Acknowledgments

Supported by NIH Grant 49288 to M.J.S. and by NIH Grant 48071 to I.P. We thank Dr. Albrecht Bergner for collecting data from lung slices.

[20] Giant Vesicles, Laurdan, and Two-Photon Fluorescence Microscopy: Evidence of Lipid Lateral Separation in Bilayers

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Introduction

The critical issues in membrane biophysics today are centered on the molecular dynamics of the bilayer structure. The interplay between lipids that results in the formation of domains on a bilayer surface and the interactions among these domains and relevant membrane-associated biomolecules is of particular interest. There has been extensive research in model systems to identify the forces involved in lipid domain formation and lipid diffusion in simple phospholipid mixtures, using nuclear magnetic resonance (NMR), electron spin resonance (ESR), infrared spectroscopy, calorimetry, and fluorescence spectroscopy. Much has been learned about lipid behavior, but even binary lipid systems are still only partially understood. Work on multicomponent bilayers and on cell membranes is difficult because of the multifaceted nature of lipid interactions. In a review article, Maggio fittingly describes the complexity of membrane dynamics as "... a complex and fascinating ecological problem at the molecular level that involves the concerted modulation of environmental, supramolecular, topological and temporal events."[1] From a biological viewpoint, we are at the beginning of our work. It will be essential to develop new techniques and new analysis strategies to effectively identify relevant local forces that give rise to the global membrane effects.

Studies of physical and chemical events in simple artificial lipid systems, in which the lipid composition and the environmental conditions (such as temperature, ionic strength, and pH) can be systematically varied, provide information that can help us understand the behavior of biological membranes. As models of cellular membranes, liposomes have had significant impact on our understanding of the molecular aspects of lipid–lipid and lipid–protein interactions since their introduction by A. D. Bangham *et al.* in 1965.[2] Liposome studies generally

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0076-6879/03 $35.00

involve aqueous suspensions consisting of small unilamellar vesicles (SUVs; mean diameter, 100 nm), large unilamellar vesicles (LUVs; mean diameter, 400 nm), and multilamellar vesicles (MLVs), these being the most popular model systems. An array of different experimental techniques (differential scanning calorimetry, fluorescence spectroscopy, NMR, low-angle X-ray scattering, and electron spin resonance, to mention a few[3–12]), including theoretical treatments using computer simulations,[13,14] have been used to determine parameters regarding the thermodynamic and kinetic aspects of liposome bilayers. However, these experimental approaches produce mean parameters on the basis of data collected from bulk solutions of many vesicles and lack information about lipid organization at the level of a single vesicle, a quality that can be provided by microscopy. In this article, we describe how microscopy techniques, which provide information about membrane microorganization by direct visualization, and spectroscopy techniques, which provide information about molecular interaction, order, and microenvironment, can be combined to give a powerful new tool to study lipid–lipid and protein–lipid interactions.

Our aim in this article is to elaborate on the use of fluorescence spectroscopy tools in fluorescence microscopy. Advances in optical methods, optical components, acquisition electronics, and detectors have greatly enhanced our ability to collect increasingly detailed spectroscopic data with the imaging optics of a light microscope. The continued development of confocal microscopy (both one-photon and two-photon approaches), which has greatly increased the information available through imaging, has allowed for rapid advances in fluorescence correlation spectroscopy and three-dimensional particle-tracking methods, both of which can now be performed in the microscope environment. At the present time, there are a number of laboratories actively advancing this concept, that is, performing spectroscopy in a microscope, for a variety of protocols and generating exciting results in studies ranging from cell physiology to the mechanics of polymer motion on surfaces.

The advantages of using a microscope as the optical arrangement are clear. The light collection efficiency of a well-designed microscope is greatly enhanced

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over other optical arrangements. Indeed, collecting every possible photon is essential for any protocol that requires single, or near-single, molecule sensitivity. In addition, the flexibility of fluorescence microscopes creates for the spectroscopist a malleable optical compartment that can be designed and readily redesigned as needed. Of equal importance as the sensitivity and flexibility of a microscope is the addition of spectroscopy to the ability to collect spatially resolved information. With a properly designed system, we can perform quantitative spectroscopic studies at each pixel and build an information image related to the sample at hand, be it a living cell, an extended surface polymer, or a giant unilamellar vesicle. Researchers have been interested in this marriage of technologies and examples can be found in the early ratio imaging studies, fluorescence lifetime imaging techniques, and fluorescence polarization imaging.

We present here examples of the use of the confocal ability of a two-photon scanning microscope with a multicolor detection attachment to study phospholipid phase behavior in unsupported lipid bilayers, giant unilamellar vesicles. We take advantage of the sectioning ability of the two-photon excitation effect to independently examine the surface and cross section of these spherical bilayers as they are perturbed by temperature and lipid makeup. The fluorescent membrane probe Laurdan [6-lauroyl-2-(*N*,*N*-dimethylamino)naphthalene; Molecular Probes, Eugene, OR] is used to monitor the membrane and changes in the membrane as perturbations are introduced. Although other probes and methodologies can be applied, we limit our discussion primarily to Laurdan and to techniques that can be used with this unique fluorophore.

Microscopy Techniques and Giant Unilamellar Vesicles

A distinct advantage of giant unilamellar vesicles (GUVs; mean diameter, 20–30 μm) over SUVs and LUVs is that GUVs can be observed under the microscope. That fact allows a variety of experiments to be performed at the level of a single vesicle.[15,16] As Menger and Keiper mentioned in their review article, GUVs are being examined by multiple disciplines with multiple approaches and objectives.[15] Admittedly, the use of GUVs in membrane biophysics is still in an early developmental stage, with only a few laboratories active in this area. Examples include elegant studies using GUVs and transmission microscope techniques to investigate changes in the physical properties of membranes through the calculation of elementary deformation parameters.[17–23] The mechanical properties of

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the lipid bilayer explored by these authors are critical for our interpretation of the morphological changes that have been observed in cell membranes.

