Visualization of lipid domains in giant unilamellar vesicles using an environment-sensitive membrane probe based on 3-hydroxyflavone

Andrey S. Klymchenko [a,*], Sule Oncul [a], Pascal Didier [a], Emmanuel Schaub [a], Luis Bagatolli [b], Guy Duportail [a], Yves Mély [a]

[a] Photophysique des Interactions Biomoléculaires, UMR 7175 du CNRS, Institut Gilbert Laustriat, Faculté de Pharmacie, Université Louis Pasteur, 67401 Illkirch, France
[b] Membrane Biophysics and Biophotonics group/MEMPHYS – Center for Biomembrane Physics, Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55 DK-5230 Odense M, Denmark

ARTICLE INFO

ABSTRACT

We characterized the recently introduced environment-sensitive fluorescent membrane probe based on 3-hydroxyflavone, F2N12S, in model lipid membranes displaying liquid disordered (Ld) phase, liquid ordered (Lo) phase, or their coexistence. Steady-state fluorescence studies in large unilamellar vesicles show that the probe dual emission drastically changes with the lipid bilayer phase, which can be correlated with the difference in their hydration. Using two-photon excitation microscopy on giant unilamellar vesicles, the F2N12S probe was found to bind both Ld and Lo phases, allowing visualization of the individual phases from the fluorescence intensity ratio of its two emission bands. By using a linearly polarized excitation light, a strong photoselection was observed for F2N12S in the Lo phase, indicating that its fluorophore is nearly parallel to the lipid chains of the bilayer. In contrast, the absence of the photoselection with the Ld phase indicated no predominant orientation of the probe in the Ld phase. Comparison of the present results with those reported previously for F2N12S in living cells suggests a high content of the Lo phase in the outer leaflet of the cell plasma membranes. Taking into account the high selectivity of F2N12S for the cell plasma membranes and its suitability for both single- and two-photon excitation, applications of this probe to study membrane lateral heterogeneity in biological membranes are foreseen.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Laterally ordered microdomains or "rafts" have received a large attention since they are believed to be involved in the regulation of various cell functions such as signal transduction, lipid trafficking and membrane protein activity [1–4]. These domains, defined as liquid-ordered phase (Lo) enriched in cholesterol, sphingomyelin and glycosphingolipids, can be observed in model membranes as "rafts" floating in a sea of loosely-packed domains enriched in unsaturated phospholipids, i.e. liquid disordered (fluid) phase (L_d) [5–8].

Visualization of lipid domains still remains a challenge especially in cellular membranes. For this purpose, several fluorescent probes have been recently developed to visualize lipid domains by fluorescence microscopy [9]. The most common one is the fluorescently labeled protein CT-B (from bacterium *Vibrio Cholerae*), which binds selectively to the ganglioside G_{M1} component associated with rafts in biomembranes [10]. On the other hand, most molecular membrane probes (lipid-like probes) are not suited for raft visualization in model systems (or cell membranes) since they are excluded from Lo phases [11]. Remarkable exceptions to this rule are provided by probes with long alkyl chains, such as L*c*TMA-DPH or diI-C20, or polycyclic probes, such as Terrylene or Naphtopyrene, which show high affinity for ordered phases in model membrane systems [11–14]. However, these probes suffer from the dependence of their partition on the lipid composition of the actual lipid domain [15]. Environment-sensitive molecular probes, such as Laurdan [15–17] and its derivatives [18,19] or di-4-ANEPPDHQ [20] constitute improved alternatives for raft visualization in model systems and cells. These probes distribute in both Ld and Lo phases of membranes and their emission color and/or lifetime depends strongly on the local polarity, that in turn is related to the actual phase state of the membrane. This property allows direct visualization of membrane lateral heterogeneities in model and native biological membranes. The search for new environment-sensitive membrane probes remains an important issue in the development of raft imaging tools for cellular applications.

