experimental design and analysis, but typically these considerations are treated implicitly. Valuable publication space is seldom used for describing the detailed nature of artifacts and how they can be overcome. In this report, we describe some especially vexing artifacts that can occur during fluorescence imaging studies of GUVs. In brief, we show here that doubt about validity of some imaging studies can be lifted when researchers recognize and explicitly address some behaviors that are special problems for GUV imaging.

2. Preparation of GUVs

Unilamellar vesicles with diameter in the approximate range of 10–100 μm (GUVs), suitable for optical microscopy, can be prepared by two different methods, each with advantages and disadvantages, as noted below. In brief, the "gentle hydration" method requires a few percent of a negatively-charged lipid, and the yield of GUVs is variable and sometimes low. In contrast, the electroformation with ITO-coated or titanium microscope slides gives high yield of GUVs, but can produce deleterious electrolysis byproducts. The electroformation method can be modified from the original procedure [2] to vary the voltage [3], or voltage and frequency [4], and thereby enable GUV formation from mixtures containing negatively charged lipids. However, here we report on GUVs prepared by only the two general methods of gentle hydration, or else electroformation using fixed voltage and frequency. Electroformation can also be achieved on non-planar substrates, specifically platinum wire [3]; however, in our studies we did not use this variation in preparing GUVs.

2.1. Gentle hydration

The gentle hydration method for making GUVs was first introduced by Reeves and Dowben [5], and significantly modified by Akashi et al. [6]. We describe below some observations and changes made to this procedure that are specifically relevant for phase behavior studies of lipid mixtures.

Preparation of liposomes based on the gentle hydration method as described by Akashi et al. [6] employs at least 10 mol% of charged lipids such as PG or PS, which enables the use of buffers at physiological ionic strength. The negatively charged lipids provide electrostatic repulsion between bilayers, which facilitates formation of unilamellar vesicles from the layers of lipid film deposited on the walls of a glass test tube. But even in perhaps the best case, in which a negatively charged PG with the same acyl chains as the cognate PC and having nearly identical gel–fluid transition temperature [7], the 10 mol% negatively charged lipid added solely for liposome preparation purpose is an impurity in the lipid mixtures studied. Recently, we have found that in lipid mixtures that contain PC and cholesterol, as little as 2 mol% PG is required to produce GUVs with good yield. The lower percentage of charged lipid requires lower ionic strength of the buffer, a compromise that is often preferable for study of the phase behavior of neutral lipid mixtures.

At each sample preparation step, the temperature chosen is important to ensure that all lipids are melted and well-mixed before and during vesicle formation. Temperature should be reduced slowly while cooling GUVs in order to achieve near-equilibrium states for phase behavior studies. It is especially important when a gel phase might be present that a temperature is chosen above the highest melting transition temperature of any lipid in the mixture. The temperatures at several steps can be adjusted depending on the lipid mixture studied, without compromising good yield. For example, vesicles that contain high-melting lipids such as DSPC and SM need to be hydrated at ~55 °C, whereas 40 °C is sufficient for samples that contain only monounsaturated lipids, such as POPC. Excessively high temperatures for long incubation times are not necessary for study of low melting lipids, especially because the high temperature accelerates oxidation and free radical chain reactions that degrade lipids. However, an exception to freely adjusting temperature is during the formation of the lipid film from chloroform/methanol solution by use of the rotary evaporator. Regardless of sample composition, we have found that at least 45–55 °C is required to form a uniform film, which is essential for good GUV yield.

One important reason behind subtle adjustments of the details in the procedure is to achieve good GUV yield. The ionic strength of the hydration buffer is a key factor influencing the yield. Although preparing liposomes at physiological salt concentrations (>100 mM salts, [6]) can be important for some studies, high ionic strength is not ideal for preparing GUVs from a series of lipid mixtures having a broad concentration range of negatively charged lipid. In general, low ionic strength solutions, even down to zero ionic strength, result in better GUV yield. Furthermore, in preparing GUVs that contain PS or PG, we find that having EDTA (e.g. at pH 7) in the hydration solution not only chelates unwanted multivalent cations, but also obviates the need for additional buffer to control pH.

2.2. Electroformation

The electroformation procedure has been described by several research groups [2–4,8–15] and we use a modified procedure described in [11]. Among the important parameters for the production of GUVs that reveal the genuine phase behavior of the lipid mixture are the electrode material and the applied voltage. Preparing GUVs by this method, in contrast to gentle hydration, does not work well with charged lipids in the mixture and requires low ionic strength in the aqueous phase (see [3,4,15] for a way to electroform GUVs that contain either negatively-charged or zwitterionic lipids).