Investigations of lipid–lipid interactions, in particular the study of phase coexistence in lipid bilayers, single components, and natural and artificial lipid mixtures, have been performed with GUVs. Although the GUV membrane model is an attractive system with which to study lipid-phase equilibria in single vesicles, using fluorescence microscopy, only a few studies have been reported in the literature.[24–32] Among these studies, we recall the seminal contribution by Haverstick and Glaser. These authors reported the first visualization of lipid domain coexistence (Ca^{2+}-induced lipid domains) in GUVs composed of artificial and natural lipid mixtures, using fluorescence microscopy and digital image processing.[24] In addition, Glaser and co-workers also studied lipid domain formation induced by addition of proteins and peptides in GUV membranes.[33–35]

In addition to their use in membrane research, GUVs have been used as membrane models in a number of studies of lipid–protein and lipid–DNA interactions.[36–40] In these reports, a novel approach was taken, consisting of depositing femtoliter amounts of DNA or protein solutions onto GUVs and then monitoring the subsequent binding events and vesicle morphology changes by optical microscopy (phase contrast or epifluorescence).[36,38–40] Membrane protein incorporation on GUVs has also been reported in the literature and offers new opportunities to address issues related to lipid–membrane protein interactions and microscopic organization at the level of single vesicles.[41]

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Fluorescence Microscopy and Lipid-Phase Coexistence

Fluorescence spectroscopy has been and still is extensively used to study the physical properties of lipid bilayers. One example is the study of the thermotropic behavior of lipid bilayers, in particular the presence of lipid-phase coexistence in model systems.[4,11,12,42] In general, experiments designed to detect lipid-phase coexistence in bilayers by fluorescence techniques (cuvette studies) are performed with liposome suspensions and, as mentioned above, these experiments do not provide direct information about the size and shape of the lipid domains and their time evolution. It is this information that allows correlation of the microscopic organization at the surface of single vesicles with the physical parameters, determined at the molecular level, of the lipid bilayer (lipid mobility, lipid hydration, etc).

Epifluorescence microscopy was applied to artificial membranes to observe phase coexistence in monolayers at the air–water interface.[43,44] In such experiments, fluorophores that display preferential partitioning into one of the coexisting phases are used to obtain information about lipid domain shape directly from the fluorescence images. Information about the lipid domain phase state is then deduced from the known partitioning of the particular fluorescent molecule. However, care must be taken when interpreting these effects. We have demonstrated that at the phase coexistence temperature region N-Rh-DPPE (Lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt), a commonly used lipid fluorophore, preferentially partitions to the fluid phase in bilayers composed of dilauroylphosphatidylcholine–distearoylphosphatidylcholine (DLPC–DSPC) mixtures whereas in those composed of DLPC–DPPC (dipalmitoylphosphatidylcholine) mixtures N-Rh-DPPE shows a high affinity for the more ordered lipid domains.[27–29] This finding clearly shows that it is difficult to directly establish, from the fluorescence image alone, the nature of the lipid-phase state, and that it is not possible to generalize the affinity of the fluorescent molecule for the different lipid phases without a careful probe characterization.[29]

Consider the two membrane fluorophores DiI C_{20} (1,1′-dieicosanyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) and Bodipy-PC [2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine], which preferentially partition into the ordered and disordered lipid phases, respectively. These probes were utilized in DLPC–DPPC–POPS (palmitoyloleoylphosphatidylserine) GUVs to directly visualize gel- and fluid-phase coexistence by confocal fluorescence microscopy.[26] Even though two different regions of the lipid membrane are clearly defined from the fluorescence images showing gel–fluid phase coexistence, the phase states of the coexisting lipid domains were determined by measuring probe translational diffusion, using

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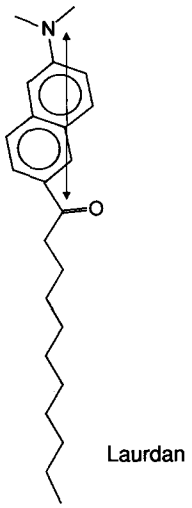

FIG. 1. Laurdan molecule. The double-headed arrow indicates the orientation of the excited state dipole.

fluorescence correlation spectroscopy (FCS).[26] In this case, additional spectroscopic methods were necessary to characterize the physical state of the membrane and to verify the impression given by the fluorescence images.

The fluorescence spectroscopic parameters measured in traditional experiments involving liposome solutions can also be measured at the level of single vesicles by using fluorescence microscopy. Fluorescence polarization, fluorescence lifetime, or phase-dependent fluorescence emission shifts can be obtained to directly determine the lipid domain phase state of the different membrane structures seen in the fluorescence images. These methods require a well-characterized fluorophore, one of the many commonly used in cuvette experiments, GUVs, a fluorescence microscope, and some way to obtain spectroscopic images. In the following sections, we focus on a particularly interesting and useful membrane probe, Laurdan, which has unique spectroscopic properties for monitoring local lipid dynamics. We emphasize how this probe can be used to simultaneously visualize regions of the membrane with different properties and characterize the properties of these regions by a relatively simple spectroscopic measurement.

Laurdan and Possibility of Performing Fluorescence Spectroscopy and Imaging Simultaneously

Laurdan (Fig. 1) belongs to the family of polarity-sensitive fluorescent probes first designed and synthesized by G. Weber for the study of the phenomenon of

solvent dipolar relaxation.[45–48] This family of probes also includes 6-propionyl-2-(*N*,*N*-dimethylamino)naphthalene (Prodan) and 2′-(*N*,*N*-dimethylamino)-6-naphthoyl-4-*trans*-cyclohexanoic acid (Danca). The advantages of Laurdan in the study of lipid–lipid interactions have been amply demonstrated by the more than 100 articles published in the 1990s. The unique characteristics of Laurdan to determine lipid lateral organization in bilayers are particularly useful in studying lipid-phase coexistence.[49,50] These characteristics can be divided into four fundamental properties[29,49,50]: (1) the electronic transition dipole of Laurdan is aligned parallel to the hydrophobic lipid chains; (2) Laurdan shows a phase-dependent emission spectral shift, that is blue in the ordered lipid phase and blue-green in the disordered lipid phase (this effect is attributed to the reorientation of water molecules present at the lipid interface near the fluorescent moiety of Laurdan); (3) Laurdan distributes equally into the solid and liquid lipid phases; and (4) Laurdan is negligibly soluble in water.