Recently, we have introduced membrane probes based on 3-hydroxyflavone, which due to their excited-state intramolecular proton transfer (ESIPT) reaction exhibit a dual emission highly sensitive to the environment [21–23]. One of these probes, F2N8, being sensitive to membrane hydration [24], could clearly distinguish
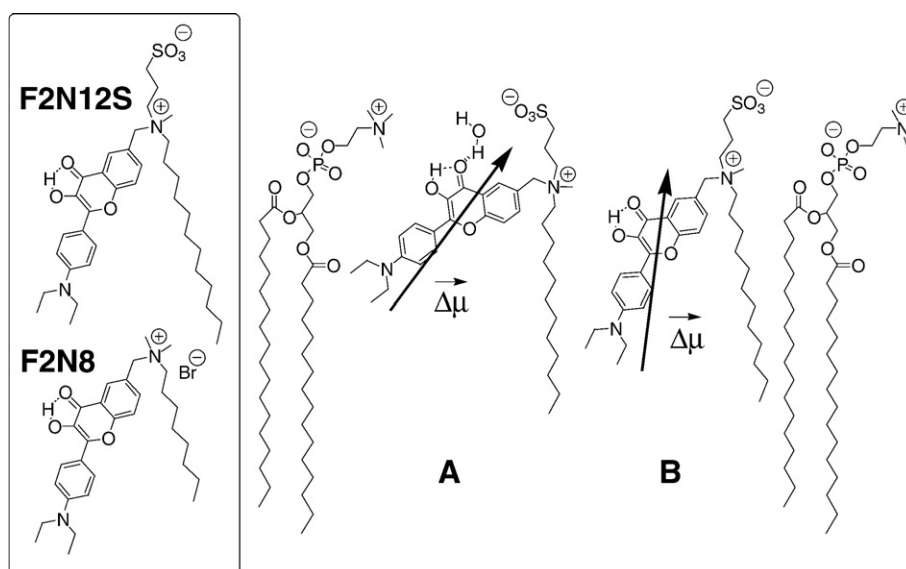
Fig. 1. Chemical structures of probes F2N12S and F2N8 and the two expected ground state forms of probe F2N12S in lipid bilayers (schematically presented as a monolayer): H-bonded with water (A) and H-bond free (B). Arrows show approximate orientation of the transition dipole moment ($\Delta\mu$) of the fluorophore.

Lo phase from Ld phase in large unilamellar vesicles (LUVs) by a dramatic variation in the ratio of its two emission bands [25]. However, fluorescence microscopy studies in giant unilamellar vesicles (GUVs) exhibiting both Lo and Ld phases, show that F2N8 partitions exclusively into the Ld phase (data not shown), and thus, cannot be applied for lipid domain imaging. In the present project, we selected the probe F2N12S (Fig. 1), a close analog of F2N8, characterized by a longer alkyl chain and a zwitterionic anchoring group. Unlike F2N8, F2N12S showed high selectivity to cell plasma membrane and was already applied for detection of apoptosis [26]. We observe that F2N12S binds both Ld and Lo phases in model systems, allowing efficient visualization of these phases by fluorescence ratiometric imaging.

2. Materials and methods

Dioleoylphosphatidylcholine (DOPC) and cholesterol were purchased from Sigma-Aldrich (Lyon, France). Bovine brain sphingomyelin was from Avanti Polar Lipids (Alabaster, USA). N-[[4′-N,N-diethylamino-3-hydroxy-6-flavonyl]-methyl]-N-methyl-N-(3-sulfopropyl)-1-dodecanaminium, inner salt (F2N12S) was synthesized as described [26]. This probe was pure according to thin layer chromatography, ^{1}H-NMR data, absorption and fluorescence spectra in organic solvents.

Large unilamellar vesicles (LUVs) were obtained by the classical extrusion method as previously described [27]. Briefly, a suspension of multilamellar vesicles was extruded by using a Lipex Biomembranes extruder (Vancouver, Canada). The size of the filters was first 0.2 μm (7 passages) and thereafter 0.1 μm (10 passages). This generates monodisperse LUVs with a mean diameter of 0.11 μm as measured with a Malvern Zetamaster 300 (Malvern, UK). LUVs were labeled by adding aliquots (generally 2 μl) of probe stock solutions in dimethyl sulfoxide to 2-ml solutions of vesicles. Since the binding kinetics is very rapid for probe F2N12S, the fluorescence experiments were performed a few minutes after addition of the aliquot. A 15 mM phosphate-citrate, pH 7.0 buffer was used in these experiments. Concentrations of the probes and lipids were 2 and 200 μM, respectively. Giant unilamellar vesicles (GUVs) were generated by electroformation in a home-built liquid cell (University of Odense, Denmark), using previously described procedures [28–30]. A 0.1 mM solution of lipids in chloroform was deposited on the platinum wires of the chamber and the solvent was evaporated under vacuum for 30 min. The chamber, thermostated at 55 °C, was filled with a 300 mM

sucrose solution, and a 2-V, 10-Hz alternating electric current was applied to this capacitor-like configuration for ca 2 h. Then, 50 ml aliquot of the obtained stock solution of GUVs in sucrose (cooled down to room temperature) was added to 200 ml of 300 mM glucose solution to give the final suspension of GUVs used in microscopy experiments. The staining of GUVs was performed by addition of an aliquot of the probe stock solution in DMSO to obtain a 0.1 μM final probe concentration (final DMSO volume < 0.25%).