2.2.1. Electrode effects

The inappropriate choice of voltage or electrode material can lead to both lipid hydrolysis and oxidation reactions, even under anaerobic

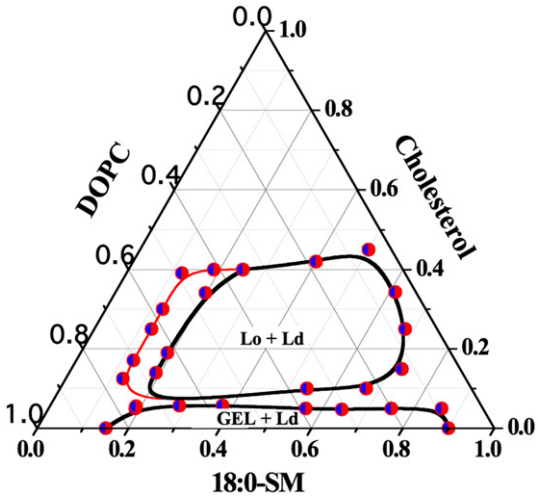

Fig. 1. Shift in phase boundaries at 22 °C. ITO with oxygen (red) versus titanium in anaerobic conditions (black) (E. Farkas and W. Webb manuscript in preparation).

conditions. Although we have not systematically examined the relationship between these unwanted chemical impurities and GUV phase behavior, awareness of these reactions can help to avoid problems. The kinetics of lipid hydrolysis are strongly dependent on pH, with the lowest rate near pH 7 [16]. This is one reason why the electrode material and the applied voltage in the electroformation procedure are so important: some materials, or too high voltage, will hydrolyze water, releasing H+ and OH− into the solution and facilitating the degradation of the lipids by hydrolysis. In addition to hydrolysis, electrode decomposition can result in lipid peroxidation: Ayuyan and Cohen [17] found that the commonly used conductive coating on glass slide, indium tin oxide (ITO) is unstable at the voltages normally applied in electroformation (1.4 to 2 Vpp).

A modified electroformation procedure in which titanium electrodes are used in place of ITO electrodes, decreased but did not eliminate the formation of lysolipid, which can be expected given the spontaneity of the hydrolysis reaction in water [16]. We found lysolipid to form even when all solutions were sparged with nitrogen and electroformation carried out in anaerobic conditions.

In a modified electroformation procedure, ~100 μL of a chloroform solution of dye and lipids is uniformly spread onto each face of two identical titanium plates (McMaster-Carr, Santa Fe Springs, CA) coated in oxide (TiO_2), under a dry nitrogen stream. Slides are separated by a Teflon spacer, and the aqueous chamber sealed by 2 o-rings. Electroformation is done using 8 to 10 Hz AC voltage at 1.0 to 1.2 Vpp. This low voltage was used to avoid hydrolysis of water and dissolution of titanium ions [18].

We observed that the $\{L_d + L_o\}$ boundary of the SM/DOPC/chol phase diagram shifted depending on the preparation procedure. For samples with SM>30%, the procedure made little difference, but for high-DOPC, low-SM samples, the phase boundary contracted by shifting toward the right (higher SM) for the samples prepared in anaerobic conditions on titanium versus those prepared in the presence of O_2 on ITO. Thus, electroformation in the presence of O_2 with ITO as the electrode effectively leads to a falsely high percentage of L_o phase at low-SM concentrations, consistent with the results found in Ayuyan and Cohen [17] and Zhao et al. [19]. This is depicted in Fig. 1. However, we have not attempted to find the relative contribution to this artifactual phase boundary shift from the presence of O_2 compared with any ITO electrode effects. But in general, it is prudent to rely on at least two different methods, e.g. GUV imaging and FRET, for reliable phase boundary determination.

2.3. Cooling rate

GUV preparations were slowly cooled for 18 to 36 h prior to any imaging. Slow cooling decreases the super-cooling [20–22] that can lead to an artifactually uniform GUV that should be phase-separated. To demonstrate this, GUVs of several compositions were quenched to 22 °C in 2–3 h and compared to vesicles of the same composition that were slowly cooled. In most cases, the quenched vesicles were either not phase separated at all, or displayed much greater dispersion in the area fractions of the two phases from vesicle to vesicle than the corresponding slowly cooled samples.

On the binary SM/DOPC axis of the phase diagram, coexistence is observed from SM/DOPC = 0.16/0.84 to 0.90/0.10 at 22 °C. It has been shown both experimentally [21,22] and theoretically [22] that very slow cooling can be necessary for gel–L_d equilibrium, on the scale of hours to days depending on the lipid mixture. An example of the observed morphology for a binary SM/DOPC sample at 22 °C is shown in Fig. 2; the composition was SM/DOPC/chol = 0.50/0.50/0.00 and the sample was prepared under anaerobic conditions on a titanium electrode.

Slow cooling, however, can cause its own type of artifact: for $\{L_d + L_o\}$ coexistence, the high SM side of the phase diagram is difficult to determine because the minority L_d phase can pinch off of the parent GUV during a lengthy cooling period. This is especially true for samples prepared using electroformation, where the vesicle osmolarity is difficult to change during the actual preparation. Thus,

