the hypertonic shock of liposomes increases the surface potential by approximately 7 mV at 25°.[33]

Final Remarks

Liposome surface properties can be measured with optical probes that may dimerize in the membrane interface. The dimerization constant may report information about changes in packing in the interface as a consequence of the chain–chain interaction, curvature (vesicle size), phase state, and osmotic state. Changes in packing are congruent with changes in polarity and membrane surface potential measured with fluorescent probes located at the interface. This could be ascribed to changes in the distribution of dipoles as measured by the dipole potential in monolayers.

[33] E. A. Disalvo, *in* "Surface Chemistry and Electrochemistry of Membranes" (T. S. Sørensen, ed.), p. 837. Marcel Dekker, New York, 1999.

[15] Thermotropic Behavior of Lipid Mixtures Studied at the Level of Single Vesicles: Giant Unilamellar Vesicles and Two-Photon Excitation Fluorescence Microscopy

By LUIS A. BAGATOLLI

Introduction

The effect of temperature on phase equilibria in lipid mixtures has been studied for almost 30 years and phase diagrams have been constructed, using both theoretical and experimental approaches.[1–12] As a consequence, important thermodynamic information is currently available for several lipid mixtures.

[1] J. H. Ipsen and O. G. Mouritsen, *Biochim. Biophys. Acta* **944,** 121 (1988).
[2] K. Jørgensen and O. G. Mouritsen, *Biophys. J.* **69,** 942 (1995).
[3] A. G. Lee, *Biochim. Biophys. Acta* **413,** 11 (1975).
[4] B. R. Lentz, Y. Barenholz, and T. E. Thompson, *Biochemistry* **15,** 4529 (1976).
[5] S. Mabrey and J. M. Sturtevant, *Proc. Natl. Acad. Sci. USA* **73,** 3862 (1976).
[6] P. W. M. Van Dijck, A. J. Kaper, H. A. J. Oonk, and J. De Gier, *Biochim. Biophys. Acta* **470,** 58 (1977).
[7] K. Arnold, A. Lösche, and K. Gawrisch, *Biochim. Biophys. Acta* **645,** 143 (1981).
[8] M. Caffrey and F. S. Hing, *Biophys. J.* **51,** 37 (1987).
[9] E. J. Shimshick and H. M. McConnell, *Biochemistry* **12,** 2351 (1973).

0076-6879/03 $35.00

One interesting temperature region in the lipid mixture phase diagram is that corresponding to the formation of a stable lipid domain structure, which was discovered in 1977.[13] This phenomenon, which involves lipid phase separation and is characterized by the coexistence of ordered (tightly packed) and disordered (loosely packed) lipid phases in the bilayer, plays a central role in the stabilization of multicomponent vesicles and in the fission of small vesicles after budding.[14] Several studies on lipid domain structure and phase connectivity in bilayers that display phase coexistence were carried out using fluorescence recovery after photobleaching (FRAP).[15–19] The information extracted from all these different experimental techniques is useful to understand, starting from simple models, the possible occurrence of phase separation phenomena in complex systems such as cell membranes. It is important to note that the presence of phase coexistence in cell membranes is still a matter of controversy. However, accumulating experimental evidence favors the presence of laterally organized domains in biological membranes.[20–24]

Direct Visualization of Lipid Domain Coexistence in Bilayers

Even though lipid phase coexistence is well accepted in model systems (monolayers and bilayers), the direct visualization of lipid domains was successfully accomplished only in lipid films at the air–water interface, using fluorescence microscopy techniques.[24–28] Changes in lateral pressure

[10] B. Maggio, *Biochim. Biophys. Acta* **815,** 245 (1985).
[11] B. Maggio, G. D. Fidelio, F. A. Cumar, and R. K. Yu, *Chem. Phys. Lipids* **42,** 49 (1986).
[12] L. A. Bagatolli, B. Maggio, F. Aguilar, C. P. Sotomayor, and G. D. Fidelio, *Biochim. Biophys. Acta* **1325,** 80 (1997).
[13] C. H. Gebhardt, C. H. Gruler, and E. Sackmann, *Z. Naturforsch.* **32C,** 581 (1977).
[14] E. Sackmann and T. Feder, *Mol. Membr. Biol.* **12,** 21 (1995).
[15] W. L. C. Vaz, E. C. C. Melo, and T. E. Thompson, *Biophys. J.* **56,** 869 (1989).
[16] W. L. C. Vaz, E. C. C. Melo, and T. E. Thompson, *Biophys. J.* **58,** 273 (1990).
[17] T. Bultmann, W. L. C. Vaz, E. C. C. Melo, R. B. Sisk, and T. E. Thompson, *Biochemistry* **30,** 5573 (1991).
[18] P. F. F. Almeida, W. L. C. Vaz, and T. E. Thompson, *Biochemistry* **31,** 7198 (1992).
[19] V. Schram, H. N. Lin, and T. E. Thompson, *Biophys. J.* **71,** 1811 (1996).
[20] J. F. Tocanne, *Commun. Mol. Cell. Biophys.* **8,** 53 (1992).
[21] K. Simons and E. Ikonen, *Nature* **387,** 569 (1997).
[22] K. Jacobson and C. Dietrich, *Curr. Opin. Cell Biol.* **9,** 84 (1999).
[23] D. A. Brown and E. London, *Annu. Rev. Cell Dev. Biol.* **14,** 111 (1998).
[24] R. G. Oliveira and B. Maggio, *Neurochem. Res.* **25,** 77 (2000).
[25] R. M. Weis and H. M. McConnell, *Nature* **310,** 47 (1984).
[26] H. Möhwald, A. Dietrich, C. Böhm, G. Brezesindki, and M. Thoma, *Mol. Membr. Biol.* **12,** 29 (1995).
[27] K. Nag, J. Perez-Gil, M. L. F. Ruano, L. A. D. Worthman, J. Stewart, C. Casals, and K. M. W. Keough, *Biophys. J.* **74,** 2983 (1998).

induce the formation of coexisting lipid phases in lipid monolayers composed of pure lipids or lipid mixtures (either artificial or natural mixtures). The direct observation of this last phenomenon, using epifluorescence microscopy, generally involves the use of different fluorescent probes with preferential partitioning to one of the coexisting lipid phases (for details see section on Fluorescent Probes).

In the past few decades, direct visualization of lipid domains in bilayers at the phase coexistence region was achieved by electron microscopy techniques.[29] As was pointed out by Raudino, however, no direct and detailed knowledge of the shape and formation of domains in bilayers was available.[30] Actually, there are few experimental approaches that allow direct visualization of lipid domain formation (shape and dynamics) in lipid bilayers under the same experimental conditions as in classic approaches (such as, e.g., differential scanning calorimetry and fluorescence spectroscopy in a cuvette).

The aim of this chapter is to introduce an experimental approach that allows visualization of temperature-dependent lipid phase equilibria at the level of single vesicles, using two-photon fluorescence microscopy. Various methods to prepare giant unilamellar vesicles (GUVs) and novel results in the direct visualization of the lipid phase coexistence at the level of single vesicles, combining the particular properties of some fluorescent probes and two-photon excitation fluorescence microscopy, are discussed in this chapter.