Fluorescence spectra were recorded on a Jobin-Yvon Fluoromax 3 (Longjumeau, France) spectrofluorometer and corrected by subtracting the spectra of the corresponding non-labeled vesicles. The excitation wavelength of F2N12S was 400 nm. Fluorescence microscopy experiments were performed by using a home-built two-photon laser scanning set-up based on an Olympus IX70 inverted microscope with an Olympus 60× 1.2NA water immersion objective [31,32]. Two-photon excitation was provided by a titanium-sapphire laser (Tsunami, Spectra Physics) and photons were detected with Avalanche Photodiodes (APD SPCM-AQR-14-FC, Perkin Elmer) connected to a counter/timer PCI board (PCI6602, National Instrument). Imaging was carried out using two fast galvo mirrors in the descanned fluorescence collection mode. Typical acquisition time was 5 s with an
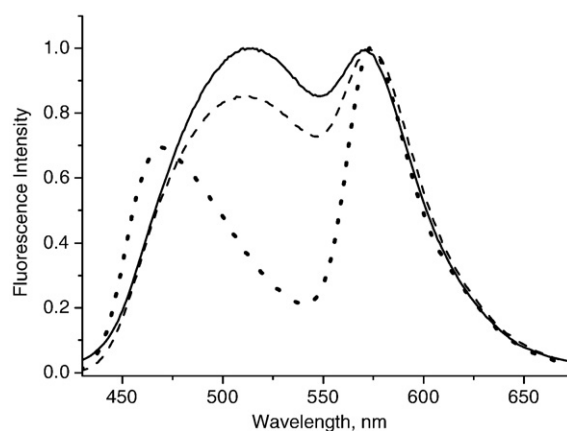

Fig. 2. Fluorescence spectra of F2N12S in LUVs composed of DOPC (solid curve), DOPC +35% Chol (dashed curve) and SM+35% Chol (dotted curve). Concentrations of lipids and probes were 200 μM, and 2 μM, respectively. Excitation wavelength was 400 nm.
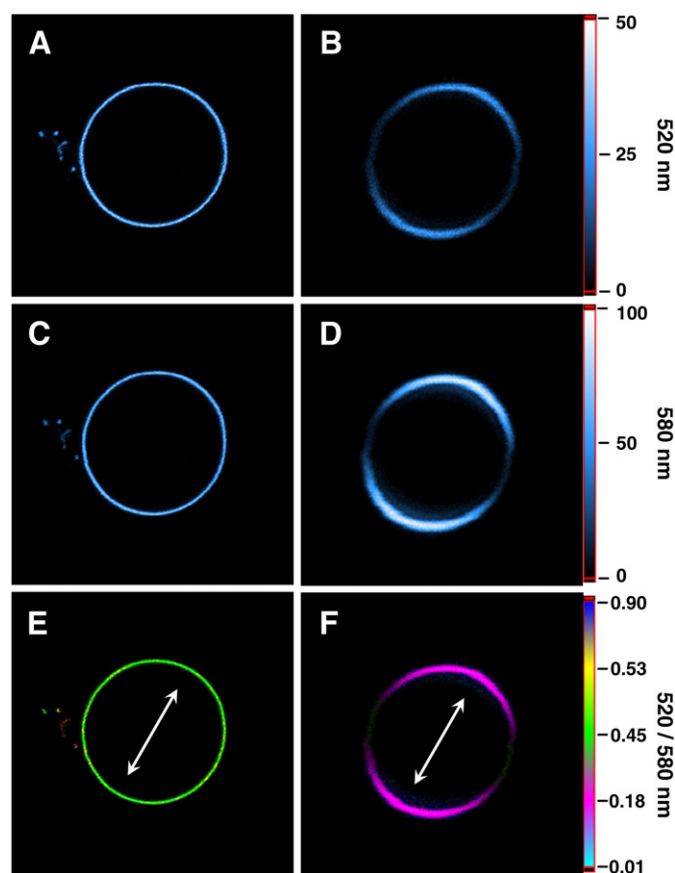
Fig. 3. Fluorescence intensity (A–D) and ratiometric (E, F) images of GUVs composed of DOPC (A, C, E) and SM+35% Chol (B, D, F). Fluorescence intensity images were recorded using 520 nm-filter (A and B) and 580 nm-filter (C, D). In the ratiometric images (E, F) the color of the pixel represents the value of the intensity ratio 520/580 nm, while the pixel intensity corresponds to the total number of photons collected at both 520 and 580 nm channels. Two-photon excitation wavelength was at 830 nm. Arrows indicate the orientation of the light polarization. Sizes of the images were 50×50 μm (A, C, E) and 15×15 μm (B, D, F). Probe concentration was 0.1 μM.