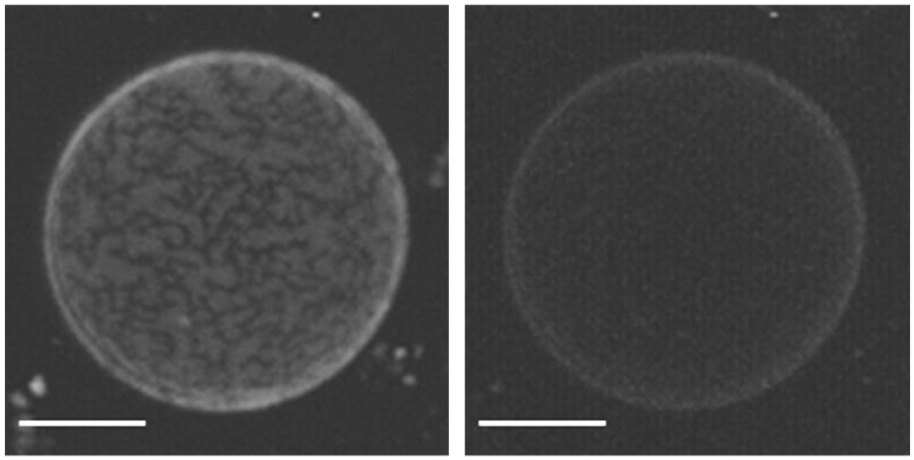

Fig. 2. Gel–L_d coexistence for binary 18:0-SM/DOPC sample at 22 °C. Composition 0.50/0.50, two-photon illumination at 780 nm, LR-DPPE (left) and naphthopyrene (right). Each label at 0.05 mol%, scale bars 10 μm.

the $\{L_d + L_o\}$ coexistence region could be even closer to the SM/chol binary edge than experimentally determined (Fig. 1) using temperature controlled two-photon microscopy.

2.4. TLC

Lipid breakdown does occur. GUV preparations should be checked for lipid breakdown products periodically using TLC (thin layer chromatography). Because a given preparation, especially from the gentle hydration method, can produce an unsatisfactory yield of GUVs, a reasonable protocol is to examine by TLC any preparation that yields useful images. Breakdown is enhanced at higher temperature during preparation, during long incubation periods, and as a result of peroxide formation or electrolysis. The result is a sample that contains unknown amounts of lipid breakdown impurities that can influence the genuine phase behavior.

Typically, both electroformation and gentle hydration yield vesicles suspended in an aqueous sucrose solution. The lipids are extracted from the aqueous solution into organic solvent before TLC testing for breakdown products. A simple and efficient method to extract lipids is the Bligh–Dyer procedure [23]. Briefly, this procedure first uses a one-phase mixed solvent of chloroform/methanol/water to solubilize the lipids. The resulting one-phase mixture is then mixed with additional chloroform and water to form a two-phase system, wherein the water-soluble salts and sucrose reside mainly in the water-rich phase, whereas the lipids partition into the chloroform-rich phase. The chloroform layer is then isolated, concentrated, and developed on activated TLC plates in the appropriate solvent systems for each lipid mixture [24], and detected using sulfuric acid-dichromate (or other detection reagents [24]). It is very important to completely remove the sucrose from the liposome samples before performing TLC: residual sucrose travels on a TLC plate very similarly to the lysolipids, one of the possible lipid breakdown products, in the chloroform/methanol/water (65/25/4) solvent system. However, we found that lyso-PC can be separated from sucrose if the chloroform/methanol/ammonia (60/30/6) solvent system is used. To avoid any ambiguity, it is best to completely remove residual sucrose before performing TLC on liposome samples.

2.5. Budding vesicles

Fission of phase domains from a GUV ("budding") leads to GUVs that are uniform at the time of observation, even though the GUVs were phase-separated before the budding. Budding occurs in GUVs with $\{L_d + L_o\}$ phase separation. The budding might well occur during the long period of slow cooling to the desired temperature. By use of refractive index difference to detect GUVs, together with fluorescence detection of complementary dyes, any pinched-off vesicles can be detected. A typical example of the budding problem is when a single dye that favors the L_d phase is used, a common situation in most reports about GUV imaging. Some vesicles with composition in the $\{L_d + L_o\}$ phase coexistence region can be observed under phase contrast, but may lack detectable florescence signal with a dye that partitions into the L_d phase, since the invisible "dark" vesicles are in the L_o phase (unpublished observations). Using phase contrast enables the observation of all vesicles present in the field of view, but does not permit detecting phase states. A combination of phase contrast detection used for locating and focusing on GUVs, together with complementary dye behavior for fluorescence detection makes it possible to see all the vesicles present and not mistakenly allow dark GUVs to go undetected (Fig. 3). The importance of detecting these dark vesicles is apparent: despite their "uniform" appearance; their presence, even as discrete vesicles, indicates $\{L_d + L_o\}$ phase separation in the mixture. Naphthopyrene and C12:0-DiI are a complementary dye pair that can be used in SM-containing mixtures; (16:0,Bodipy)-PC and C20:0-DiI work well for DSPC-containing mixtures (Table 1).