Some Considerations about Giant Unilamellar Vesicles

To mimic the lipid lateral organization of the cell plasma membrane, GUVs are the proper choice as a model membrane system (mean diameter, $\sim 20\,\mu$m). These ''cell-sized vesicles'' are attractive experimental systems to study the phase equilibria of pure lipid systems and lipid mixtures by fluorescence microscopy techniques, mainly because single vesicles can be observed under the microscope. Surprisingly, few studies based on fluorescence microscopy and GUVs have described lipid phase coexistence. In this sense, it is important to remark on the seminal contribution of Haverstick and Glaser, who achieved the first visualization of lipid domains in GUVs by fluorescence microscopy with digital image processing.[31] These

[28] R. Veldhuizen, K. Nag, S. Orgeig, and F. Possmayer, *Biochim. Biophys. Acta* **1408,** 90 (1998).
[29] E. Sackmann, *Ber. Bunsenges. Phys. Chem.* **82,** 891 (1978).
[30] A. Raudino, *Adv. Colloid Interface Sci.* **57,** 229 (1995).
[31] D. M. Haverstick and M. Glaser, *Proc. Natl. Acad. Sci. USA* **84,** 4475 (1987).

authors directly visualized Ca^{2+}-induced lipid domains in erythrocyte ghosts, GUVs formed of mixtures of phosphatidylcholine (PC) and acidic phospholipids, and GUVs formed from natural lipids from the erythrocyte membrane at constant temperature.[31] In addition, Glaser and co-workers also studied lipid domain formation caused by addition of proteins and peptides to GUVs.[32–34]

GUVs have been objects of intense scrutiny in diverse areas that focus on membrane behavior.[35] In particular, the advantages of direct observation of a single giant vesicle, using transmission and fluorescence microscopy, was well appreciated in areas such as chemistry, physics and biophysics. In this sense, an excellent review by Menger and Keiper[35] about GUVs and a book completely devoted to giant vesicles, edited by P. L. Luisi and P. Walde,[36] provide the reader with an overview of the initial stages in GUV research. For example, an intense study of membrane physics has been done with GUVs, in particular studies on the mechanical properties of model membranes.[35–42] These studies revealed the physical properties of the membranes through the calculation of elementary deformation parameters. Studies of lipid–lipid, lipid–protein, and lipid–DNA interactions were also performed with GUVs.[43–56] Still, the enormous potential capabilities of this model system are in an early stage of development.

[32] D. M. Haverstick and M. Glaser, *Biophys. J.* **55,** 677 (1989).
[33] M. Glaser, *Commun. Mol. Cell. Biophys.* **8,** 37 (1992).
[34] L. Yang and M. Glaser, *Biochemistry* **34,** 1500 (1995).
[35] F. M. Menger and J. S. Keiper, *Curr. Opin. Chem. Biol.* **2,** 726 (1998).
[36] P. L. Luisi and P. Walde, "Giant Vesicles." John Wiley & Sons, London, 2000.
[37] E. Evans and R. Kwok, *Biochemistry* **21,** 4874 (1982).
[38] D. Needham, T. M. McInstosh, and E. Evans, *Biochemistry* **27,** 4668 (1988).
[39] D. Needham and E. Evans, *Biochemistry* **27,** 8261 (1988).
[40] P. Meléard, C. Gerbeaud, T. Pott, L. Fernandez-Puente, I. Bivas, M. D. Mitov, J. Dufourcq, and P. Bothorel, *Biophys. J.* **72,** 2616 (1997).
[41] P. Meléard, C. Gerbeaud, P. Bardusco, N. Jeandine, M. D. Mitov, and L. Fernandez-Puente, *Biochimie* **80,** 401 (1998).
[42] E. Sackmann, *FEBS Lett.* **346,** 3 (1994).
[43] L. A. Bagatolli and E. Gratton, *Biophys. J.* **77,** 2090 (1999).
[44] L. A. Bagatolli and E. Gratton, *Biophys. J.* **78,** 290 (2000).
[45] L. A. Bagatolli and E. Gratton, *Biophys. J.* **79,** 434 (2000).
[46] L. A. Bagatolli, E. Gratton, T. K. Khan, and P. L. G. Chong, *Biophys. J.* **79,** 416 (2000).
[47] C. Dietrich, L. A. Bagatolli, Z. Volovyk, N. L. Thompson, M. Levi, K. Jacobson, and E. Gratton, *Biophys. J.* **80,** 1417 (2001).
[48] L. A. Bagatolli and E. Gratton, *J. Fluoresc.* **11,** 141 (2001).
[49] D. P. Pantazatos and R. C. MacDonald, *J. Membr. Biol.* **170,** 27 (1999).
[50] R. Wick, M. I. Angelova, P. Walde, and P. L. Luisi, *Chem. Biol.* **3,** 105 (1996).
[51] S. Sanchez, L. A. Bagatolli, E. Gratton, and T. Hazlett, *Biophys. J.* **82,** 2232 (2002).
[52] M. L. Longo, A. J. Waring, L. M. Gordon, and D. A. Hammer, *Langmuir* **14,** 2385 (1998).

General Considerations for Preparation of Giant Unilamellar Vesicles

In general, experimental conditions, such as ionic strength, pH, lipid composition, substrate on which to dry the lipid film, and addition of some sugars, seem to be critical parameters to obtain giant vesicles.[38,57–61] Nevertheless, there is no general agreement about ''unique'' conditions to obtain such vesicles, mainly because the mechanism of giant vesicle formation is still obscure. A major consequence of the lack of precise knowledge is that many different methods to obtain GUVs have been described.[38,57–70] For instance, low ionic strength (below 10 m*M*) in aqueous solution is required to successfully prepare giant vesicles.[38,58,59,61] Alternatively, as reported by Akashi *et al*., physiological conditions can be used to obtain the giant lipid structures, using a percentage of charged lipids in the sample.[60] As a general rule two important conditions are required to prepare GUVs: (1) the temperature during vesicle preparation must be higher than the phase transition temperature of the lipids used to form the GUVs (this condition also operates in the formation of multilamellar, small unilamellar, and large unilamellar vesicles), and (2) to form large structures agitation of the samples during the vesicle formation must be prevented.

[53] P. Bucher, A. Fischer, P. L. Luisi, T. Oberholzer, and P. Walde, *Langmuir* **14,** 2712 (1998).
[54] M. I. Angelova, N. Hristova, and I. Tsoneva, *Eur. Biophys. J*. **28,** 142 (1999).
[55] J. Korlach, P. Schwille, W. W. Webb, and G. W. Feigenson, *Proc. Natl. Acad. Sci. USA* **96,** 8461 (1999).
[56] G. W. Feigenson and J. T. Buboltz, *Biophys. J*. **80,** 2775 (2001).
[57] A. Moscho, O. Orwar, D. T. Chiu, B. P. Modi, and R. N Zare, *Proc. Natl. Acad. Sci. USA* **93,** 11443 (1996).
[58] J. P. Reeves and R. M. Dowben, *J. Cell. Physiol*. **73,** 49 (1969).
[59] J. Käs and E. Sackmann, *Biophys. J*. **60,** 825 (1991).
[60] K. Akashi, H. Miyata, H. Itoh, and K. Kinosita, Jr., *Biophys. J*. **71,** 3242 (1996).
[61] M. I. Angelova and D. S. Dimitrov, *Prog. Colloid Polym. Sci*. **76,** 59 (1988).
[62] L. Yang and M. Glaser, *Biochemistry* **35,** 13966 (1996).
[63] M. I. Angelova and D. S. Dimitrov, *Faraday Discuss. Chem. Soc*. **81,** 303 (1986).
[64] D. S. Dimitrov and M. I. Angelova, *Prog. Colloid Polym. Sci.* **73,** 48 (1987).
[65] D. S. Dimitrov and M. I. Angelova, *Biolectrochem. Bioenerget*. **19,** 323 (1988) [a section of *J. Electroanal. Chem.* constituting Vol. 253].
[66] M. I. Angelova, S. Soléau, P. Meléard, J. F. Faucon, and P. Bothorel, *Prog. Colloid Polym. Sci*. **89,** 127 (1992).
[67] M. I. Angelova and D. S. Dimitrov, *Prog. Colloid Polym. Sci*. **76,** 59 (1988).
[68] D. D. Lassic, *Biochem. J*. **256,** 1 (1988).
[69] M. Winterhalter and D. D. Lassic, *Chem. Phys. Lipids* **64,** 35 (1993).
[70] F. M. Menger and M. I. Angelova, *Acc. Chem. Res*. **31,** 789 (1998).