The characteristics of Laurdan encourage its use in fluorescence microscopy, in particular to resolve different domains by simple analysis of Laurdan fluorescence images at the level of single vesicles. Unfortunately, the extent of Laurdan photobleaching under the epifluorescence microscope is severe, making it almost impossible to collect images of Laurdan-labeled specimens for more than few seconds.[29] However, the use of two-photon excitation fluorescence microscopy[51,52,53] helps circumvent this problem. The fact that two-photon absorption in the microscope is confined to the focal volume without excitation in areas above and below the focal plane (because of insufficient photon flux) dramatically reduces the extent of probe photobleaching during image collection. In addition, Laurdan has a good two-photon absorption cross section, which permits collection of Laurdan fluorescence. Furthermore, the sectioning (confocal) effect of two-photon excitation allows the researcher to collect images from different focal planes; a useful feature that was an essential component in a number of studies aimed at visualizing lipid-phase coexistence in GUVs.[25,27–29,53,54]

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Using Laurdan Imaging to Define Lipid Domain Shape and Phase State

In addition to the phase-dependent emission spectral shift of Laurdan, the location of the Laurdan excited state dipole, which is parallel to the lipids in the bilayer, offers an additional advantage to ascertain lipid-phase coexistence from the fluorescence images, using the photoselection effect.[25,27–29,31,54] Because of the photoselection effect, only those fluorophores that are aligned parallel, or nearly so, to the plane of polarization of the excitation light are excited. For this reason, when observing the top or bottom surface of a spherical lipid vesicle displaying gel- and fluid-phase coexistence, only fluorescence coming from the fluid part of the bilayer can be observed (Fig. 2). In the fluid phase, a component of the transition dipole of Laurdan is always parallel to the excitation polarization because of the relatively low lipid order, that is, the wobbling movement of the Laurdan molecule. In contrast, Laurdan molecules present in the lipid gel phase have a greatly restricted wobbling motion so that few molecules are excited on the top or bottom GUV surface and little fluorescence intensity is observed. This photoselection effect exists only at the top and bottom surface of the vesicle, where the Laurdan molecules are oriented along the z axis (the excitation light polarization plane is defined as the x–y plane and light propagates in the z direction).

GUVs displaying fluid-ordered/fluid-disordered and gel/fluid lipid-phase coexistence are shown in Fig. 3. In Fig. 3, we exploit the photoselection effect to recognize the tightly and loosely packed bilayer regions occurring at the phase coexistence temperatures.[27–29,31] In the case of gel- and fluid-phase coexistence (Fig. 3B) Laurdan fluorescence originates from the fluid region even though Laurdan is homogeneously distributed in the bilayer (see above). In this case, the fluid region is associated with a red-shifted fluorescence emission (high extent of water dipolar relaxation). In the GUV displaying fluid-ordered/fluid-disordered phase coexistence (Fig. 3A), the less ordered region displays enhanced intensity compared with the more ordered region because of the photoselection effect. In addition, the fluorescence emission of Laurdan at the fluid-ordered bilayer regions is blue shifted, when compared with the emission from the fluid-disordered bilayer regions, allowing identification of the phase of the lipid domain directly from the fluorescence image.[29,31]

Phase State and Shape of Lipid Domains

When fluid domains are embedded in a fluid environment, circular domains will form because both phases are isotropic and the line energy (tension), which is associated with the rim of two demixing phases, is minimized by reducing the area-to-perimeter ratio. This last situation is observed in the DOPC–cholesterol–sphingomyelin mixture (Fig. 3A) and is consistent with considerations about the lipid domain phase state obtained from fluorescence images using Laurdan.[29,31]

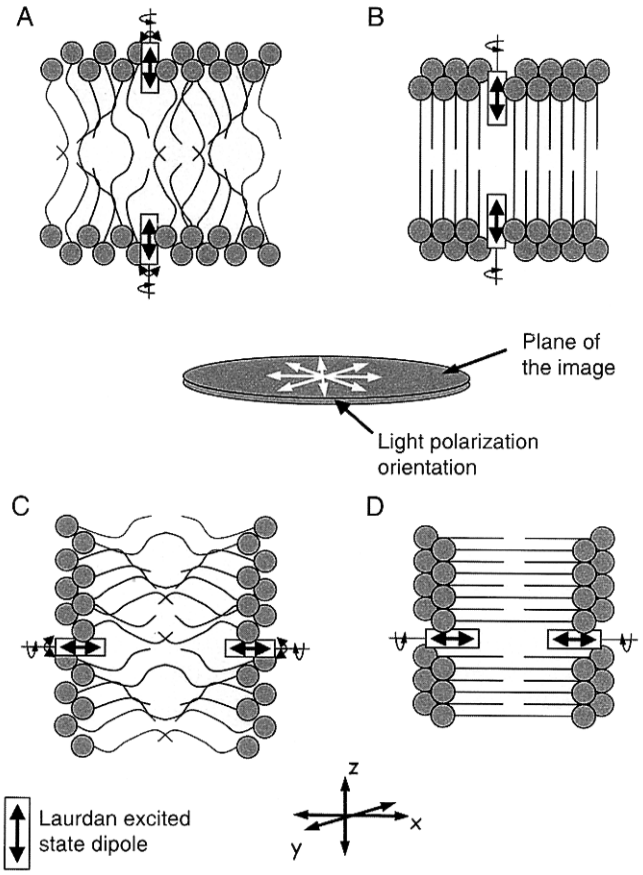

FIG. 2. Sketch of the orientation of Laurdan in the lipid bilayer with respect to the polarization plane of the excitation light (circular polarized light). (A and C) Fluid-phase (loosely packed) bilayer observed at the polar region (A) and in the equatorial region of the GUV (C). In these last two situations green emission is observed in both cases—the emission intensity is lower in (A) with respect to (C) because of the photoselection effect. (B and D) Gel-phase bilayer observed at the polar region (B) and in the equatorial region of the GUV (D). In this case only blue emission is observed in (D) [being zero in (B)] because of the photoselection effect (see text).