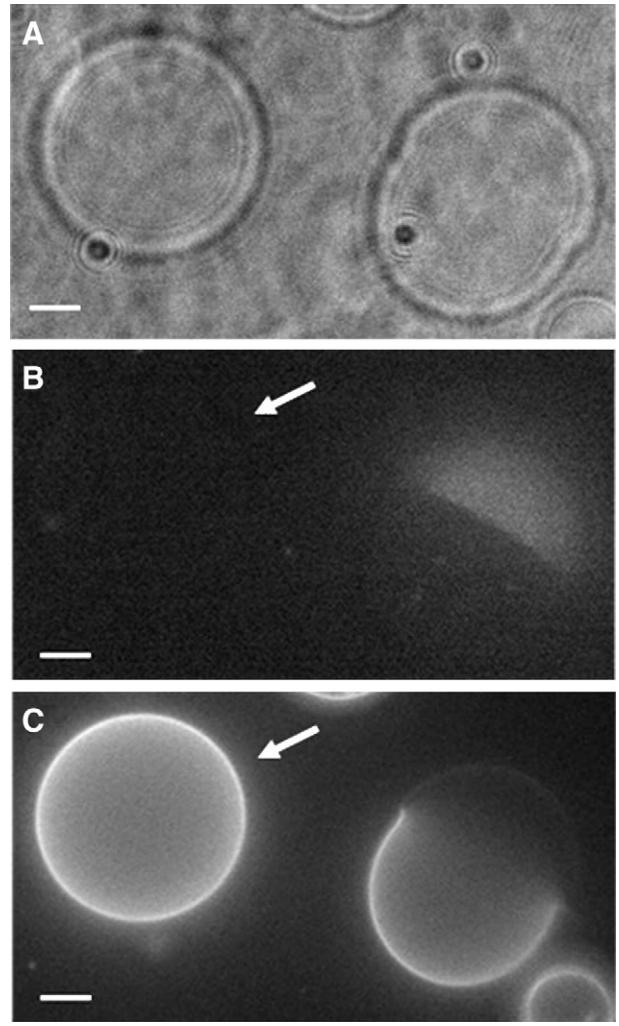

Fig. 3. GUVs of b-SM/(18:0,22:6)-PC/chol can lack fluorescence and appear invisible, or "dark" in fluorescence imaging. Composition 0.375/0.375/0.25, 100 mM sucrose inside and 100 mM glucose outside. Phase contrast image shows all GUVs present in the field of view (A); Fluorescence image of same field of view with C12:0-DiI 0.04 mol%, partitioning into L_d phase does not show all vesicles (arrow) (B); Fluorescence image with naphthopyrene 0.1 mol%, partitioning into L_o phase reveals the "dark" vesicle in B (arrow shows the same GUV that is dark in B) (C). Wide-field illumination, temperature 23 °C, scale bars 10 μm.

2.6. Osmotic effects

Pressure difference across the bilayer can play a role in the observed phase morphology, especially for $\{L_d + L_o\}$ coexistence. Osmotic pressure influences whether a lamellar system should be described by 2D or 3D models due to out-of-plane curvature [25] and correlations of out-of-plane fluctuations [26]. This is important because curvature can induce phase separation [27] and trap vesicles in metastable morphologies [28]. If the domains are small enough that they are below optical resolution, there is the potential to observe what appears to be a lower miscibility transition, which might actually be a sample kinetically trapped from undergoing complete (macroscopic) phase separation [20,29].

The change in morphology due to osmotic differences is readily demonstrated by adding 2 μL of water to a GUV suspension in 100 mM sucrose. With the same sucrose concentration inside and outside, the GUVs are flaccid; a majority adopts a hexagonally-packed arrangement of domains. This morphological pattern can persist for days. The

Table 1
Fluorescent dye partitioning in ternary lipid mixtures.

Probe	Phase preference	Lipid mixture
Cm:0-DiI[a]	L_d	SM/DOPC/chol[b]
C12:0-DiI	L_d	DSPC/DOPC/chol[b]
C16:0-DiI	L_d	DSPC/DOPC/chol[b]
C18:0-DiI	L_β	DMPC/DOPC/chol
	L_o	DSPC/DOPC/chol[b]
C18:1-DiI	L_d	SM/DOPC/chol[b]
C20:0-DiI	L_β	DSPC/DLPC/chol, DPPC/DLPC/chol, DPPC/POPC/chol, DSPC/POPC/chol
	L_β, L_o	DSPC/DOPC/chol, DSPC/SOPC/chol
C22:0-DiI	L_o	DSPC/DOPC/chol[b]
Fast DiO	L_d	SM/DOPC/chol[b]
(16:0,Bodipy)-PC	L_d	DMPC/DOPC/chol, DPPC/DLPC/chol, DPPC/POPC/chol, DSPC/POPC/chol, DSPC/DOPC/chol, DSPC/SOPC/chol, DSPC/DLPC/chol, SM/DOPC/chol[b]
Bodipy ceramide	L_d	SM/DOPC/chol[b]
SM Cn bodipy[c]	L_d	SM/DOPC/chol[b]
Cholestatrienol, terrylene	L_o	SM/DOPC/chol[b]
NBD-chol, "PERY", R18	L_d	SM/DOPC/chol[b]
Naphthopyrene	L_o	SM/DOPC/chol[b]
Perylene, Rubicene, DPH	L_d, L_o	SM/DOPC/chol[b]
X-DOPE[d], X-DPPE[d], Y-DPPE[e]	L_d	SM/DOPC/chol[b]

[a] $m = 12, 16, 18, 20, 22$.
[b] [32].

addition of water to the external sucrose solution creates an osmotic gradient, and water flows into the vesicle, swelling it outward so that it is spherical. A sample viewed 2 h after the addition of water reveals that most vesicles now show only two large domains (Fig. 4).

2.7. Grease-sealed chamber

Careful preparation of the slides, glass cover and grease chamber is important to successful GUV microscopy. A 1000-μL Hamilton syringe is used to deposit a thin and uniform layer of Apiezon vacuum grease (type N or L) onto a microscope slide or coverslip making a square grease cage with dimensions slightly smaller than the glass coverslip (Corning cover glass #1). The grease provides a protective "cushion" and sufficient space to safely hold GUVs without rupturing them. Once the cage is prepared, an aqueous GUV suspension is added to its center using a large orifice pipette tip (to ensure that GUVs do not rupture in the process of extraction). The volume of sample added to the center of the cage varies, but a typical sample volume is on the order of 5 μL. The cage is then completed by placing a coverslip on top, and pressing gently and uniformly on all sides to ensure a good seal.