Comparison of Methods Used to Generate GUVs

One of the first experimental protocols reported (and the most popular) to generate giant vesicles was based on the exposure of dried lipid films to aqueous solution for a long time (up to 24 h) at temperatures above the lipid phase transition.[58] Some modifications of this last technique were reported to improve the yield of single-walled liposomes.[34,38,59,60] In all cases, giant vesicles with a mean diameter of approximately 15 to 20 μm can be obtained. Another technique based on organic solvent evaporation in aqueous solution was presented by Moscho *et al*. to prepare GUVs.[57] One of the advantages of this last method, compared with that presented above, is the short time required to obtain the vesicles (a few minutes). In this case vesicles up to 50 μm in diameter are obtained.[57] One of the drawbacks of these two techniques is that the yield of GUVs is low (no more than 20%). This last fact makes finding a single-walled vesicle under the microscope a time-consuming process. In addition, the vesicle size distribution obtained by these methods is broad and a significant number of multilamellar vesicles as well as other types of lipid structures (such as lipid tubes or vesicles with internal structures) are observed at the end of the formation process.[71]

An interesting technique to generate GUVs was introduced by Dimitrov and Angelova in 1986, consisting of hydration of the dried lipid films above to the lipid phase transition in the presence of electric fields.[63–67] In this last case, a high yield of GUVs (~95%), with a narrow size distribution (5 to 60 μm) with respect to the distributions obtained by the other methods previously described, is achieved in a reasonable period of time (60 to 90 min).[67,72] Figure 1 shows pictures of the GUV-generating chamber designed in our laboratory,[44,45] together with an image of the border of the Pt wire after vesicle formation. Once the vesicles are formed the AC field is turned off and the vesicles remain adsorbed to the Pt wires. This last fact is remarkable and allows a single vesicle to be monitored for long periods of time without vesicle drifting.[43–46] The many advantages of the electroformation method over other protocols have been discussed.[71] In particular, Bagatolli *et al*.[71] compare different preparation techniques and emphasize the necessity of evaluating the characteristics of the entire sample. In general, all the studies already made on such samples focus only on the GUVs, and do not consider the rest of the sample.[71] In Fig. 2, two-photon excitation fluorescence images of various lipid structures obtained by various GUV formation protocols are presented for comparison.

[71] L. A. Bagatolli, T. Parasassi, and E. Gratton, *Chem. Phys. Lipids* **105,** 135 (2000).
[72] L. Mathivet, S. Cribier, and P. F. Devaux, *Biophys. J*. **70,** 1112 (1996).

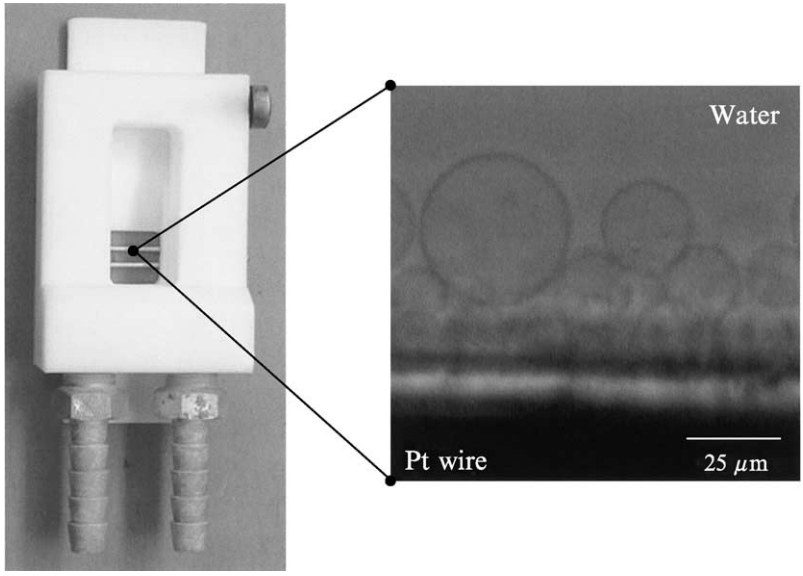

FIG. 1. Homemade chamber for GUV electroformation. The chamber is made of Teflon (inert material). The bottom of the unit has the same dimensions as a standard microscope slide. The separation between the Pt wires (diameter, 1 mm) is 3 mm (center to center) and the distance from the bottom of the chamber to the center of the Pt wires is minimal (100 μm in this case). The total cost of the equipment is relatively low, making this method efficient for generating GUVs. *Inset:* Transmission microscope image taken with a CCD color video camera (CCD-Iris; Sony) in an inverted microscope after vesicle formation.

Why Two-Photon Excitation Microscopy?

Two-photon excitation is a nonlinear process in which a fluorophore absorbs two photons simultaneously. Each photon provides half the energy required for excitation. The high photon densities required for two-photon absorption are achieved by focusing a high peak power laser light source on a diffraction-limited spot through a high numerical aperture objective.[73] Therefore, in the areas above and below the focal plane, two-photon absorption does not occur, because of insufficient photon flux. This phenomenon allows a sectioning effect without using emission pinholes as in one-photon confocal microscopy.[73]

Studies of lipid–lipid interactions in GUVs, using two-photon excitation[43–46,74] or conventional confocal[55,56] fluorescence microscopy, have been

[73] W. Denk, J. H. Strickler, and W. W. Webb, *Science* **248,** 73 (1990).

