Controlling and Measuring Local Composition and Properties in Lipid Bilayer Membranes

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Abstract. Local composition, structure, morphology, and phase are interrelated in lipid bilayer membranes. This gives us the opportunity to control one or more of these properties by manipulating others. We investigate these relationships with combinations of simultaneous two-color widefield fluorescence imaging, three-dimensional rendering of vesicle domains, and manipulation of the vesicle morphology via optical trapping and micropipette aspiration. We describe methods to modulate, to measure, and to probe the local structure of model membranes through control of membrane curvature in liposomes.

Key words: fluorescence imaging, phase domains, vesicle curvature

1. Introduction

Lateral heterogeneity and order of lipid bilayers are known to play critical roles in many biological membrane functions [1–4]. In order to understand and to control membrane function at the molecular level, it is necessary to understand and to control the lateral structure. We have hypothesized that a simple and powerful means to control local structure is to control the curvature of the bilayer. Curved membrane surfaces are often the norm for biological systems, and wide ranges of curvatures are observed [5, 6]. Many cellular functions necessitate dramatic changes in membrane contour, often resulting in regions having very high radii of curvature; such functions include exocytosis, endocytosis, pathogen scavenging, and adhesion to surfaces and other cells [4].

While a substantial body of theoretical work addresses the relationship between compositional variations within membranes and their curvature [7–18], most experiments in this area have focused on selecting lipid composition and solution conditions to drive compositional heterogeneity and shape changes at equilibrium [19–24]. We have chosen to examine the reciprocal effect, manipulating the curvature to cause changes in the local structure in a biphasic system. The theoretical treatments referenced above apply to both approaches. Not only are we investigating the coupling of local structure and curvature, but we also have the advantage of examining this interaction at biologically relevant states far from equilibrium.

Figure 1. Differential interference contrast optical micrograph of a phase segregating 0.54:0.46 molar ratio DLPC:DPPG vesicle at ambient conditions. At this temperature, the DPPG is in the gel-phase and the DLPC is in the fluid-phase. A phase sensitive probe labels the DLPC rich phase, as displayed in the fluorescence image (right). The domain structure, phase separation and the membrane curvature are interdependent.

Novel imaging techniques, micropipette aspiration, and optical trapping are some of the many tools that have been developed in order to investigate the properties of biomimetic membranes. Imaging and measurement techniques have been developed by the Feigenson, Webb, and Gratton groups [25–29]. Evans and Needham have pioneered measurements of the material properties of vesicles, comparing stress applied via micropipettes to the resulting strain in vesicle morphology [4, 30–34]. Recent studies use similar methods to study vesicle networks and endocytosis [35]. Several groups have described the manipulation of membranes using optical trapping [36–40].

This report describes our efforts to combine established tools with new imaging strategies to develop techniques useful in controlling, probing, and measuring the local structure as we manipulate vesicle morphology. Our long-term goals are to understand the relationship between and to control both the structure and the curvature of biomimetic membranes in order to gain spatial control over membrane functions such as adhesion and transport. A complete understanding of phase domains and lipid interactions in vesicles requires three-dimensional (3D) dynamic measurements. Combinations of simultaneous, two-color, 3D widefield fluorescence imaging, micropipette aspiration and optical trapping are used here to investigate lipid bilayers.

An example of the relationship between phase segregation, local structure and vesicle morphology is displayed in Figure 1. The differential interference contrast (DIC) optical micrograph (left) shows the morphology of a phase-segregating vesicle from the organization of two types of lipids, dipalmitoylphosphoglycerol (DPPG) and dilaurylphosphocholine (DLPC), into their domain-rich phases. The bright areas of the fluorescence optical micrograph (right) indicate the presence of a phase sensitive probe, known to associate with the fluid phase [41]. Here we

have used 6-(9-anthroyloxy)stearic acid, which preferentially partitions into the fluid DLPC-rich phase, at a molar concentration ratio of 640:1 DLPC:probe. The localization of the fluorophore demonstrates that the dissimilar lipids segregate due to phase and local packing structure.