This contrasts starkly with that found in the gel–fluid coexistence where round domains are not observed (Fig. 3B).[27,29]

Laurdan Generalized Polarization Function

A way to quantify the extent of water dipolar relaxation, which in turn is related to the phase state of the lipid interfaces, is based on a useful relationship between the emission intensities obtained at the blue and red sides of the Laurdan emission

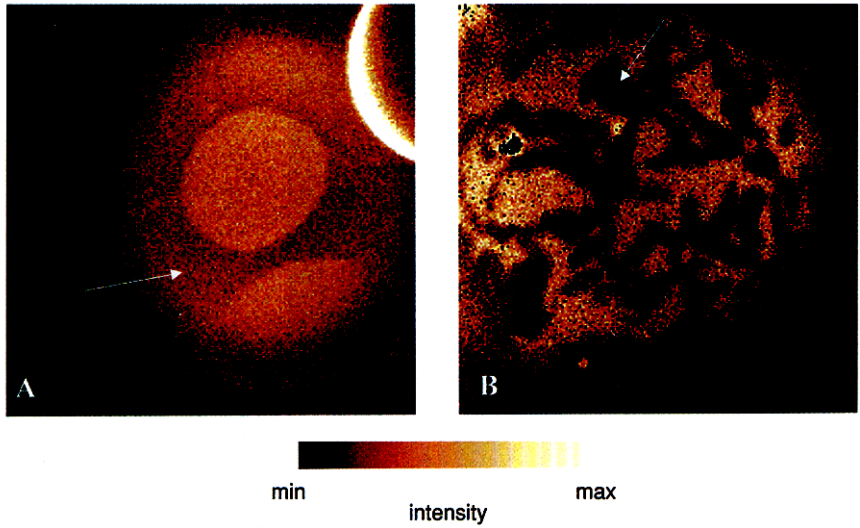

FIG. 3. Two-photon excitation fluorescence intensity images (false color representation) of GUVs formed of DOPC–cholesterol–sphingomyelin (1 : 1 : 1, mol/mol) (A) and DMPC–DSPC (1 : 1, mol/mol) (B) labeled with Laurdan. The images were taken at the top part of the GUV at temperatures corresponding to the phase coexistence region (24° for DOPC–cholesterol–sphingomyelin and 45° for DMPC–DSPC). The arrows indicate the fluid-ordered (DOPC–cholesterol–sphingomyelin) and gel (DMPC–DSPC) domains. GUVs diameter $\sim 30\ \mu$m.

spectrum. This relationship, called generalized polarization (GP),[49,50] was defined in analogy with the fluorescence polarization function as

$$\mathrm{GP} = \frac{I_B - I_R}{I_B + I_R} \tag{1}$$

In this function, the relative parallel and perpendicular orientations in the classic polarization function were substituted by the intensities at the blue and red edges of the emission spectrum [I_B and I_R, respectively, in Eq. (1)], using a given excitation wavelength.[49,50] The generalized polarization parameter contains information about solvent dipolar relaxation processes that occur during the time that Laurdan is in the excited state, and it is related to water penetration in the phospholipid interfaces (see above). Therefore, GP images can be constructed from the intensity images obtained with blue and green bandpass filters on the microscope, allowing further characterization of the phase state of the coexisting lipid domains.[25,27–31] It is important that the GP images be obtained at the equatorial region of the GUVs to avoid artifacts due to the photoselection effect because of the particular locations of the Laurdan probe in the lipid bilayer (see above).[29]

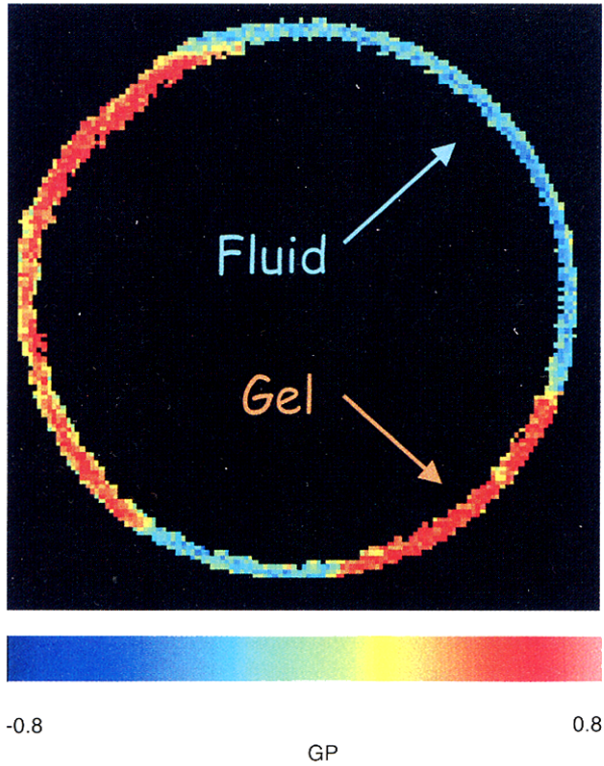

FIG. 4. Two-photon excitation Laurdan generalized polarization (GP) image of a single GUV composed of DLPC and DAPC (1 : 1, mol/mol), obtained with circular polarized light. The temperature was 55°, corresponding to the gel- and fluid-phase coexistence temperature regime. The image corresponds to the equatorial region of a 30-μm-diameter vesicle. The arrows show the fluid and gel lipid domains spanning the bilayer.

As an example, the GP image of DLPC–DAPC (diarachydoylphosphatidylcholine) is shown in Fig. 4. In this phospholipid binary mixture the GP measured in the tight- and loose-packed regions of the lipid bilayer corresponds to that observed at temperatures below and above the main phase transition temperature in GUVs composed of a single phospholipid component. The fact that in the DLPC–DAPC mixture the physical characteristics of the coexisting phases resemble those observed in the "pure" gel- and fluid-phase states suggests highly energetic and compositional differences between the coexisting lipid domains (low miscibility between the components of the binary mixture).[28,29] Interestingly enough, the observation that lipid domains span the lipid bilayer is obtained directly from the GP images (see Fig. 4). This finding is in agreement with the observations of GUVs composed of DLPC–DPPC–POPS mixtures at room

temperature, using standard one-photon confocal microscopy.[26] Lipid domain spanning the lipid bilayer is a general phenomenon in samples displaying fluid/fluid and gel/fluid phase coexistence (GUVs composed of artificial and natural lipid mixtures).[27–29,31] This last feature suggests a mechanism for how differentiated domains in the outer monolayer of biological membranes are coupled to cytoplasmic signal transduction pathways: a question that has eluded simple answers.[31]

Laurdan Generalized Polarization Images Providing Information about Domains Smaller than Microscope Resolution