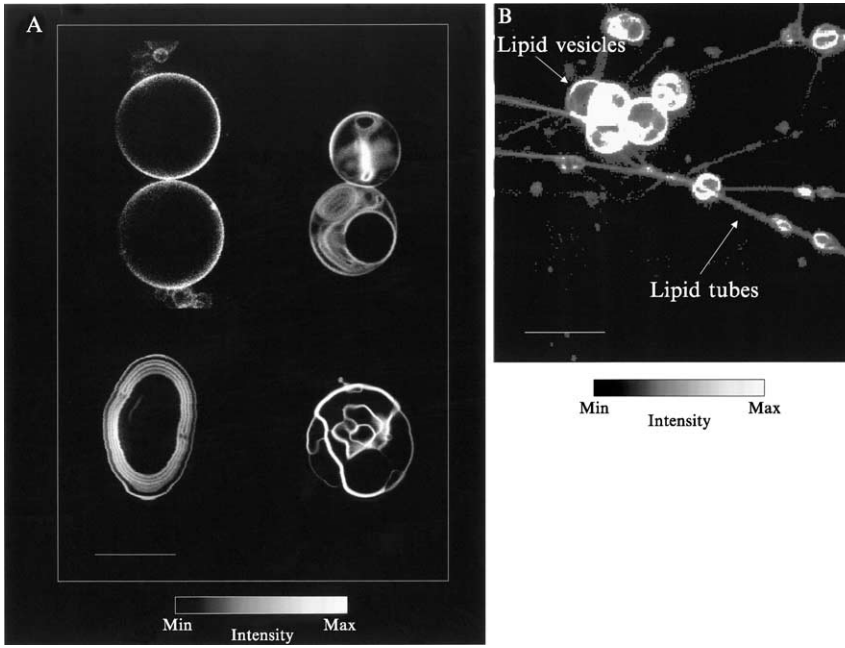

FIG. 2. (A) Fluorescence images of various lipid structures obtained by two-photon excitation microscopy. These lipid structures were obtained by different GUV formation protocols. *Top left:* Giant unilamellar vesicles obtained by the electroformation method. These vesicles are also visualized by the gentle hydration and solvent evaporation methods, but their occurrence is considerably reduced with respect to the electroformation method (see text). *Top right* and *bottom left:* Liposomes with internal structure obtained by the gentle hydration method. *Bottom right:* Liposomes with internal structure obtained by the solvent evaporation method. These structures occur frequently in samples obtained by the gentle hydration and solvent evaporation methods. (B) Two-photon excitation fluorescence image of lipid vesicles connected by lipid tubes. This image occurs frequently in samples obtained by the gentle hydration method. LAURDAN was used as the fluorescent probe. Scale bar: 20 μm.

reported. The direct visualization of lipid domain coexistence obtained by these last experimental approaches opened a fascinating new window on the topological features of the lipid bilayer when two different lipid phases coexist (see later). The advantages and disadvantages between using confocal or two-photon excitation microscopy have been well reviewed[73,75–77]; this chapter focuses instead on why two-photon excitation fluorescence

[74] K. Nag, J. S. Pao, R. R. Harbottle, F. Possmayer, N. O. Petersen, and L. A. Bagatolli, *Biophys. J.* **82,** 2041 (2002).

microscopy is used in the author's laboratory. An important advantage of two-photon excitation is the low extent of photobleaching and photodamage above and below the focal plane. This last point is crucial for experiments done with rapidly fading ultraviolet (UV) probes[78] such as 6-lauroyl-2-(*N*, *N*-dimethylamino)naphthalene (LAURDAN), 6-propionyl-2-(*N*, *N*-dimethylamino)naphthalene (PRODAN), or 1,6-diphenyl-1,3,5-hexatriene (DPH). For instance, the photobleaching effects on LAURDAN fluorescence (a favorite fluorescent molecule in the study of lipid–lipid interactions) are dramatic when using epifluorescence microscopy.[48] However, when using two-photon excitation, LAURDAN-labeled giant vesicles can be imaged for hours. The other important aspect in the author's experimental approach is the sectioning effect that allows observation of the vesicle surface without background contributions; this is essential to observe the formation of lipid domains (see later). This effect can also be accomplished by conventional confocal microscopy, using fluorescent probes that are excited in the visible wavelength range.[55,56] Another attractive advantage of two-photon excitation is that one wavelength can be used to excite many different fluorescent molecules simultaneously. Technically, this possibility can be exploited to perform multiple emission color experiments without changing the excitation wavelength.[76]

Fluorescent Probes

Several fluorescent probes have been reported to be useful in the study of the physical characteristics of lipid bilayers [see, e.g., the Molecular Probes (Eugene, OR) handbook], using mainly cuvette studies. In particular, to ascertain lipid domain coexistence in lipid bilayers or monolayers by fluorescence microscopy, fluorescent molecules that show different partition properties between the ordered and disordered phases are used.[24–27,44,45,55,56] In general, the fluorescence intensity images display the shape of the lipid domains, but additional techniques are necessary to

[75] P. T. C. So, T. French, W. M. Yu, K. M. Berland, C. Y. Dong, and E. Gratton, *in* "Fluorescence Imaging Spectroscopy and Microscopy" (X. F. Wang and B. Herman, eds.), Chemical Analysis Series, Vol. 137, p. 351. John Wiley & Sons, New York, 1996.

[76] B. R. Master, P. T. C. So, and E. Gratton, *in* "Fluorescent and Luminescent Probes," 2nd Ed., p. 414. Academic Press, New York, 1999.

[77] P. T. C. So, T. French, W. M. Yu, K. M. Berland, C. Y. Dong, and E. Gratton, *Bioimaging* **3,** 49 (1995).

[78] T. Parasassi, L. A. Bagatolli, E. Gratton, M. Levi, F. Ursini, W. Yu, and H. K. Zajicek, *in* "Confocal and Two-Photon Microscopy: Foundations, Applications and Advances" (A. Diaspro, ed.), p. 469. John Wiley & Sons, New York, 2001.

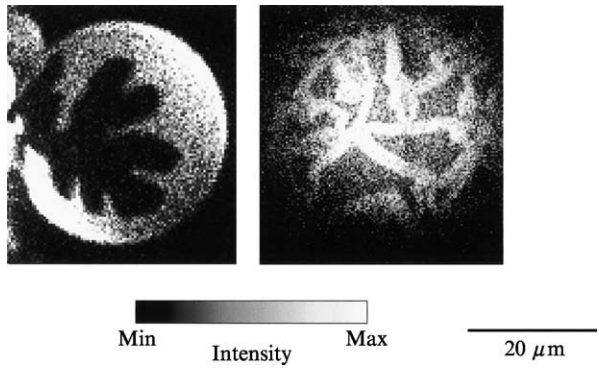

FIG. 3. Two-photon excitation fluorescence intensity images of *N*-Rh-DPPE-labeled GUVs composed of DPPE–DPPC (7:3, mol/mol) (*left*) and DLPC–DPPC (1:1, mol:mol) (*right*).

further evaluate the local physical characteristics of the observed lipid domains.[55,79]

In general, there is a tendency to classify fluorescent molecules that present different partition properties between coexisting phases [as in the case of Lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (*N*-Rh-DPPE)] as gel- or fluid-like phase probes. Figure 3 shows a comparison between *N*-Rh-DPPE–labeled GUVs composed of dilauroylphosphatidylcholine and dipalmitoylphosphatidylcholine (DLPC–DPPC) and dipalmitoylphosphatidylethanolamine (DPPE)–DPPC at the phase coexistence temperature region. Even though it is well known from the phase diagram of the phospholipid binary mixture that both samples display gel/fluid-phase coexistence, it is difficult after examination of Fig. 3 to determine which domain corresponds to the gel or fluid phase in each sample. This last finding shows clearly that it is difficult to establish directly from the GUV fluorescent image the lipid domain phase state, using fluorescent probes that show only differential partition between the coexisting phases (such as *N*-Rh-DPPE),[44,48] and that it is not wise to generalize the affinity of a fluorescent molecule for the different lipid phases without a careful probe characterization. However, it is possible to circumvent this last problem by choosing particular fluorescent probes with particular fluorescence properties, as described in the next section.

[79] R. G. Oliveira and B. Maggio, *Biochim. Biophys. Acta* **1561,** 238 (2002).