2. Experimental

Phospholipids DLPC (1,2-dilauroyl-*sn*-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), DPPG (1,2-dipalmitoyl-*sn*-glycero-3-*rac*-(1-glycerol)) and EggPC (Egg Phosphatidylcholine or Lysolecithin) were purchased from Avanti (Alabaster, AL, USA). Lipid purity was examined using thin layer chromatography on activated silica plates (Keystone Scientific, Bellefonte, PA, USA), with a (64:25:4) chloroform:methanol:water (v:v:v) solution and developed in an iodine chamber. The fluorescent probes diI (1,1'-didodecyl-3,3,3',3'-tetramethylindocarbocyanine), diD (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indodicarbocyanine), and 6-(9-anthroyloxy)stearic acid were purchased from Molecular Probes (Eugene, OR, USA). Chloroform and methanol were of spectroscopic grade (EMScience, Hawthorne, NY, USA). All water used was filtered through a Barnstead NANOPURE filter and had a resistivity greater than 18.0 M Ω ·cm (Boston, MA, USA).

We form vesicles using the gentle hydration method, which has been described in detail elsewhere [37, 42]. Briefly, 0.25 mg of lipid in chloroform are added to a test tube with an appropriate volume of fluorophore. Typical lipid:fluorophore molar ratios are 1000:1 for DPPC:diD and 5000:1 for DLPC:diI. A larger concentration of diD is necessary due to reduced camera sensitivity in the emission range of the probe. The fluorescent probes diI and diD have been shown to associate preferentially with DLPC and DPPC, respectively [41, 43].

The lipid is dried under an argon stream to create a thin lipid layer inside the test tube, followed by vacuum desiccation for 6 hours to remove residual solvent. A 5 ml aliquot of water, heated to 50 °C, is carefully added to the test tube. The solution is blanketed with argon, sealed under foil, incubated for 4 hours at 55 °C, and then slowly cooled in a thermally insulating box to room temperature overnight.

All experiments are carried out on a modified inverted microscope (TE300 Quantum, Nikon, Melville, NY, USA). Much of the microscope has been modified to allow for the micropipette and optical trapping components to be incorporated, based on the designs of Prof. Steven Block [44, 45]. These modifications enable us to use multi-beam optical trapping, dual micropipette aspiration, and multi-color fluorescence imaging simultaneously. The objectives used are strain-free high-resolution 1.4 NA oil immersion 60 $\times$ or 100 $\times$ infrared. Images are obtained either with trans-illuminated Nomarski DIC or epi-fluorescence. Fluorescence exposure times are synchronized with a high-speed shutter (Uniblitz, Vincent Associates, Rochester, NY, USA). A custom fluorescence cube (CY3/CYS BS&X, Chroma, Brattleboro, VT, USA) allows for the simultaneous excitation of diI and

diD. The fluorescence emission is sent through a branched beamsplitter (W-View, Hamamatsu, Bridgewater, NJ, USA) used to separate the diI and diD signals and projects them onto separate halves of a CCD camera (Orca-100, Hamamatsu). The W-View is equipped with custom dichroic beamsplitters and standard emission filters. Emission filters used are a 585/30 nm filter for diI and a 695 nm long pass filter for diD (Chroma). Images are acquired with Image Pro Plus capture software (Media Cybernetics, Des Moines, IA, USA).

Stage motion and focus are controlled via a three-axis Inchworm stage, used for long-range, fast, precision motion (TSE-150H, Burleigh Instruments, Fishers, NY, USA). A joystick has been interfaced for simple, fast, computer control of the entire system. Using the joystick and our interface program, we have control over the speed, position, and focus of the stage, as well as the exposure time, operation of multiple shutters, lamp source (epi-fluorescence vs. transillumination), and fluorescence cube selection.