When the size of the lipid domain is smaller than the size of the image pixel, it is not possible to determine the lipid domain shape by the above-mentioned experimental approach. However, it is possible to determine lipid domain coexistence by obtaining Laurdan GP images at the equatorial region of the GUV, using linear polarized light.[25,54] If the lipid domain size is smaller than the microscope resolution, each pixel of the image (we assume a pixel has a size comparable to the microscope resolution) will present an average GP value in the equatorial section of the GUV. We can discriminate pixels with high and low GP values because the polarized light, which photoselects appropriately oriented Laurdan molecules, also selects Laurdan molecules associated with high GP values. Essentially, if the image contains separate domains (pixels) of different GP values, because of lipid-phase coexistence, the higher GP value domains appear parallel to the orientation of the polarized excitation light and not in the perpendicular direction. This effect was used to ascertain lipid domain coexistence in model and natural membranes[54–56] and in GUVs composed of pure phospholipids.[25]

To further illustrate this effect, let us analyze the fluorescence intensity and the GP function of Laurdan in the GUV equatorial plane (Fig. 5). If we excite with polarized light (the plane of polarization corresponds to the direction 0° axis in Fig. 5), the intensity will vary as we go around the equatorial section and the extent of change in the intensity will depend on the local orientation of the probe. Let us first consider the case in which the bilayer is homogeneous. In the gel phase, the intensity will greatly vary as we go around the section, but in the liquid–crystalline phase the intensity will vary much less. If the probe is randomly oriented, the intensity will not change as we go around the section. If we now look at the GP changes (Fig. 5), again assuming that the sample is homogeneous, the GP should be independent of the intensity. However, if the sample displays microheterogeneity, the GP value will also change as we go around the GUV

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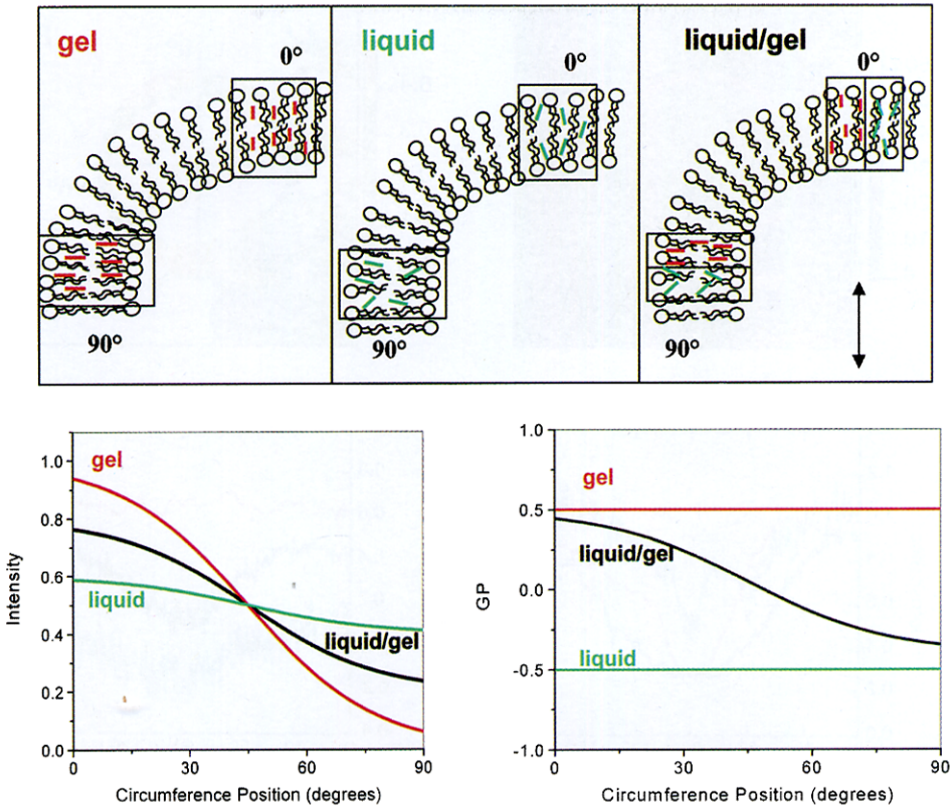

FIG. 5. *Top:* Schematic drawing of the orientation and mobility of Laurdan within a phospholipid bilayer under gel, liquid, and phase coexistence conditions. The excitation light is assumed to be polarized along the 0° axis and the excited state Laurdan molecules are indicated in red (gel) and green (fluid). *Bottom:* Laurdan intensity and Laurdan GP are plotted as a function of the perimeter of a circular vesicle. The curves are labeled to match the lipid states illustrated (*top*).

perimeter because, along the polarization axis, we are preferentially exciting the gel phase with high GP, but in the perpendicular direction we are exciting only the liquid–crystal microdomains.

Measurements of a GUV composed of a simple lipid above, at, and below the phase transition is shown in Fig. 6A and B. At low temperature, the GUV is in the gel phase. There is little microheterogeneity because the intensity varies greatly but the GP is essentially constant around the equatorial section (red line in Fig. 6D). Instead, at the phase transition and above, both the intensity and the GP vary (black and green lines respectively in Fig. 6C and D). As we further increase the temperature, the membrane heterogeneity decreases gradually and even at a temperature well above the main transition temperature, we can still observe the membrane microheterogeneity. This unexpected result suggests that there is an