Extracting Lipid Domain Phase State Information Directly from Fluorescence Intensity Images

An easy way to extract phase state information directly from fluorescence intensity images is to take advantage of the particular fluorescent properties of LAURDAN. This molecule belongs to the family of polarity-sensitive fluorescent probes, first designed and synthesized by G. Weber for the study of dipolar relaxation of fluorophores in solvents, bound to proteins, and associated with lipids.[80–83] When inserted in lipid membranes, LAURDAN displays unique characteristics compared with other fluorescent probes,[48,84–88] namely (1) homogeneous probe distribution on lipid membranes displaying phase coexistence, (2) a phase-dependent emission spectral shift, that is, LAURDAN's emission is blue in the ordered lipid phase and greenish in the disordered lipid phase (this last effect is attributed to the reorientation of water molecules present at the lipid interface near the LAURDAN fluorescent moiety), and (3) parallel alignment of the electronic transition dipole of this molecule with the hydrophobic lipid chains in lipid vesicles.

Because LAURDAN is distributed homogeneously between the coexisting lipid phases in the membrane, the less ordered domains have a red-shifted emission compared with the more ordered domains.[43–45,48,84–88] This last fact can be observed easily by using a blue (or green) bandpass filter to obtain fluorescence intensity images at the equatorial region of the vesicle.[44–46,48] Figure 4 shows an image of LAURDAN-labeled GUVs composed of DPPE–DPPC, taken at the equatorial region of the vesicle with a blue bandpass filter. The high-intensity regions on the lipid bilayer correspond to the gel phase and LAURDAN is present in both coexisting domains. For comparison, a fluorescence image of *N*-Rh-DPPE–labeled GUVs composed of DPPE–DPPC is included in Fig. 4. Note that in this case the fluorescent molecule is excluded from the gel phase because this particular probe shows different partitioning between the coexisting phases.[44,45,48]

[80] G. Weber and F. J. Farris, *Biochemistry* **18,** 3075 (1979).
[81] R. B. MacGregor and G. Weber, *Nature* **319,** 70 (1986).
[82] T. Parasassi, F. Conti, and E. Gratton, *Cell. Mol. Biol.* **32,** 103 (1986).
[83] M. Lasagna, V. Vargas, D. M. Jameson, and J. E. Brunet, *Biochemistry* **35,** 973 (1996).
[84] T. Parasassi and E. Gratton, *J. Fluoresc.* **5,** 59 (1995).
[85] T. Parasassi, E. Krasnowska, L. A. Bagatolli, and E. Gratton, *J. Fluoresc.* **8,** 365 (1998).
[86] L. A. Bagatolli, E. Gratton, and G. D. Fidelio, *Biophys. J.* **75,** 331 (1998).
[87] T. Parasassi, G. De Stasio, G. Ravagnan, R. M. Rusch, and E. Gratton, *Biophys. J.* **60,** 179 (1991).
[88] T. Parasassi, G. De Stasio, A. d'Ubaldo, and E. Gratton, *Biophys. J.* **57,** 1179 (1990).

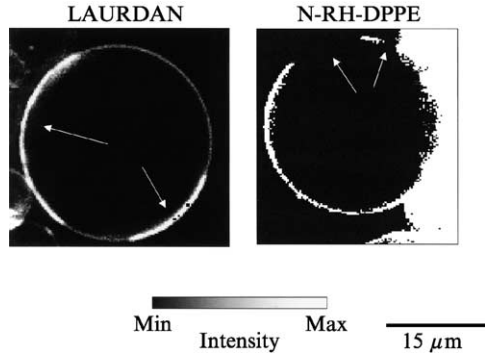

FIG. 4. Two-photon excitation fluorescence intensity images of LAURDAN- and *N*-Rh-DPPE–labeled GUVs composed of DPPE–DPPC (7:3, mol/mol) at the phase coexistence temperature region. The images have been taken at the equatorial region of the GUV. The open arrows indicate gel phase regions (see text). Note that the gel domains span the lipid bilayer. The probe concentration was below 0.25 mol%.

Photoselection Effect and LAURDAN Generalized Polarization Function

In addition, the parallel location of the electronic transition dipole of LAURDAN with respect to the lipid chains in the bilayer offers an important advantage to ascertain lipid phase coexistence from the fluorescence images, using the photoselection effect.[43–45,48,89] The photoselection effect is dictated by the fact that only those fluorophores that possess the electronic transition dipole aligned parallel, or nearly so, to the plane of polarization of the excitation light become excited. Using circular polarized excitation light (on the x–y plane) and observing the polar region of a LAURDAN-labeled GUV displaying phase coexistence, only fluorescence emanating from the fluid part of the bilayer is expected because the relatively low lipid order allows a component of the probe's electronic transition dipole to parallel the polarization plane of the excitation light. This last fact is well represented in Fig. 5, which shows fluorescence images of LAURDAN-labeled GUVs composed of DLPC–DPPC and DPPE–DPPC at the phase coexistence temperature region; the images were taken at the top part of the vesicle. The line-shaped domains in DLPC–DPPC and the leaf-shaped domains in DPPC–DPPE are gel-phase domains as judged from the LAURDAN fluorescence image (there is a strong photoselection effect in these regions).[44,45] Taking into account this last fact, *N*-Rh-DPPE partition is favored to the gel phase in DLPC–DPPC and to the fluid phase

[89] T. Parasassi, E. Gratton, W. Yu, P. Wilson, and M. Levi, *Biophys. J.* **72,** 2413 (1997).

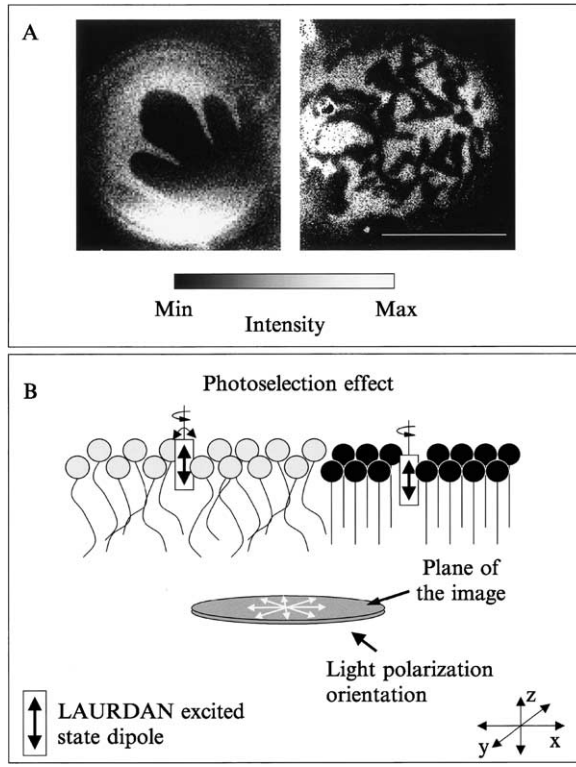

FIG. 5. (A) Two-photon excitation fluorescence intensity images of LAURDAN-labeled GUVs composed of DPPE:DPPC (7:3, mol/mol) (*left*) and DLPC:DPPC (1:1, mol/mol) (*right*). (B) Sketch of LAURDAN orientations on the lipid bilayer with respect to the polarization plane of the excitation light. The lipid molecules in the fluid and gel phases were colored gray and black, respectively, as observed in the two-photon excitation images (A). Note that the wobbling movement of LAURDAN due to the loosely packed lipid organization in the fluid phase allows excitation of a component of the probe's transition dipole. This last phenomenon is not observed in the gel phase (photoselection effect, see text).

in DPPC–DPPE (compare Figs. 3 and 5).[44,45] It is important to remark that the photoselection effect in the case of LAURDAN is evident only at the polar region of the vesicle, where the probe molecules are located parallel to the lipid chains along the z axis (the polarization plane of the excitation light is on the x–y plane).[48]