To image a vesicle in 3D, we use a 60 $\times$ APO lens and the W-view. A xenon arc-lamp is used for fluorescence excitation. We acquire a stack of images as we step through the focal planes of the vesicle, with a typical step size of 0.30 μ m. We correct for vesicle translation, ensuring the images are concentric, then pseudocolor and combine the complementary images to form a complete image using matrix-based image processing software (Matlab, The MathWorks, Natick, MA, USA). We perform 60 iterations of deconvolution to remove a large portion of the out of plane scatter with a commercial software package (AutoDeblur, AutoQuant, Watervliet, NY, USA). We use bi-linear interpolation to smooth the areas between data planes. The stack of XY images is then rendered into a 3D voxel image and rotated to visualize the XZ axis (AutoVisualize, AutoQuant).

The optical trapping data shown here uses a 647 nm Kr⁺ laser line (I-70, Coherent, Santa Clara, CA, USA). Approximately 300 mW of laser power is introduced into the microscope. Vesicle deformation is accomplished either by translating the stage or with a homebuilt flow cell, consisting of a parallel series of capillaries embedded in a glass chamber using standard coverglasses as windows (number 1.5, Fisher Scientific, Hanover Park, IL, USA). Flow rate is controlled with a syringe pump (EW-74900-00, Cole-Palmer, Vernon Hills, IL, USA) connected to the capillaries via manifold and HPLC fittings (Upchurch Scientific, Oak Harbor, WA, USA). Temperature control is achieved with an objective heater, accurate to 0.2 °C (Bioptechs, Butler, PA, USA). Latex beads were purchased commercially and used as received (Bangs Laboratories, Fishers, AL, USA).

Micropipette aspiration experiments utilize oil-cartridge fine motion controllers (MMO-203, Narishige, East Meadow, NY, USA) and tips pulled to four microns inner diameter using a Narishige PC10 puller and forged to a smooth edge with a M900 microforge. Relative applied pressure is controlled using a homebuilt manometer system to a resolution of microns of water height and measured with a variable reluctance pressure transducer (P55, Validyne, Northridge, CA, USA).

Figure 2. Traditional *widefield* fluorescence microscopy may be used to acquire simultaneous, two-color images to map phase domains in two-lipid systems. The two insets show fluorescence micrographs – diI-labeled DLPC (left) and diD-labeled DPPC (right). The main image is a composite of the insets. The phases display complementary spatial distributions. This is accomplished by acquiring two-color images *simultaneously* with a single high-resolution CCD (see text). This micrograph is a single image, focused on the top portion of the vesicle, rather than at the equator.

3. Results and Discussion

We have acquired simultaneous two-color fluorescence images of vesicle domains with a widefield fluorescence microscope, with the use of a branching emission splitter, as shown in Figure 2. This optical component spatially separates the fluorescence emission of the two probes and projects the image onto separate halves of an ordinary CCD chip. Imaging vesicles in this way yields more reliable informa-

Figure 3. Three-dimensional voxel rendering of one hemisphere of a 70:30 molar ratio DLPC:DPPC vesicle. The optical axis is vertical in this rendering. The dark stripe represents the diD-labeled DPPC gel-phase, while the lighter areas represent the diI-labeled DLPC fluid phase. We acquired a series of two-color fluorescence images, each of a different focal plane of the vesicle at ambient conditions. The stack of images was aligned, deconvoluted, and rendered, yielding a 3D model, which can be viewed from any angle. The vesicle is cropped near the top and the bottom, as the small signal is lost in the out-of-plane fluorescence scatter. Although the individual images were aligned to correct for translation, we have not yet accounted for rotation during imaging. This may explain the jaggedness of the gel-phase domain.

tion regarding the phase domains. The lack of fluorescence signal in one channel does not necessarily indicate the presence of the other phase.

We have expanded this two-color fluorescence capability, creating three-dimensional renderings of an entire vesicle, as shown in Figure 3. This figure displays a stacked series of images in the XY plane, rotated here as if to view one hemisphere of the vesicle from a point to the side of the vesicle, perpendicular to the optical axis (see below). We acquired a series of two-color fluorescence images at regular step intervals as we focused through the vesicle. We process the images to remove translation and drift occurring in the XY plane during acquisition; currently, we do not account for rotation of the vesicle or diffusion of the domains that may occur. The stack of images is then deconvoluted with commercial software to remove out-of-focus and background fluorescence scatter. We process this deconvoluted stack of images into a 3D rendering, which we rotate to visualize. This technique provides a view of the vesicle from any vantage point, enabling us to explore the 3D distribution of topologically two-dimensional domains. Such 3D visualization can

be accelerated beyond this initial demonstration in order to observe phase dynamics in 3D on the ~ 1 second timescale.