In general, grease cage construction is a straightforward process with variables that depend on the type of microscope used (e.g. inverted vs. upright) as well as the unique experimental conditions. Its advantages are ease of construction, lack of contamination of the GUVs, and enhanced mechanical protection of the GUVs. Furthermore, for temperature-dependent studies one must be sure to choose a grease type with a very small coefficient of thermal expansion.

3. Light-induced phase separation and shape changes

Light-induced domains are artifacts that appear in fluorescently labeled uniform GUVs, typically under the intense illumination of the wide-field fluorescence microscope. The artifactual nature of these round liquid-appearing domains is apparent when GUVs of the same composition are found to be uniform when examined by multiphoton excitation, at low probe concentrations (e.g. <0.1 mol%), or with near-zero pre-exposure to light. Chemical reactions of unsaturated lipids, likely to be free radical-induced polymerization initiated by photochemical free radical formation via the excited state of fluorescence probes, seem to be the cause for the formation of such light-induced domains [19]. The domains are without doubt artifacts, because they are not present after many hours of sample incubation in the dark, and strongly depend on probe concentration and illumination intensity. Even without a complete analysis of the details of their chemical nature, they mislead phase studies of lipid mixtures. The artifactual domains differ from genuine phase separated domains, because they are neither formed during the slow cooling of the GUV samples nor during subsequent incubation at 23 °C for up to several days. Furthermore, this unwanted phenomenon can be minimized by reducing the illumination of the dyes (e.g. by use of multiphoton excitation or simply neutral density filters during wide-field illumination) or by reducing the dye concentration.

As to the types of mixtures that can show this artifact, reports in the last few years indicate that a bilayer mixture that contains unsaturated lipid species like POPC, DOPC, (18:0,22:6)-PC, together with a fluorescent dye, exhibit light-induced domains. The formation rate depends upon illumination intensity, probe concentration, and location of sample composition within the phase diagram. Different fluorescence probes, e.g. C12:0-DiI, C18:0-DiI, C20:0-DiI, (16:0, Bodipy)-PC, naphthopyrene, and TR-DHPE, have all proven to give rise to light-induced domains at various speeds.

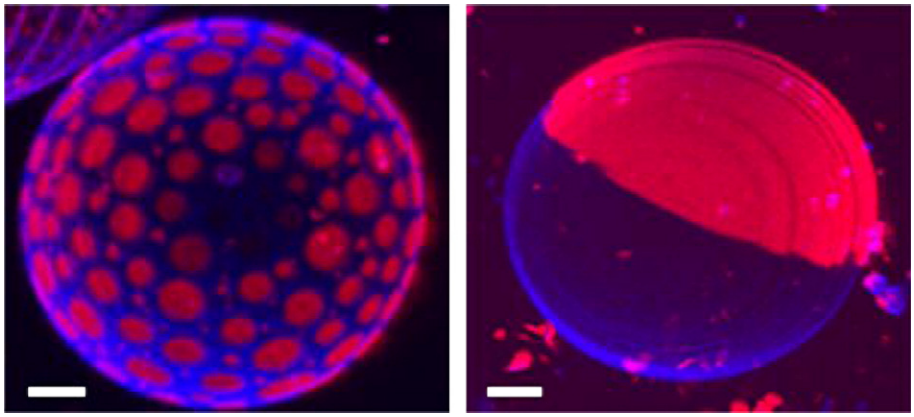

Fig. 4. Change in domain morphology following osmotic swelling. 18:0-SM/DOPC/chol = 0.56/0.24/0.20; Left: no swelling, 100 mM sucrose inside and outside GUV; Right: 2 h after addition of 2 μL water to 8 μL GUV suspension in 100 mM sucrose. The sample was prepared in anaerobic conditions on titanium electrodes and imaged using laser scanning two-photon microscopy with an excitation wavelength of 780 nm. Red false color corresponds to the LR-DPPE and blue to naphthopyrene. Each label at 0.05 mol%, scale bars 5 μm.

3.1. Fluorescent dye

A previous report revealed that TR-DHPE showed ~50× faster formation of artifactual domains at the 5× higher dye concentration of 0.8 mol% compared with 0.15 mol%, in the system of 16:0-SM/POPC/chol = 0.33/0.34/0.33 [19]. At the lower dye concentration (0.15 mol%), GUVs appeared uniform at the earliest observation times studied (a few seconds of illumination in a wide-field fluorescence microscope). As long as it is within the detection range of the instrument, lowering the dye concentration is an easy and practical step to minimize light-induced domains. Other low concentrations of dyes are noted here, where GUVs appeared uniform at the earliest time and were still sufficiently bright for observation: naphthopyrene 0.1 mol%; (16:0,Bodipy)-PC 0.033 mol%; 0.02 mol% for C12:0-DiI, C18:0-DiI and C20:0-DiI. Although we have not examined this aspect systematically, we observe that lower wavelength excitation, e.g. ~450 nm for naphthopyrene or perylene, leads to faster light-induced domain formation than the ~500 nm illumination used for Bodipy, DiI, or rhodamines.