The fact that LAURDAN has different emission spectra between the coexisting lipid phases is well exploited by calculating LAURDAN generalized polarization (GP) function images.[43–48,89] The GP is a useful

relationship between the emission intensities obtained from the blue and red sides of the emission spectrum and contains information about solvent dipolar relaxation processes that occur during the time that LAURDAN is in the excited state, and is related to water penetration in phospholipid interfaces and therefore is related to the phase state of the lipid membrane.[84,85] The bluer the emission spectrum the higher the GP value. Calculations of LAURDAN GP images are confined to images obtained at the equatorial region of vesicles to avoid artifacts introduced by the photoselection effect, that is, the same excitation efficiency is expected for LAURDAN molecules at the equatorial region of GUVs displaying phase coexistence when using circular polarized excitation light because all LAURDAN molecules have the transition dipole aligned parallel to the polarization plane of the excitation light.[48] This last effect is well represented in Fig. 6, which shows a coexistence between two different fluid phases [dioleoylphosphatidylcholine (DOPC)–cholesterol–sphingomyetin (1:1:1, mol/mol)].[47] Note that the photoselection effect produces a progressive decrease in LAURDAN GP value in each different lipid phase, being stronger in the more ordered phase as the pole of the vesicle is approached

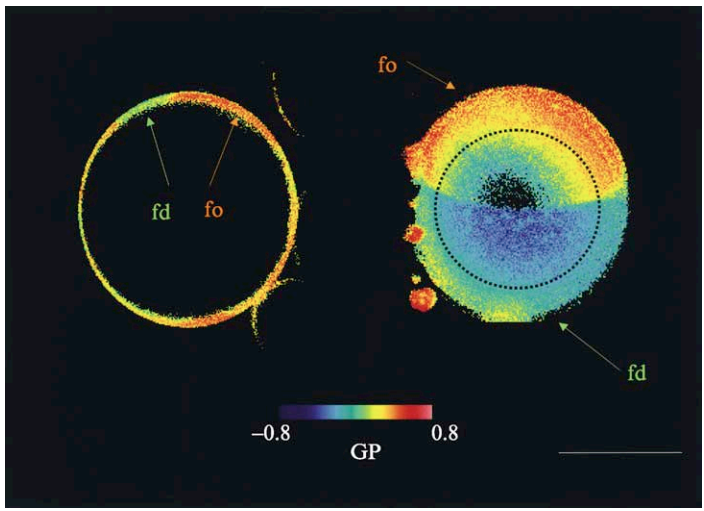

FIG. 6. Two-photon excitation GP images (false color representation) of LAURDAN-labeled GUVs composed DOPC–cholesterol–sphingomyelin (1:1:1, mol/mol) displaying fluid-ordered (fo)/fluid-disordered (fd) phase coexistence (false color representation). GP images obtained at the equatorial region of the GUV, where the photoselection effect is prevented (*left*) and at the polar region of the GUV, where the photoselection operates (*right*). Note the influence of the photoselection effect on the LAURDAN calculated values (dotted black circle) in the image obtained at the polar region of the GUV. Scale bar: 20 nm.

(Fig. 6, right, dotted circle). In addition, Fig. 6 (left) shows a GP image taken at the equatorial region of the GUV, where the photoselection effect is prevented.

LAURDAN GP images provide information about phase coexistence, even though the lipid domains are smaller than the microscope resolution, by using linearly polarized excitation light. The reader is encouraged to explore Refs. 43 and 89, which confirm the advantages of using this experimental approach in different lipid systems. To summarize this section, when the lipid domains are bigger than the microscope resolution ($\sim$0.3 μm), exciting LAURDAN with circularly polarized light allows one to extract information about the lipid domain phase state and shape by (1) the orientation of the lipids in a particular domain (polar region, photoselection effect) and (2) the extent of water dipolar relaxation processes in the different lipid domains (equatorial region, GP).[48] LAURDAN and also PRODAN[44,48,90] are unique fluorescent probes that enable the extraction of simultaneous information about lipid domain phase state and shape from fluorescence images.

Direct Visualization of Lipid Domains

The quality and novelty of the information extracted using the experimental approach described previously is remarkable. For example, one of the most relevant observations made through the direct visualization of domain coexistence in the many different mixtures (artificial or natural lipid mixtures) studies is that the lipid domains span the lipid bilayer.[44,45,47,48,55,74] Two important observations, independent of the nature of the fluorescent probe or lipid mixture studied, support the last conclusion: (1) in observing Fig. 4, for example, it is clear that both *N*-Rh-DPPE and LAURDAN—which are localized in both leaflets of the bilayer—show the spanning of the lipid domain at the equatorial region of the vesicle because *N*-Rh-DPPE is excluded from the gel phase and LAURDAN displays different emission spectra from each lipid phase; and (2) the domain spanning the lipid bilayer is extracted from fluorescence images taken at the polar region of the GUVs. Namely, the lipid domain shape does not change, regardless of the fluorescent probe used, at constant temperature for a long period of time. This last fact is well illustrated in Fig. 7, in which fluorescence images obtained at the polar region of a giant multilamellar vesicle (GMV, two bilayers in this particular case) observed as a function of time are compared with those obtained in GUVs at constant

[90] E. K. Krasnowska, L. A. Bagatolli, E. Gratton, and T. Parasassi, *Biochim. Biophys. Acta* **1511,** 330 (2001).

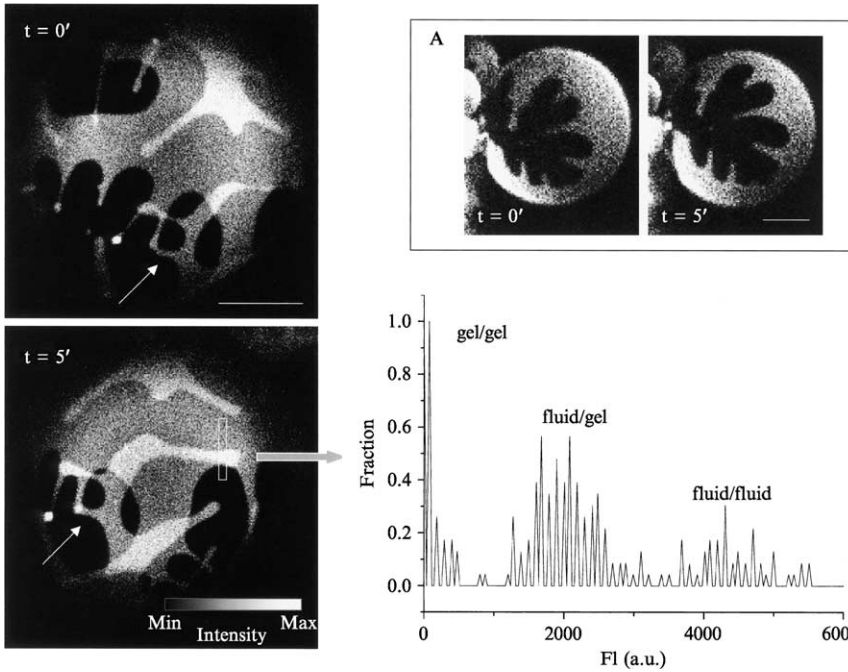

FIG. 7. Two-photon excitation fluorescence intensity images of *N*-Rh-DPPE–labeled giant multilamellar vesicle (GMV, two bilayers in this case) composed of DPPE–DPPC (7:3, mol/mol) at the phase coexistence temperature region. The images have been taken at the top part of the GMV. The open arrow in the GMV fluorescence image indicates independent movement of the two bilayers, showing the particular pattern of uncoupled lipid layers. This particular pattern is not observed in GUVs (*inset A*), showing that the gel domains in both leaflets of the bilayer are coupled. The intensity histogram obtained from the boxed region (bottom GMV image) shows three different situations: (1) overlap of two gel bilayers (black), (2) overlap of gel and fluid bilayers (gray), and (3) overlap of two fluid bilayers (intense gray). Note that the mean intensity value in the intense gray area duplicates that obtained in the gray area (see text). Scale bar: 10 μm.