excitation power around 2.5 mW (λ=830 nm) at the sample level. Images corresponding to the short-wavelength and the long-wavelength emission bands were recorded simultaneously using a dichroic mirror (Beamsplitter 550 DCXR) and two band-pass filters (Brightline HC 520/20 and HQ 585/40) in front of the APDs. The images were processed with a home-made program under LabView that generates a ratiometric image by dividing the image of the short-wavelength channel by that of the long-wavelength channel. For each pixel, a pseudo-color scale was used for coding the ratio, while the pixel intensity was defined by the integral intensity recorded for both channels at the corresponding pixel.

3. Results and discussion

On binding to LUVs, probe F2N12S shows more than 100-fold increase in the fluorescence intensity, so that the recorded fluorescence spectra correspond exclusively to the emission of the probe bound to the vesicles. Fluorescence spectrum of F2N12S in DOPC LUVs is characterized by a broad short-wavelength band and a narrow long-wavelength band (Fig. 2) which, according to our previous studies of 3-hydroxyflavone dyes in lipid vesicles [24], can be assigned to the emission of the normal N* (H-bonded and H-bond free) and tautomer (T*, ESIPT product) emissive species, respectively. This spectrum is very similar to that observed for its F2N8 analog [24], indicating that

these two probes display a similar location in the lipid bilayer, as expected from their high structural similarity (Fig. 1) Therefore, in analogy with F2N8 [24], the two ground states of the F2N12S fluorophore (A and B, Fig. 1) are thought to be located in the region of the ester groups of the lipids with slightly different positions and orientations. Indeed, the H-bonded form (with water molecule) exhibits probably a rather shallow location with a tilted orientation in respect with the bilayer (A) while the H-bond free form is likely deeper with a more vertical orientation (B).

In DOPC vesicles containing 35% of cholesterol (DOPC/Chol), representing bilayers in a Ld phase, the short-wavelength band slightly decreases (Fig. 2), indicating a slight decrease in both polarity and hydration of the lipid bilayer [24,25]. Importantly, in LUVs composed of sphingomyelin+35% cholesterol (SM/Chol), where a homogeneous Lo phase is expected, F2N12S shows a dual emission differing from that in the Ld phase by a large blue-shift of the short-wavelength band and a strong decrease of its relative intensity (Fig. 2). According to our previous studies with probe F2N8, the strong differences in the spectra of F2N12S in the two phases indicate a significantly decreased hydration of the Lo phase as compared to the Ld phase [25]. These large differences in the spectra prompted us to apply the F2N12S probe to visualize lipid rafts in giant unilamellar vesicles (GUVs).

We first studied GUVs composed either of DOPC or SM/Chol, representing pure Ld and Lo phases, respectively. The images were obtained using a linearly polarized IR femtosecond laser source to perform two-photon excitation of the probe. Two images were recorded simultaneously at 520 and 580 nm, the two emission maxima of the probe in DOPC vesicles. In our conditions (2.5 mW excitation power at 830 nm, with 10–50 μs integration time per pixel), the signal/noise ratio (>50) allows reliable ratiometric imaging. Moreover, images could be recorded for minutes without significant loss of fluorescence intensity, indicating a moderate photo-bleaching of F2N12S with two photon excitation. The present results are the first demonstration of the suitability of a 3-hydroxyflavone-based dye (F2N12S) for two-photon excitation microscopy. From the images at two different wavelengths (Fig. 3A–D), a ratiometric images were obtained, where the color of each pixel was associated with the I_{520}/I_{580} intensity ratio, while its intensity was associated with the number of counted photons (see Materials and methods). The intensity ratio coded in color is homogeneously distributed in both types of vesicles (Fig. 3E, F). In full line with the spectroscopic data (Fig. 2), the I_{520}/I_{580} ratio is more than two-fold larger in DOPC GUVs than in SM/Chol GUVs.