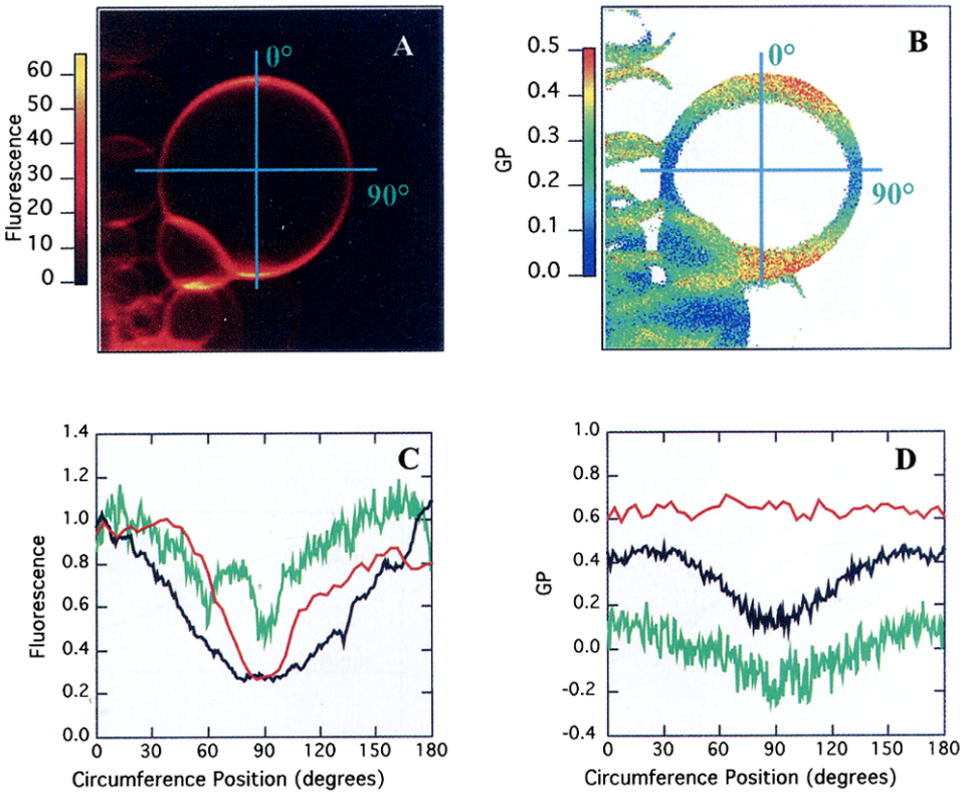

FIG. 6. Fluorescence intensity (A) and GP (B) images are shown for a Laurdan-labeled DMPC giant unilamellar vesicle at 25°, near the main phase transition temperature of 24.5°. The excitation light was polarized along the 0–180° axis. Fluorescence intensity (C) and GP (D) as a function of GUV circumference position are shown for Laurdan-labeled vesicles in liquid phase, DMPC at 41° (green line), in the phase transition region, DMPC at 25° [black line, from the images in (A) and (B)], and in gel phase, DPPC at 32° (red line).

intrinsic microheterogeneity for phospholipids in the fluid phase[28] that does not appear to be true for the gel-phase phospholipid domains.

Laurdan Generalized Polarization Providing Information about Phase State of Lipid Membrane during Action of Interfacial Hydrolysis: Case of Phospholipase A_2

In this section, we show the use of the microscopy techniques described above to study a particular problem of an enzymatic reaction in lipid vesicles. Laurdan was used to characterize the changes in the phase state of vesicles during enzymatic hydrolysis of membrane phospholipids.

Secreted phospholipase A_2 ($sPLA_2$) has been shown to directly hydrolyze phospholipids of GUVs made by the electroformation process.[36] It is not surprising that this enzyme was chosen for examination of the interactions between protein and GUVs because this class of enzyme is particularly active against aggregated phospholipids, hydrolyzing the *sn*-2-acyl chain of phospholipids to produce free fatty acid and lysophospholipid. As the substrate is hydrolyzed the reaction products become new membrane components, giving rise to hydrolysis-associated changes in the bilayer that can be explored through the use of membrane-reporting fluorophores. Although seemingly simple, the hydrolysis of a phospholipid bilayer is a complex, nonequilibrium process. The rapid production of fatty acid and lysolipid by the enzyme creates a complex membrane dynamic that, in turn, will be self-perturbing and affect continued $sPLA_2$ actions. Indeed, $sPLA_2$ activity is sensitive to the membrane character and is modified by a host of membrane factors that include the phospholipid headgroup size, headgroup charge, phospholipid phase state, phospholipid dynamics, and membrane curvature.[57–63] This interaction between organized phospholipid substrate and $sPLA_2$ has long intrigued researchers and has made these enzymes ideal model systems for examining protein and membrane interactions.

Direct visualization of $sPLA_2$ action on unsupported bilayer membranes has primarily come from images of lipid vesicles demonstrating morphological changes, using electron microscopy techniques.[63,64] Atomic force microscopy has also been used to visualize the effect of $sPLA_2$ hydrolysis on supported bilayers.[65] Images of the time-dependent changes in palmitoyloleoylphosphatidylcholine (POPC) GUVs due to the presence of $sPLA_2$ have been reported by Wick and co-workers.[36] The approach taken by these authors consisted of injecting femtoliter volumes of enzyme solution into, or onto, GUVs by use of a microinjector and then monitoring the influence of $sPLA_2$ on single vesicles, using conventional microscope techniques (phase contrast). This elegant work presents a simple, yet powerful experimental arrangement to study the interactions between proteins and lipid interfaces. In describing the $sPLA_2$ attack on GUVs, Wick and co-workers reported two kinds of effects: gradual shrinking of the GUV and immediate destruction of the GUV.[36] In our hands, using this same general approach, we have found