temperature. As observed in Fig. 7, the fluorescence intensity where two fluid bilayer regions are superimposed is twice the value of a fluid bilayer region superimposed with a gel bilayer region. In addition, a lack of fluorescence intensity is observed only when two gel bilayer regions are superimposed. GMV images are clear examples of uncoupled lipid layers. This last is not observed in GUVs[44,45,47,48,55,74] (see inset A in Fig. 7), supporting the fact that the domain spans the lipid bilayer. Domains spanning the membrane confirm the existence of epitactic coupling in free-standing bilayers. Direct epitactic coupling has also been demonstrated in studies of

supported membranes by Merkel *et al.*,[91] who showed that laterally ordered domains in one monolayer induce solidification of domains of identical topology in the juxtaposed monolayer transferred from the fluid state. Because this phenomenon is independent of the composition[44,45,47,48,55,74] it is possible that this last feature could provide a mechanism for how events that occur in the outer monolayer of a biological membrane are coupled to cytoplasmic components of signal transduction pathways—a question that has eluded simple answers.[92]

Lipid Domain Shape Information

The phase coexistence observed at the level of single vesicles can be divided into three different groups: gel/fluid-phase coexistence,[44,45,74] fluid-ordered/fluid-disordered phase coexistence,[47,93] and a peculiar phase coexistence observed in binary mixtures composed of bipolar lipids from thermoacidophilic archaebacteria, for example, *Sulfolobus acidocaldarius*.[46]

The main differences observed between gel/fluid and fluid-ordered/ fluid-disordered phase coexistence is related to the domain shape and the behavior of LAURDAN[48] (Fig. 8). Two general trends related to the shape of lipid domains and LAURDAN fluorescence properties are observed in samples displaying gel/fluid-phase coexistence, namely, gel domains with irregular shape and a lack of fluorescence intensity due to strong effect of photoselection (Fig. 8A). In addition to well-characterized phospholipid binary mixtures, which display gel/fluid-phase coexistence,[44,45] natural samples such as bovine lipid extract surfactant[74] or brush border membrane lipid extracts after cholesterol extraction[47] can be included in this group even though these last samples display a heterogeneous lipid composition (see Fig. 8A). Also, important subtle differences among all these mixtures can be extracted on the basis of LAURDAN GP images, as described in the next section.

On the other hand, a common characteristic of cholesterol-containing mixtures (natural and artificial lipid mixtures)[47] is the circular shape of the domain at the phase coexistence temperature region, something that is not observed in samples displaying gel/fluid-phase coexistence (compare Fig. 8A and B). 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 optimizing the area-to-perimeter ratio.[47] Moreover, the strong photoselection effect observed in the gel domains is not observed in the fluid-ordered domains in GUVs composed of

[91] R. Merkel, E. Sackmann, and E. Evans, *J. Phys. France* **50,** 1535 (1989).
[92] D. A. Brown and E. London, *Annu. Rev. Cell Dev. Biol.* **14,** 111 (1998).
[93] S. L. Veatch, and S. L. Keller, *Phys. Rev. Lett.* **89**, 268101 (2002).

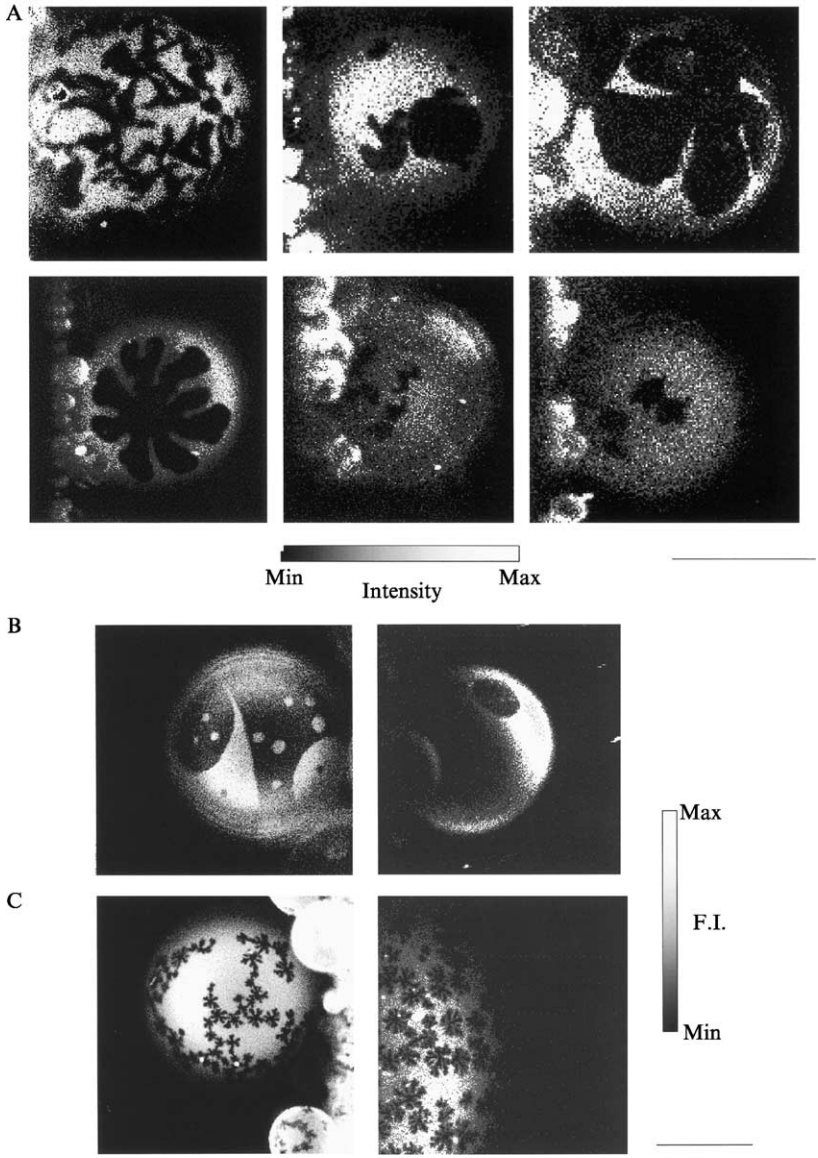

FIG. 8. Two-photon excitation fluorescence intensity images of LAURDAN-labeled GUVs at the phase coexistence temperature region. The images have been taken at the equatorial region of the GUV. (A) Mixtures displaying gel/fluid phase coexistence: DLPC/DPPC, DLPC/DSPC, and DLPC/DAPC (1:1, mol:mol) (*top*, from left to right); DMPC/DMPE (1:1, mol/mol), cholesterol-depleted brush border membrane lipid extract and bovine

DOPC–cholesterol–sphingomyelin mixtures, indicating that the fluorescent probe lies in an ordered but fluid environment (see Fig. 8B, left image).