While the fluorescence intensity of F2N12S is homogeneously distributed all over the DOPC GUVs (Fig. 3A, C, E), it depends strongly on the orientation of the bilayer plane with respect to the polarization plane of the excitation light in GUVs composed of SM/Chol (Fig. 3B, D, F). The fluorescence intensity is the highest when the light polarization is perpendicular to the lipid bilayer plane, while it is almost negligible when the light polarization is parallel to the bilayer plane. Similar effects of the light polarization on the fluorescence intensity of membrane probes were already reported for other environment-sensitive probes, Prodan and Laurdan [15]. These two probes exhibit a relatively large wobbling freedom in DOPC vesicles, due to the loose lipid packing of the fluid (Ld) phase, and, therefore, almost no preferential orientation of the probe was observed. In this case, the polarization direction of the excitation light has little influence on the fluorescence intensity of the probe. In sharp contrast, a liquid ordered or gel phase imposes a nearly vertical orientation of Laurdan and Prodan (and thus of their transition moments) with respect to the plane of the lipid bilayer, giving a characteristic intensity distribution close to that observed in Fig. 3 (B, D, C, F) [15]. Similarly to Prodan, the transition dipole moment in F2N12S is nearly parallel to the fluorophore (Fig. 1), due to the electron transfer from the dialkylamino group to the carbonyl group [33,34]. In DOPC vesicles, the fluorophore displays both tilted (H-bonded, A) and nearly vertical (H-bond free, B)
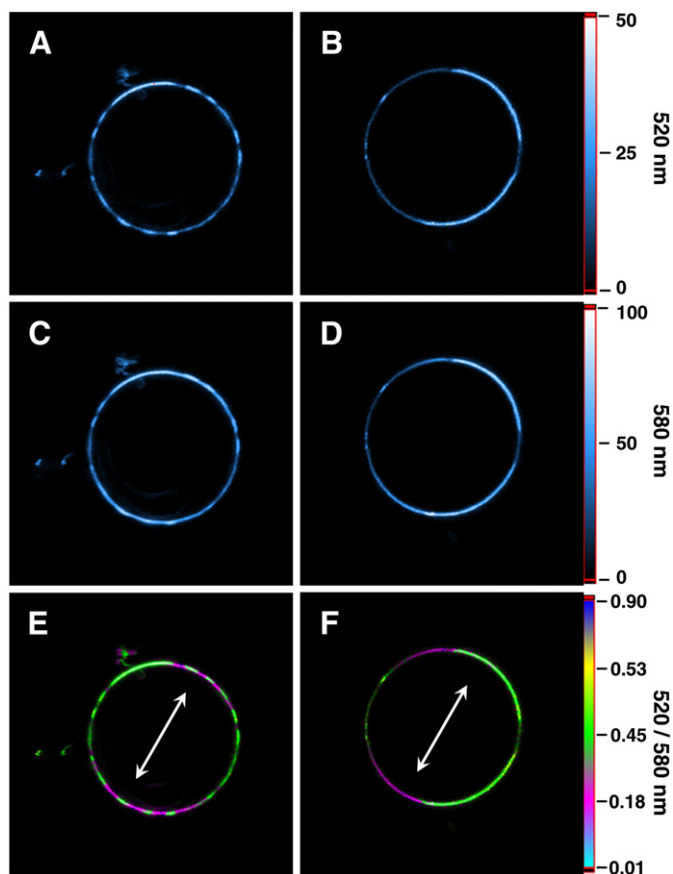
Fig. 4. Fluorescence intensity (A–D) and ratiometric (E, F) images of GUVs composed of DOPC/SM/Chol in the molar ratios 1/1/0.7. Two different individual GUVs are presented showing small (A, C, E) and large (B, D, E) lipid domains. Experimental conditions are as in Fig. 3. Arrows indicate the orientation of the light polarization. Size of the images was 45×45 μm.