The jagged morphology shown in the gel-phase domain in Figure 3 is due to rotation of the vesicle during imaging. We concluded this after examining each of the individual images of the stack. In each slice, there are two gel-phase domains of approximately the same size, which remained diametrically opposite to one another regardless of rotation. Also, when we imaged the top portion of the vesicle, the gel-phase domain appeared straight with straight boundaries. The top and bottom of the vesicle in Figure 3 appear truncated, as we do not image past these regions. The fluorescence signals in these regions are lost in the background scatter and provide no usable information.

Confocal imaging has the advantages of removing nearly all the scatter from an image slice, acquiring simultaneous two-color images, and the capability to image phase domains of lipid vesicles in three dimensions [25]. However, confocal microscopes are cost prohibitive, complex, and slow. A laser is required for excitation, possibly limiting fluorescent probe choice. Furthermore, the laser must be rastered across the surface to create the image, increasing acquisition times. Using our system as described above, we are able to acquire simultaneous two-color images of vesicle domains using traditional widefield microscopy at a greatly reduced cost. The entire plane is imaged at one time, shortening acquisition time. With our system, processing a stack of these two-color images also yields a 3D rendering. However, our microscope requires deconvolution software to remove background scatter and suffers from a somewhat poorer image quality compared to a confocal system. Our technique demonstrates the capability of serving as a relatively inexpensive method of obtaining simultaneous two-color fluorescence images with a traditional fluorescence microscope.

Optical trapping is a non-invasive method of applying forces on a microscopic dielectric. Our optical trapping experiments have been described previously in detail [37]. Briefly, we encapsulate a $2\ \mu\text{m}$ latex bead within the vesicle to serve as a handle for the laser trap. The vesicle is separated a distance from the head by a lipid ‘tether’. The optical trap anchors the bead while fluid flow via a flowcell, or viscous drag by translating the stage, deforms the vesicle. We have succeeded in combining optical trapping, DIC microscopy, temperature, and environmental flow control, as shown in Figure 4. The composition of the displayed vesicle is 1:1 molar ratio DLPC:DPPC at $43\ ^\circ\text{C}$ held by an optical trap in a fluid flow rate of $0.25\ \text{ml/hr}$, yielding a linear velocity of $\sim 30\ \mu\text{m/sec}$. A wide variety of curvatures are noted in this image: ‘regular’ vesicle curvature for the bulk of the membrane, negative curvature at the neck of the tether, and very tight curvature in the tether itself. Another advantage of optical trapping is that we may apply deformation forces to the membrane without physically touching the sample. This reduces the likelihood of imaging artifacts, which may occur from interactions between the lipids and the surface used to manipulate the membrane. However, there are fundamental difficulties that must be considered when combining optical trapping based

Figure 4. DIC micrograph of a 1:1 molar ratio DLPC:DPPC vesicle at 42 °C. A 2.6 μm latex head has been encapsulated by the vesicle and is held in the grasp of an optical trap. The vesicle is deformed by fluid flow from a flowcell. The fluid flow separates the main body of the vesicle from the bead, forming a lipid tether between the two.

deformation and 3D imaging. One of these issues is that the optical trap occurs a fixed distance from the objective and that changing the focus to acquire a series of images in regular step intervals will also move the trap. Two objectives, one for trapping and one for imaging, may be necessary to circumvent this issue [46].