A particular phase coexistence was observed in a binary mixture composed of bipolar lipids extracted from *S. acidocaldarius* [polar lipid fraction E (PLFE) lipids].[46] The reason this particular phase coexistence is classified in a third group is mainly because the PLFE GUV membrane is a monolayer. In this case lipid domains having a ''snowflake'' shape are detected (see Fig. 8C). The LAURDAN location in these membranes is also peculiar, that is, LAURDAN has the transition dipole aligned parallel to the vesicle surface and the chromopore moiety lies at the level of the polar head groups.[46] Following the LAURDAN GP a phase transition related to the reorientation of the polar head groups was detected at 50° in this mixture. However, the appearance of phase coexistence was detected around 20°, with LAURDAN being excluded from the snowflake domains. These results suggest the possibility of lipid lateral segregation in the presence of rare lipid species and open new questions about the biological relevance of such phenomena in complex lipid systems.

LAURDAN GP Can Be Related to Compositional Differences between Coexisting Lipid Domains

In addition to domain shape information and qualitatively inferences about the lipid domain phase state, using the photoselection effect, LAURDAN GP images can offer important subtle information about particular characteristics of the coexisting phases. An interesting example is that reported for binary phospholipid mixtures that contain DLPC.[45] In this experiment the hydrophobic mismatch of the phospholipid binary mixtures was increased by using DPPC, distearsylphosphatidylcholine (DSPC), and diarachidonylphosphatidylcholine (DAPC) as the second lipid component. The first comparison among these mixtures reveals that the lipid domain shape changes as the hydrophobic mismatch of the binary mixtures increases. This last conclusion is accompanied by additional qualitative information extracted from the LAURDAN intensity images taken at the polar region of the GUVs (where the photoselection effect operates), that is, gel and fluid domains coexist in the lipid bilayer. An important question to be answered is whether the general characteristics of the phase coexistence picture obtained present any correspondence among the different lipid mixtures.

lipid extract surfactant (*bottom*, from left to right). (B) Mixtures that display fluid-ordered/fluid-disordered phase coexistence: DOPC–cholesterol–sphingomyelin (1:1:1, mol/mol) with 1 mol% of ganglioside G_{M1} (*left*) and brush border membrane lipid extract (*right*). (C) PLFE lipids displaying phase coexistence. The probe concentration was below 0.25 mol%. Scale bar: 30 micrometers.

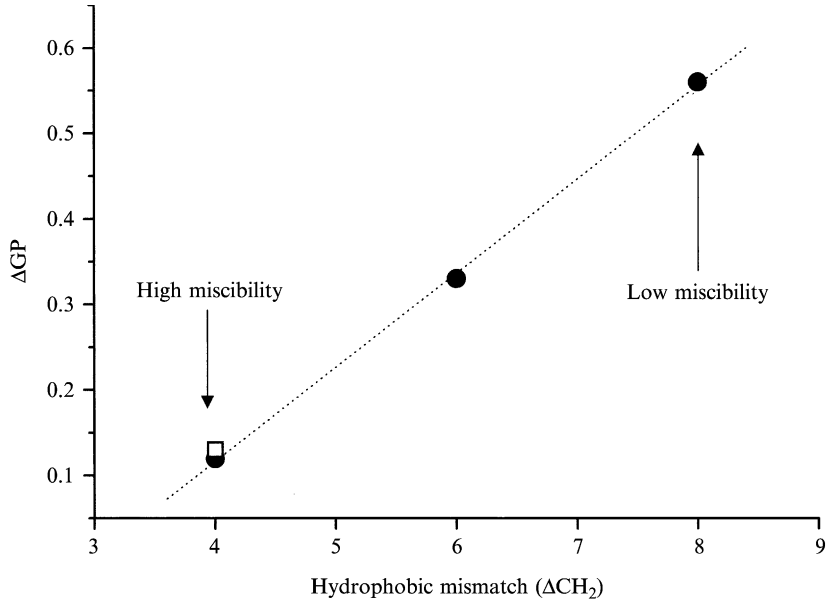

FIG. 9. LAURDAN GP differences between the fluid- and gel-phase domains (ΔGP) plotted versus the hydrophobic mismatch between the components of the binary mixtures. (●) DLPC-containing GUVs [DLPC–DPPC, DLPC–DSPC, and DLPC–DAPC (1:1, mol/mol), hydrophobic mismatch equal to 4, 6, and 8 respectively]; (□) DMPC–DSPC mixture (1:1, mol/mol; hydrophobic mismatch equal to 4). The ΔGP between the gel and fluid components for DMPE–DMPC and DPPE–DPPC (different polar head group/same lipid chain length) was similar to that observed for DLPC–DAPC, showing similar leaf shape (see Fig. 8A).

Subtle differences in phase coexistence among the different mixtures can be obtained from GP images. For example, differences in GP values between the gel and fluid regions of the lipid bilayer (ΔGP) plotted versus the hydrophobic mismatch (ΔCH_2) of DLPC-containing binary phospholipid mixtures shows a linear relationship, that is, the difference in the extent of water dipolar relaxation between the gel and fluid domains increases as the hydrophobic mismatch increases (see Fig. 9).[45] This last result is explained by taking into account the progressive increment in the compositional and energetic differences between the gel and fluid domains as the miscibility between the binary phospholipid mixture components decreases.[48] As shown in Fig. 9, similar ΔGP values are obtained for dimyristoylphosphatdylcholine (DMPC)–DSPC and DLPC–DPPC, in agreement with the fact that these mixtures have the same hydrophobic mismatch. Other samples that display low miscibility, such as DPPC–DPPE and DMPC–DMPE, display a ΔGP similar to that observed for

DLPC–DAPC. Interestingly, these low-miscibility phospholipid mixtures also present a similar shape for the gel domain (leaf shape).[45]

Conclusions

A new experimental approach to study lipid–lipid interactions has been presented in this chapter. The enormous advantages in using the fluorescent probe LAURDAN as a tool to study lipid phase coexistence is noteworthy. Important novel information such as lipid domains spanning the lipid bilayer, correlation of domain shape with lipid mixture composition, and comparisons of lipid domain compositional and energetic differences among different phospholipid mixtures is extracted directly from fluorescence images. The experiments at the level of single vesicles undoubtedly open new ways to study the thermotropic behavior of lipid bilayers, including the possibility to explore lipid–protein interactions.[51,94]

Acknowledgments

This research was supported by Fundacion Antorchas (Argentina), Beca de Investigación Carrillo-Oñativia, Ministerio de Salud de la Nación (Argentina), and RR03155 (which supports the Laboratory for Fluorescence Dynamics). The author thanks Dr. E. Gratton for stimulating opinions about the experimental results and for support during the author's postdoctoral research in his laboratory, and Dr. D. M. Jameson for critical reading of the manuscript. MEMPHYS-Center for Biomembrane Physics is supported by the Danish National Research Foundation.

[94] L. A. Bagatolli, S. Sanchez, T. Hazlett, and E. Gratton, *Methods Enzymol.* **360,** 481 (2003).

[16] Determination of Liposome Surface Dielectric Constant and Hydrophobicity

By S. OHKI and K. ARNOLD

Introduction

Phospholipids of biological origin usually form stable bilayer membranes in aqueous solutions, exposing their polar groups to the surrounding aqueous phases and enclosing their nonpolar hydrocarbon tails in the interior of the membrane.[1] When such bilayers are in an aqueous solution, they

[1] R. Harrison and G. G. Lunt, "Biological Membranes." Blackie, London, 1980.

0076-6879/03 $35.00