strong dependence of the fluorescence intensity on the direction of the polarization of the excitation light, in contrast to the regions of high I_{520}/I_{580} ratios, where the light polarization effects are much less pronounced (Fig. 4). The differences in the fluorescence intensity between the two phases (Fig. 4A–D) reflect also the higher affinity of the probe for the Ld phase as compared to the Lo phase. The preference for the Ld phase is a common property of most fluorescent dyes, since this phase, being less densely packed, exhibits a larger number of binding sites for guest molecules. Nevertheless, in contrast to F2N8 that binds exclusively to the Ld phase (data not shown), the partitioning of F2N12S into both Lo and Ld phases is sufficient for their successful visualization by ratiometric imaging. The significantly higher affinity of F2N12S compared to F2N8 towards Lo phase can be explained by its longer saturated alkyl chain (dodecyl vs. octyl) and the neutral (zwitterionic) nature of the anchor group. Thus, probe F2N12S, being partitioned in both Ld and Lo phases, allows their direct recognition by means of its intensity ratio signal. The ratiometric response of the probe is especially attractive for imaging applications in model and cellular membranes, because unlike the intensity-based imaging, the ratio-metric imaging is not affected by inhomogeneous probe distribution, photobleaching and power of the excitation light source [35].

The present results shed light on our previous data obtained with probe F2N12S in living cells, where it was shown to bind selectively the outer leaflet of the plasma membrane [26]. The fluorescence spectrum of probe F2N12S in CEM cells showed a remarkable separation of the two emission bands (see Fig. 2 in ref [26]), indicating a relatively low hydration of the probe surrounding. Thus, the position of the short-wavelength band of F2N12S at 483 nm in cells is closer to that in the Lo phase (LUVs made of SM/Chol, 470 nm) than in the Ld phase (LUVs made of DOPC and DOPC/Chol, 511 and 513 nm, respectively). This suggests that the Lo phase may constitute a large fraction of the outer leaflet of the cell plasma membrane, in line with the high sphingomyelin and cholesterol content reported for this leaflet in normal eukaryotic cells [36]. However, the quantitative description of the fraction of Lo phase in the outer leaflet is not possible at this step since the influence of membrane proteins on the probe response was not accounted in these experiments. Further cellular studies using F2N12S are needed to better understand the distribution of the Lo phase in the cell membranes.

4. Conclusions

The recently developed membrane probe F2N12S exhibits strong differences in its dual emission in liquid ordered and liquid disordered phases. Two-photon fluorescence microscopy studies in GUVs show that the probe partitions in both phases and allows distinguishing them using ratiometric imaging. Moreover, the effects of light polarization suggest that in the liquid ordered phase, the fluorophore displays a nearly parallel orientation with respect to the lipid chains of the membrane, while in the fluid phase no predominant orientation is observed. Thus, F2N12S probe, being a selective fluorescent marker of the cell plasma membrane [26], appears promising for investigating lipid rafts in living cells by ratiometric imaging using either single- or two-photon excitation. Cellular studies with F2N12S probe are currently in progress and will be reported in due course.

orientations, explaining its poor sensitivity to the light polarization. In contrast, in vesicles composed of SM/Chol, representing Lo phase, the H-bond free form with a vertical orientation largely prevails (Fig. 1B). Moreover, in Lo phase characterized by high order and rigidity, a minimal wobbling motion of the probe in the ground state is expected. Therefore, in the case of Lo phase the vertical orientation of the F2N12S fluorophore (particularly its transition dipole, Fig. 1B) in the lipid bilayers should be rather uniform, so that the probe is efficiently excited only when the light polarization is perpendicular to the bilayer plane. Importantly, the observed color (i.e. the I_{520}/I_{580} ratio) of the GUV membrane in Lo phase is independent from the strong variation of the intensity (see Fig. 3F, the only exceptions are the small regions of the membrane parallel to the light polarization, where the intensity is close to the background), thus, showing a key advantage of the ratiometric signal of the probe to identify the membrane phase.

In a second set of experiments, we imaged GUVs composed of a ternary mixture of DOPC/SM/Chol with molar ratios 1/1/0.7. The intensity-based images and the ratiometric images are clearly hetero-geneous, showing domains with different intensities and I_{520}/I_{580} ratios (Fig. 4). By comparison with the ratiometric images in Fig. 3E, F, Ld phase regions corresponding to high I_{520}/I_{580} ratio (green) can be easily distinguished from Lo phase regions, characterized by low I_{520}/I_{580} ratio (pink). According to the images, the I_{520}/I_{580} ratio in Ld phase regions is 2–3 times as large compared to Lo phase regions, which is in line with the 2.8 times change of the I_{520}/I_{580} ratio (DOPC/Chol vs SM/Chol) calculated from the corresponding fluorescence spectra (Fig. 2). Moreover, as expected from the different orientations of the probe in the two phases, the regions displaying low I_{520}/I_{580} ratios also show a

References

[1] K.A. Field, D. Holowka, B. Baird, Compartmentalized activation of the high affinity immunoglobulin E receptor within membrane domains, J. Biol. Chem. 272 (1997) 4276–4280.