Micropipette aspiration provides a tool to measure the material properties of membranes as well as for a strong holding force independent from the microscope objective focus. A fluorescence image of a partially aspirated vesicle is shown in Figure 5, which displays a 3:1 molar ratio DLPC:DPPG vesicle labeled with diI. A brightfield overlay of the micropipette tip is included for clarity. We have yet to successfully label DPPG gel-phase domains with a fluorescent probe. Although we observe the presence of lipid in rigid areas of DLPC:DPPG vesicles with DIC microscopy, all of our probes designed for dipalmitoyl phospholipids in the gel-phase have been excluded from these regions. These include diD, a fluorescent lipid with an NBD-labeled headgroup (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)), a fluorescent lipid with a rhodamine-labeled headgroup (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)), and a phosphoglycerol with an NBD-labeled tailgroup (1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-[phospho-rac-(1-glycerol)]). When incorporated into a 1:1 molar ratio DLPC:DPPG vesicle, the fluorescence signal from each of these probes was observed in the fluid phase, as determined by the presence of diI (data not shown). We tentatively attribute this to the DPPG lipids packing more tightly than other dipalmitoyl phospholipids, therefore excluding the bulky fluorescent probe.

Figure 5. Fluorescence micrograph of a partially aspirated 3:1 molar ratio DLPC:DPPG vesicle. The bright areas are diI-labeled DLPC domains. We have been unable to label the DPPG gel-phase domains with a fluorescence probe. The gray arrows indicate the dark DPPG gel-phase domains, while the white arrow points to the aspirated portion of the vesicle. A brightfield overlay of the pipette tip has been included for clarity and has an inner diameter of 4.5 μm .

Domain patches, consistent with DPPG gel-phase domains, are noted with gray arrows, and the aspirated portion of the vesicle is marked with a white arrow. Comparing the applied suction pressure to the length of membrane that is aspirated yields measurements of the membrane material properties, including expansion modulus and tensile strength. We compare the material properties to the naturally occurring deformations of the vesicles in order to investigate the coupling between properties and curvature. We assign a simple numerical value to the observed naturally occurring deformations to assist in the correlations [47]. For two dimensions, the equation used is:

$$D = \frac{C^2}{4\pi A}$$

Where D is the deformation value, C is the vesicle circumference, and A is the planar area of the vesicle slice. We will expand this numerical measurement to 3D in future experiments by comparing the surface area to the encapsulated volume.

We have also used micropipette aspiration to create tethers in vesicles. Using two independent pipettes, we capture the vesicle from either side and stretch the membrane to form a tether, as demonstrated in Figure 6. This image is a fluorescence micrograph of EggPC labeled with diI to enhance visualization of the tether. The aspirated portions of the vesicle can be seen at the far ends of the vesicle. Two small vesicles have formed on the thin tether connecting the main portions of the vesicle. Several smaller vesicles, encapsulated by the original, are visible on the

Figure 6. Fluorescence micrograph of a diI-labeled EggPC vesicle stretched between two micropipettes. Two small vesicles have formed on the thin tether connecting the two main portions of the vesicle. Several smaller vesicles, encapsulated by the original, are visible on the right and did not appear to affect the tether formation. The inner diameters of the pipette tips are 4.5 μm .

right and did not appear to affect the tether formation. One issue with micropipette aspiration is non-specific binding occurring between the lipids and the micropipette glass. This issue is averted by coating the inside of the micropipette with a protein, such as bovine serum albumin (BSA).

There are several advantages to using micropipettes for the deformation of vesicles. Most notably, the holding force is strong and stable. Also, the micropipette does not rely on fluid flow for creating tethers and is independent of the focus of the microscope objective. These benefits make micropipette aspiration a prime choice for holding vesicle deformation while we image in 3D.

4. Conclusions

We have been successful in our efforts to obtain simultaneous two-color widefield fluorescence images and to use stacks of these images to create 3D renderings of two-phase vesicles. We have also been successful in manipulating the curvature of a membrane with both optical trapping and micropipette aspiration. Combining these imaging and manipulation techniques will yield a powerful tool useful for studying biomimetic membranes. We will utilize this tool to manipulate, to measure, and to probe the inter-dependent relationship between the local structure, phase segregation, and membrane morphology of lipid vesicles. These studies are relevant to many cellular processes, including endocytosis, exocytosis, diffusion of membrane bound species, and ligand-receptor binding.

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