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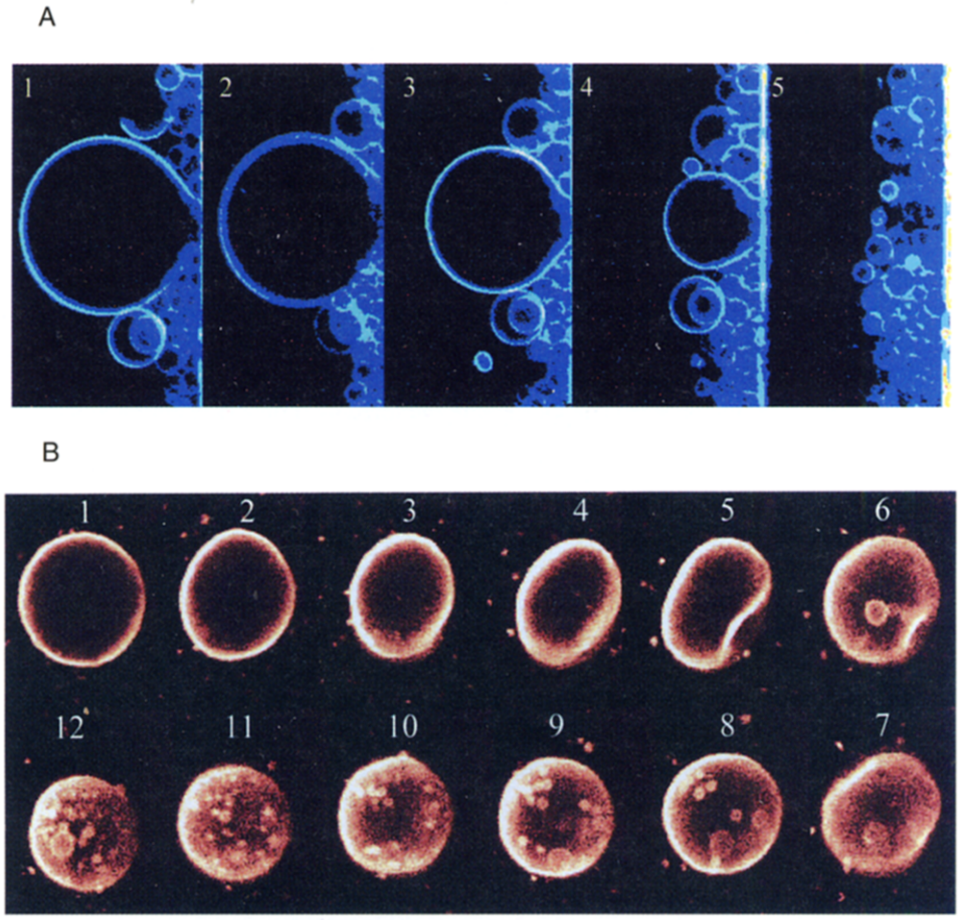

FIG. 7. $sPLA_2$-induced morphology changes in GUVs labeled with Laurdan. $sPLA_2$, isolated from *Crotalus atrox* venom, was added to the bulk solution as indicated. The images shown are as follows: (A) POPC vesicles at 20° and (B) DMPC vesicles at 31°.

that the most common effect is the steady reduction in size of the GUV, presumably following the time course of hydrolysis of the membrane phospholipids (Fig. 7A). The shrinking is surprising for a number of reasons. Within the phospholipase research community, the assumption has long been made that in the hydrolysis of vesicles only the outer layer of the vesicle is hydrolyzed and the vesicle remains intact.[66–68] The evidence to support these claims appears to be solid, although

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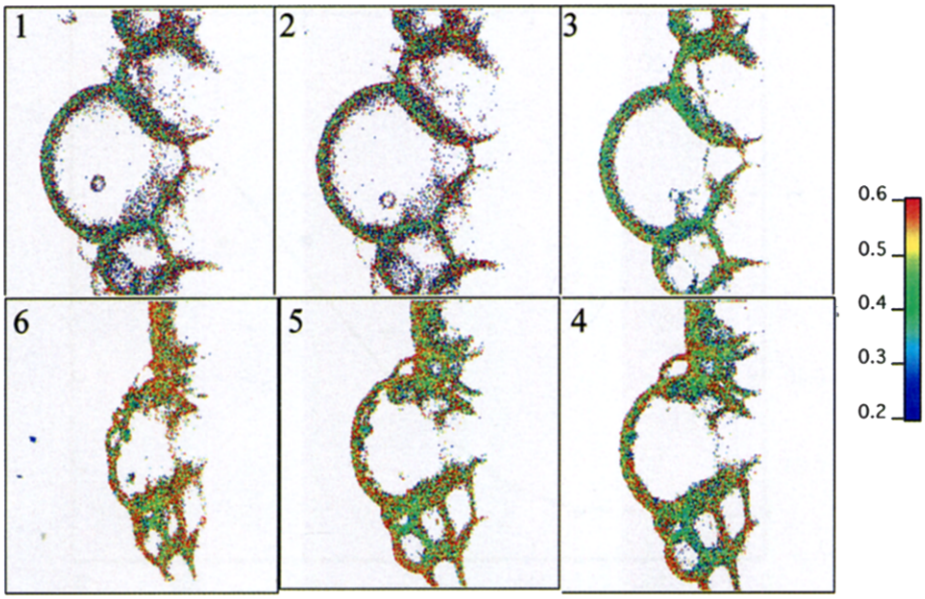

FIG. 8. $sPLA_2$-induced Laurdan GP changes in DMPC GUVs equilibrated at 26°. *Crotalus atrox* venom $sPLA_2$ was added to the bulk solution immediately before the initial image. The buffer contained 0.5 m*M* Tris (pH 8.0) and 0.2 m*M* $CaCl_2$.

there have been suggestions that vesicle morphology may be significantly affected by $sPLA_2$ lipid hydrolysis.[59,69] What are the forces involved and why should the GUV system, which is merely another model membrane bilayer, show different characteristics than any other bilayer system?

Shrinking is not the only effect that can be observed with $sPLA_2$ hydrolysis of the GUV membrane surface. On occasion, a gross distortion of GUV shape after $sPLA_2$ addition can be seen. Although not a common observation, this distortion underscores the fact that significant structural forces are induced through phospholipid hydrolysis and the creation of products in the membrane. A clear example of this effect is shown in Fig. 7B. The formation of vesicles inside the primary GUV during the hydrolysis is apparent in this sequence (Fig. 7B) and suggests that membrane stress can be relieved through expulsion of smaller vesicles. How can the nature of these changes be examined? Membrane probes, such as Laurdan, offer the researcher one method to dissect time-dependent fluctuations in membrane water penetration, which, in turn, are related to the packing and kinetics of the membrane phospholipids (see above). A GP image series monitoring the time-dependent changes in a GUV membrane after $sPLA_2$ addition is shown in Fig. 8. Laurdan had been added to the GUVs after their formation and allowed