[2] D.A. Brown, E. London, Functions of lipid rafts in biological membranes, Ann. Rev. Cell Dev. Bio. 14 (1998) 111–136.

[3] D.A. Brown, E. London, Structure and origin of ordered lipid domains in biological membranes, J. Membr. Biol. 164 (1998) 103–114.

[4] K. Simons, E. Ikonen, How cells handle cholesterol, Science 290 (2000) 1721–1726.

[5] C. Dietrich, L.A. Bagatolli, Z.N. Volovyk, N.L. Thompson, M. Levi, K. Jacobson, E. Gratton, Lipid rafts reconstituted in model membranes, Biophys. J. 80 (2001) 1417–1428.

[6] S.L. Veatch, S.L. Keller, Organization in lipid membranes containing cholesterol, Phys. Rev. Lett. 89 (2002) 268101.

[7] S.L. Veatch, S.L. Keller, Separation of liquid phases in giant vesicles of ternary mixtures of phospholipids and cholesterol, Biophys. J. 85 (2003) 3074–3083.

[8] T. Baumgart, S.T. Hess, W.W. Webb, Imaging coexisting fluid domains in biomembrane models coupling curvature and line tension, Nature 425 (2003) 821–824.

[9] D.M. Owen, M.A.A. Neil, P.M.W. French, A.I. Magee, Optical techniques for imaging membrane lipid microdomains in living cells, Semin. Cell Dev. Biol. 18 (2007) 591–598.

[10] P.W. Janes, S.C. Ley, A.I. Magee, Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor, J. Cell. Biol. 147 (1999) 447–461.

[11] T. Baumgart, G. Hunt, E.R. Farkas, W.W. Webb, G.W. Feigenson, Fluorescence probe partitioning between Lo/Ld phases in lipid membranes, Biochim. Biophys. Acta 1768 (2007) 2182–2194.

[12] A. Beck, D. Heissler, G. Duportail, Influence of the length of the spacer on the partitioning properties of amphiphilic fluorescent membrane probes, Chem. Phys. Lipids 66 (1993) 135–142.

[13] X. Xu, R. Bittman, G. Duportail, D. Heissler, C. Vilchéze, E. London, Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts). Comparison of cholesterol to plant, fungal, and disease-associated sterols and comparison of sphingomyelin, cerebrosides, and ceramide, J. Biol. Chem. 276 (2001) 33540–33546.

[14] J. Korlach, P. Schwille, W.W. Webb, G.W. Feigenson, Characterization of lipid bilayer phases by confocal microscopy and fluorescence correlation spectroscopy, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 8461–8466.

[15] L.A. Bagatolli, To see or not to see: lateral organization of biological membranes and fluorescence microscopy, Biochim. Biophys. Acta 1758 (2006) 1541–1556.

[16] T. Parasassi, E. Gratton, W.M. Yu, P. Wilson, M. Levi, Two-photon fluorescence microscopy of Laurdan generalized polarization domains in model and natural membranes, Biophys. J. 72 (1997) 2413–2429.

[17] K. Gaus, E. Gratton, E.P.W. Kable, A.S. Jones, I. Gelissen, L. Kritharides, W. Jessup, Visualizing lipid structure and raft domains in living cells with two-photon microscopy, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 15554–15559.

[18] H.M. Kim, H.-J. Choo, S.-Y. Jung, Y.-G. Ko, W.-H. Park, S.-J. Jeon, C.H. Kim, T. Joo, B.R. Cho, A two-photon probe for lipid raft imaging: C-Laurdan, ChemBioChem 8 (2007) 553–559.

[19] H.M. Kim, B.H. Jeong, J.-Y. Hyon, M.J. An, M.S. Seo, J.H. Hong, K.J. Lee, C.H. Kim, T. Joo, S.-C. Hong, B.R. Cho, Two-photon fluorescent turn-on probe for lipid rafts in live cell and tissue, J. Am. Chem. Soc. 130 (2008) 4246–4247.