3.2. Composition dependence of artifact formation

The rate of formation of light-induced domains can refer to either the time during which GUVs appear uniform before the onset of light-induced domains, or to the time-dependent variation of domain size during observation [19], i.e. faster domain appearance does not always lead to a faster increase of domain size. And both times can vary independently depending on the lipid composition. We have noticed also dramatic differences in the rate of formation of the artifacts among different compositions within a given ternary lipid mixture such as DSPC/POPC/chol, DSPC/SOPC/chol, or SM/POPC/chol. In bilayer mixtures with unsaturated lipid species, light-induced phase separation generally happens faster at those compositions in the one-phase L_d or L_o region but that are close to $\{L_d + L_o\}$ boundaries. Thus far, we could not establish any other relationships between the mixture composition and the rate of light-induced phase separation.

3.3. Light-induced shape changes

Another light-induced effect is a GUV shape change or membrane stiffening upon exposure to intense light from an arc lamp in the presence of fluorophores. When flaccid, phase separated vesicles are exposed to light during wide-field illumination, they ceased to undulate [30] and adopt a spherical shape, as depicted in Fig. 5. This effect happened in GUVs containing intercalating PAH probes (perylene and naphthopyrene at 405 nm excitation), and with vesicles containing only a headgroup-labeled probe (LR-DPPE, at 568 nm excitation). A somewhat similar phenomenon is reported in Bruckner et al. [30], which attributed GUV shape changes to a transient change in bending elasticity caused by the formation of excimers of the pyrene dye used as a label. However, in the present case, the sphericalization was irreversible on a much longer time scale (hours versus minutes) than observed in Bruckner et al. [30], and thus likely indicative of chemical changes [31]. Such light-induced shape changes can be avoided by the use of two-photon illumination or phase contrast microscopy as discussed below.

3.4. Use of free radical scavenger

Ayuyan and Cohen [17] reported that the free radical NPG can yield GUVs that show no phase separations at compositions where the light-induced artifact would otherwise appear. However, mixtures containing NPG can be misleading in their own way because the high concentrations of NPG that are needed can alter the phase behavior [19].

3.5. Refractive index difference for locating GUVs and focusing

Even an experienced microscopist requires a few seconds to find a candidate GUV and to focus on some part of that vesicle. Yet, some lipid mixtures at certain compositions will have artifactual domain formation in much less than 1 s using wide-field illumination, even at dye concentration of less than 0.02 mol%. The light source for phase contrast microscopy, usually a tungsten filament or halogen lamp, is much weaker than the light source for wide-field microscopy, usually a mercury or xenon arc lamp. Phase contrast conditions fully enable both focusing on GUVs without initiating the formation of light-induced domains, and also identification of unilamellar vesicles, as described by Akashi et al. [6]. One can search carefully for unilamellar vesicles without disturbing the true phase behavior of domain formation. Once a suitable field of view is found, the camera is started, and only then is the shutter opened to intense illumination. With this procedure, the sample has been illuminated for less than 100 ms (using that shutter open time) for the first image taken. This first image therefore has special value, being minimally exposed to exciting light. It is straightforward to adjust the focus using phase contrast such that the surface of a chosen GUV is in focus in this very first image taken with intense illumination. It is easier to detect the light-induced artifact in an image of the vesicle surface rather than its equator. Besides distinguishing authentic phase separation from light-induced artifacts, one can also capture the evolution of the artifact domains with time (Fig. 6), which is sometimes useful to distinguish genuine phase separation from artifactual domains: genuine phase-separated

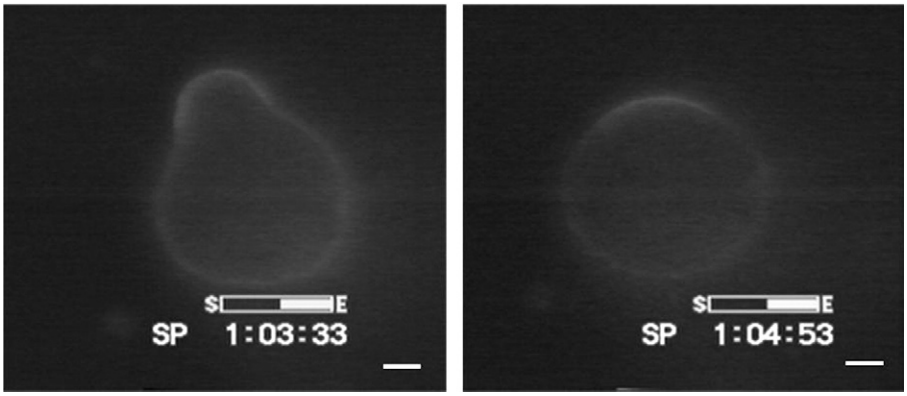

Fig. 5. GUV becomes spherical after illumination. 18:0-SM/DOPC/chol = 0.22/0.48/0.30, naphthopyrene 0.1 mol%, illuminated using wide-field at 405 nm. Left, illumination turned on. Right, 1 min 20 s after illumination start. Temperature 22 °C, scale bars 5 μm.