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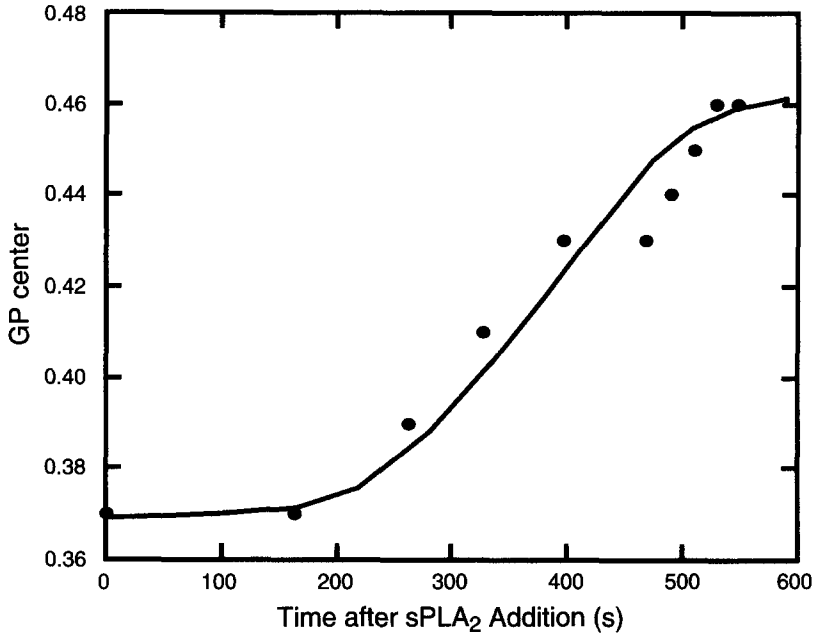

FIG. 9. The membrane GPs were well fit to a single Gaussian distribution model. The distribution center is given as a function of time after *Crotalus atrox* venom $sPLA_2$ addition. These data are derived from the image series that is selectively represented in Fig. 8.

to disperse throughout the sample chamber for approximately 15 min. GP images of the vesicles were then calculated from the simultaneously collected red and blue images, as described earlier in this article. The shrinking is readily apparent, although not as extreme as in other cases that we have observed. As hydrolysis proceeds, the average GP shifts from approximately 0.37 to 0.46, indicating an overall decrease in membrane polarity. The results are shown in the plot of the average GP as a function of time in Fig. 9. These data are consistent with results from bulk solution studies of large unilamellar vesicles.[58,70,71] In our case, however, we have the additional ability to examine the membranes of individual vesicles and observe the ongoing events at the local microscopic level. The GP images in Fig. 8 show the progressive formation of small solid domains (red-yellow), showing us that the shift in average GP values (Fig. 9) is the consequence of small solid domains formed as hydrolysis proceeds. The melting temperature of

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myristic acid is significantly higher than that of the root phospholipid and would be expected to give rise to higher GP values as its concentration builds in the membrane. The increase in average GP can be due to interdigitation of the fatty acid in the membrane, reducing water penetration, or fatty acid domain formation. The fact that small multipixel domains with higher GP are observed suggests that the fatty acid is not homogeneously distributed and small, fatty acid-rich regions are the source of the GP change. The other hydrolysis product, lysophospholipid, is relatively water soluble and would largely partition into the bulk phase (see below).[72–74] Phospholipid monolayer studies by Grainger and co-workers show a similar phenomenon through monitoring of fluorophore-labeled fatty acid and, interestingly, the authors also observed that the fatty acid coalesced with $sPLA_2$ to form enzyme–product domains.[75–77] Our hydrolysis-induced higher GP microdomains are consistent with this hypothesis, but we have not as yet been able to identify preferential binding of the *Crotalus atrox* $sPLA_2$ to these regions.

The shape changes shown in Fig. 7 are complex and can be linked to what we have already inferred from the Laurdan GP images. As already stated, the $sPLA_2$ hydrolysis of bilayer phospholipids generates two products: fatty acid and lysophospholipid. At the pH used in these studies, pH 8.0, both products have highly favorable partition coefficients into the lipid phase. However, under the conditions of the GUV system the concentration of lipid is extremely low, on the order of 0.3 μM. This creates a large volume for the soluble phase in proportion to the lipid, hydrophobic phase. The equilibration of fatty acid and lysolipid into the soluble phase creates structural stress on the outer leaflet of the membrane, where $sPLA_2$ initially has access and begins active lipid hydrolysis. Given the partition coefficients for myristoyl-lysophosphatidylcholine (0.5×10^6)[76] and myristic acid (2.6×10^6),[78]the products would be expected to partition into the aqueous phase. In contrast, bulk solution measurements with large/medium/small unilamellar vesicles are usually performed at lipid concentrations of >100 μM, where a significant fraction of the products would be expected to remain in the bilayer. We would not expect to observe as great a degree of vesicle distortion in these systems. However, the fact that we see a GP change in the GUVs as hydrolysis proceeds suggests that fatty acid concentration is, in fact, building up in the membrane, despite its partition coefficient. It would be reasonable to assume that kinetic effects and organizational forces of the membrane help to

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retain product, at least on the time scale of these experiments (minutes). Clearly, the interaction of $sPLA_2$ and a lipid bilayer is complex and there is much more to be done to understand the microscopic scenario that is now unfolding. The Laurdan GP images have been instrumental in our interpretation of the hydrolysis events and have provided clues as to the fate of the individual hydrolysis products. Further work with other fluorophores and under varying experimental conditions should prove productive in our studies of the intricate lipid dynamics involved with this and similar systems. Readers are encouraged to explore Ref. 79 that compares the action of $sPLA_2$ on GUVs composed of single-component phospholipids and phospholid binary mixtures displaying phase coexistence.

Conclusions

In this article we have pointed out the importance of generating new experimental tools to explore the concerted phenomena occurring at different mesoscopic levels of the lipid membrane. The combination of Laurdan, GUVs, and two-photon fluorescence microscopy has been extremely useful in producing a microscopic picture of lipid-phase coexistence in the GUV bilayer model system. Laurdan is a unique probe, giving simultaneous information about morphology and phase state of lipid domains from fluorescence images. After 30 years of study of the physical and chemical aspects of lipid–lipid interactions (in particular the lateral lipid separation) in model systems, the importance of the relationship between lipid composition and membrane organization is now well recognized.[80] We have shown with several examples that it is possible to visualize and characterize regions of different properties in artificial membranes. It is important to realize that the same kind of studies can be done in cells. The characterization of Laurdan parameters in different cell membranes is a necessary step that will help to understand the role of lipid composition and organization in biological membranes.

Acknowledgments

This work is supported by a grant from the National Institutes of Health (RR03155 to S.A.S., E.G., and T.L.H.) and the Fundacion Antorchas (to L.A.B.). MEMPHYS is supported by the Danish National Research Foundation.

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