[20] L. Jin, A.C. Millard, J.P. Wuskell, H.A. Clark, L.M. Loew, Cholesterol-enriched lipid domains can be visualized by di-4-ANEPPDHQ with linear and nonlinear optics, Biophys. J. 89 (2005) L4–L6.

[21] A.S. Klymchenko, G. Duportail, T. Oztürk, V.G. Pivovarenko, Y. Mély, A.P. Demchenko, Novel two-band ratiometric fluorescence probes with different location and orientation in phospholipid membranes, Chem. Biol. 9 (2002) 1199–1208.

[22] A.S. Klymchenko, G. Duportail, Y. Mély, A.P. Demchenko, Ultrasensitive two-color fluorescence probes for dipole potential in phospholipids membranes, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 11219–11224.

[23] V.V. Shynkar, A.S. Klymchenko, G. Duportail, A.P. Demchenko, Y. Mély, Two-color fluorescent probes for imaging dipole potential of cell plasma membranes, Biochim. Biophys. Acta 1712 (2005) 128–136.

[24] A.S. Klymchenko, Y. Mély, A.P. Demchenko, G. Duportail, Simultaneous probing of hydration and polarity of lipid bilayers with 3-hydroxyflavone fluorescent dyes, Biochim. Biophys. Acta 1665 (2004) 6–19.

[25] G. M'Baye, Y. Mély, G. Duportail, A.S. Klymchenko, Liquid ordered and gel phases of lipid bilayers: fluorescent probes reveal close fluidity but different hydration, Biophys. J. 95 (2008) 1217–1225.

[26] V.V. Shynkar, A.S. Klymchenko, C. Kunzelmann, G. Duportail, C.D. Muller, A.P. Demchenko, J.-M. Freyssinet, Y. Mély, Fluorescent biomembrane probe for ratiometric detection of apoptosis, J. Am. Chem. Soc. 129 (2007) 2187–2193.

[27] M.J. Hope, M.B. Bally, G. Webb, P.R. Cullis, Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential, Biochim. Biophys. Acta 812 (1985) 55–65.

[28] M.I. Angelova, D.S. Dimitrov, Liposome electroformation, Faraday Discuss. 81 (1986) 303–311.

[29] M. Fidorra, L. Duelund, C. Leidy, A.C. Simonsen, L.A. Bagatolli, Absence of fluid-ordered/fluid-disordered phase coexistence in ceramide/POPC mixtures containing cholesterol, Biophys. J. 90 (2006) 4437–4451.

[30] N. Kahya, D. Scherfeld, K. Bacia, B. Poolman, P. Schwille, Probing lipid mobility of raft-exhibiting model membranes by fluorescence correlation spectroscopy, J. Biol. Chem. 278 (2003) 28109–28115.

[31] J.-P. Clamme, J. Azoulay, Y. Mély, Monitoring of the formation and dissociation of polyethylenimine/DNA complexes by two photon fluorescence correlation spectroscopy, Biophys. J. 84 (2003) 1960–1968.

[32] J. Azoulay, J.-P. Clamme, J.-L. Darlix, B.P. Roques, Y. Mély, Destabilization of the HIV-1 TAR structure by the nucleocapsid protein through activation of conformational fluctuations, J. Mol. Biol. 362 (2003) 691–700.

[33] S.O. Yesylevskyy, A.S. Klymchenko, A.P. Demchenko, Semi-empirical study of two-color fluorescent dyes based on 3-hydroxychromone, J. Mol. St.: Theochem. 755 (2005) 229–239.

[34] P.-T. Chou, S.-C. Pu, Y.-M. Cheng, W.-S. Yu, Y.-C. Yu, F.-T. Hung, W.-P. Hu, Femtosecond dynamics on excited-state proton/charge-transfer reaction in 4'-N,N-diethylamino-3-hydroxyflavone. The role of dipolar vectors in constructing a rational mechanism, J. Phys. Chem. A 109 (2005) 3777–3787.

[35] R.B. Silver, Ratio imaging: practical considerations for measuring intracellular calcium and pH in living tissue, Methods Cell. Biol. 56 (1998) 237–251.

[36] R.F. Zwaal, A.J. Schroit, Pathophysiologic implications of membrane phospholipid asymmetry in blood cells, Blood 89 (1997) 1121–1132.