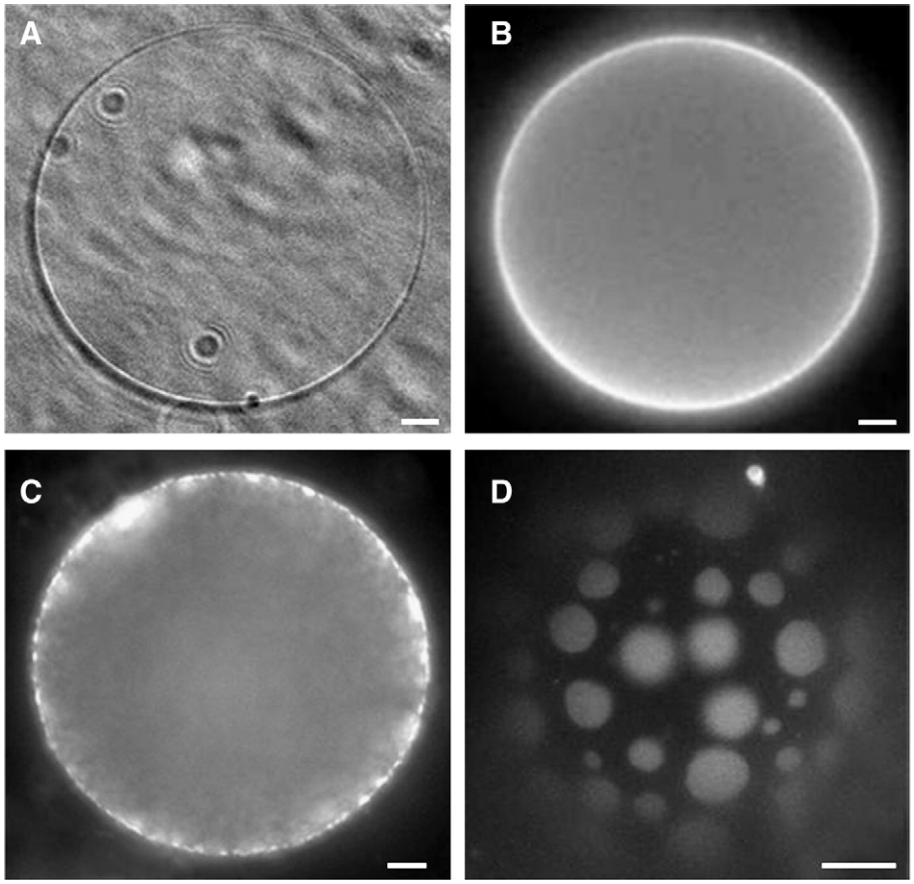

Fig. 6. Light-induced artifact domains can appear soon after start of illumination. Phase contrast image of a GUV with composition b-SM/DOPC/SOPC/chol = 0.38/0.15/0.22/0.25 (A); first fluorescence image collected at less than 100 ms, the shutter open time (B); ~30 s later (C); ~10 min later (D). Vesicle is labeled with C12:0-DiI at 0.03 mol%, wide-field illumination, temperature 23 °C, scale bars 10 μm.

domains that have equilibrated for many hours do not suddenly start to fuse and enlarge within seconds of starting illumination.

Finding GUVs in a large sample chamber is facilitated by use of phase contract microscopy of samples having an internal and an external solution with different refractive index. Solutions of sucrose and glucose at the same concentration have sufficiently different refractive index for this purpose. Regardless of the preparation method (i.e. electroformation or gentle hydration), GUVs can be formed in a sucrose solution, e.g. 100 mM. In order to create the refractive index difference in samples prior to imaging, the GUVs formed in sucrose are harvested and then diluted in an equal osmolarity glucose solution. However, for GUVs made using the gentle hydration method, it is important to gently mix the "lipid cloud" in the glucose solution in order to uniformly disperse the lipid. Once mixed, the sucrose-containing vesicles settle a few centimeters to the bottom of a glass tube within about a half hour. For preparing microscope slides for observations, samples are then gently pipetted from the very bottom of the tube.

3.6. Complementary dyes: detecting the minor phase

To reliably visualize the separation of coexisting domains in GUVs, it can be useful to have two dyes with different spectral properties as well as different preference for the coexisting phases. For example, (16:0,Bodipy)-PC and C20:0-DiI favor L_d and L_o phases, respectively, in the ternary lipid mixture of DSPC/DOPC/chol, whereas in the case of SM/DOPC/chol, (16:0,Bodipy)-PC or one of the DiI probes together with naphthopyrene are a complementary pair. We use such dye pairs to detect the coexisting $\{L_d + L_o\}$ liquid domains in DSPC/DOPC/chol = 36/28/36 at 23 °C. In addition, complementary dyes pairs are especially useful in detecting the minor phases at compositions close to a phase boundary, revealing the minor phase as a bright spot instead of the dark spot seen when only a single dye is used.

3.6.1. Dye partition

Complementary partitioning of a dye pair in one lipid mixture can be dramatically different in another lipid mixture[32–34]. For example, whereas the dye pair of (16:0,Bodipy)-PC and C20:0-DiI lights up different phases in DSPC/DOPC/chol mixtures, both dyes partition into the L_d phase in the mixture of SM/DOPC/chol [32]. Table 1 gathers the phase preference of some probes (pairs) in a few 3-component lipid mixtures.

In bilayer mixtures with genuine phase separation, in favorable cases fluorescent dyes can reveal both whether a phase is solid or liquid, as well as the ratio of the two types of phases. Yet, a simple correspondence between observed domain area and lyotropic phase extent does not happen in light-induced phase separation, where partitioning of fluorescent dyes between the artifactual domains does not share those key features of genuine phase-separated domains. Dye partitioning in light-induced phase separation is not simply related to the phase being liquid or solid, and the area ratio of light-induced domains does not follow the expected dependence on lipid composition, and even dependence of lipid species. For example (16:0,Bodipy)-PC and C20:0-DiI exhibit complementary partitioning in genuine coexisting phase separation of DSPC/DOPC/chol, and also both dyes enter different phases in the same mixture when light-induced domains appear. The same probe pair again shows complementary partitioning in the 4-component system of DSPC/DOPC/

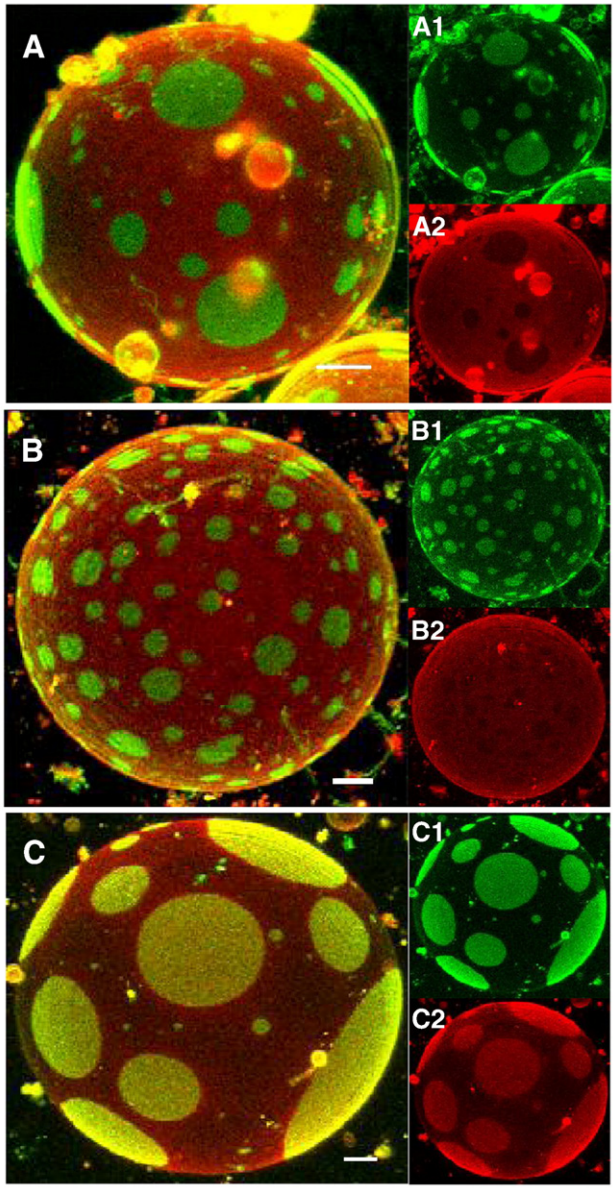

Fig. 7. Dye partitioning is unreliable when light-induced domains occur. Complementary partitioning in both genuine coexisting phase separation of DSPC/DOPC/chol = 0.42/0.2/0.38 (A)[1] and light-induced domains with composition of DSPC/DOPC/chol = 0.36/0.24/0.4 (B)[2], but same-phase partitioning in light-induced domains of DSPC/DOPC/POPC/chol = 0.5/0.16/0.16/0.18 (C). Fluorescent dyes are (16:0, Bodipy)-PC (green) and C20:0-DiI (red), both at 0.1 mol%, 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 5 μm. [1][35], [2][19].

POPC/chol, yet here both dyes favor the same domain in the same mixture when light-induced domains appear, as shown in Fig. 7.

4. Conclusions

Imaging of GUVs provides information about lipid mixing and phase separation that is not readily available from other methods. However, care is required in order to obtain the most reliable images.

1. The two different GUV preparation methods each have advantages and disadvantages: gentle hydration enables GUV formation in mixtures that contain negatively-charged lipids, but yields can be low; electroformation on ITO-coated or titanium slides gives high yield of GUVs, but is less successful with mixtures containing negatively-charged lipids unless voltage [3] or voltage and frequency [4] are varied;
2. the ITO electrodes can give rise to lipid breakdown products;
3. slow cooling of GUV preparations is helpful for providing close to equilibrium conditions, especially important when a gel is one of the equilibrium phases. However, vesicles formed from budding of coexisting liquid phases can occur;
4. thin layer chromatography should be used routinely when possible to examine GUV preparations;
5. an osmotic pressure difference across the GUV can induce small domains to fuse into large domains;
6. light-induced artifactual domain formation can be minimized. Low dye concentration, e.g. 0.02 mol% lipids greatly lessens this artifact, as does several ways to reduce the light intensity experienced by the GUV preparation (multiphoton vs. wide-field illumination; neutral density filters; and finding and focusing on GUVs by use of phase contrast rather than fluorescence, opening the shutter to intense illumination only just before taking the first image).

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