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Photoconversion of Bodipy-Labeled Lipid Analogues

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Lipid membrane heterogeneity (the physical basis of membrane rafts) is vital for many membrane-related cellular processes.[1,2] Liquid–liquid phase coexistence—as occurs in synthetic membranes where saturated lipids and sterols form a liquid-ordered (Lo) phase immiscible with an unsaturated lipid-rich liquid-disordered (Ld) phase—has been a widely used model for the coexistence of membrane domains in live cells.[3] The most common tools to probe lateral heterogeneity, both in model[4–9] and live-cell membranes,[10] are fluorescent lipid analogues. The influence of the membrane environment on the photophysical properties of these analogues (e.g., fluorescence lifetime and quantum yield) has been exploited to probe the structure of the membrane;[9,11,12] however, this environmental sensitivity can also lead to erroneous interpretation if not properly controlled.

An example of a potentially confounding photophysical phenomenon of fluorescent molecules is photoconversion, that is, light-induced change in excitation and/or emission spectra, as has been reported for several fluorescent proteins;[13,14] however, in spite of their widespread use, this phenomenon has not yet been investigated for fluorescent lipid analogues. Here we present examples of significant lipid-environment-dependent photoconversion in commonly used Bodipy-labeled lipids. These so-far unreported effects significantly impact experiments where the probes' spectral properties influence the result (e.g., ratiometric studies), and they need to be taken into account in future studies. Moreover, these effects may even be exploited as new tools when thoroughly controlled.

To investigate photoconversion of membrane-embedded Bodipy FL, time–intensity profiles through green (excitation = 488 nm; emission = 505–530 nm) and red (excitation = 543 nm; emission = 580–615 nm) fluorescence channels (see the Supporting Information for details) were measured with giant unilamellar vesicles (GUVs) prepared from an unsaturated zwitterionic lipid (DOPC) doped with 0.5 mol% Bodipy FL–monosialotetrahexosylganglioside (BD-GM1; structure in Figure S1 in the Supporting Information) upon illumination with unattenuated wide-field blue light (450–490 nm). UV (300–400 nm) and green (530–560 nm) illumination showed similar effects, though smaller in magnitude. Respective dual-color images of GUVs taken before illumination showed no signal in the red channel (Figure 1 A), consistent with the expected emission spectrum of BD-GM1. After 5 seconds of blue-light illumination, the signal in the green channel decreased, while the signal in red channel increased dramatically (Figure 1 B), thus suggesting green-to-red fluorescence photoconversion of BD-GM1. The same effect was observed for a different Bodipy-labeled sphingolipid analogue (Bodipy FL-sphingomyelin) but not for one with a different fluorescent moiety (NBD-GM1, data not shown). Efficient photoconversion was also observed for BD-GM1 embedded in living cell membranes (Figure 1 C and D). Multispectral analysis with a 16-channel metadetector was applied to better resolve the spectral differences upon illumination. An increase in the red region of the spectrum confirmed photoconversion (Figure 1 E and F).

We first tested how this fluorescence shift is affected by fluorophore concentration. Therefore, the same experiment was carried out at different concentrations of BD-GM1. We found that the light-induced fluorescence shift occurred at fairly low concentrations of BD-GM1 (around 0.05 mol%, Figure S2). However, we did not observe it below 0.01 mol%. Thus, one possible reason for this shift is short-lived excimer formation, which usually only happens when Bodipy analogues are used at extremely high concentrations (10-50 mol%).[15] In order to test whether the red-emitting molecules are "light-induced" excimers, we checked the excitation spectra and lifetimes of newly generated red molecules. If the reason for the fluorescence shift is light-induced excimer formation, the existence of red fluorescence would be dependent on continuous excitation with 488 nm light, as green molecules must be excited to form the excited dimer.[16] However, after initial illumination with blue wide-field light to stimulate the photoconversion, a red signal was observed, even in the absence of 488 nm laser excitation. Moreover, no red signal was observed in the absence of 543 nm laser excitation. Even after 15 min of equilibration (when short-lived excimers would relax to the ground state), 543 nm laser was necessary and sufficient to obtain the red signal without further blue-light illumination (Figure S3). This shows that red-emitting molecules are not short-lived excimers but permanent entities whose excitation and emission spectra differ from those of the precursor molecules.

Secondly, we investigated the dependence of photoconversion on the lipid environment of the fluorescent analogue. To this end, we measured BD-GM1 green-to-red photoconversion in a heterogeneous lipid environment, the most common mini-

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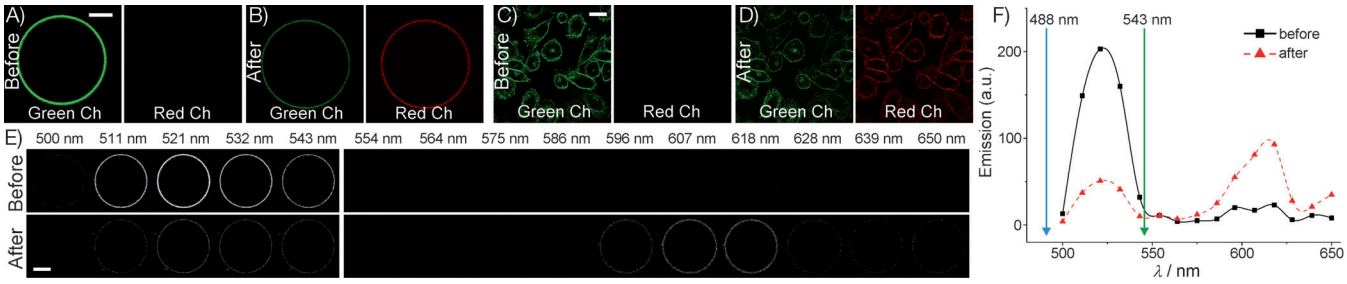

Figure 1. Photoconversion of BD-GM1. Green- and red-channel images of BD-GM1 in DOPC GUVs: A) before, and B) after 5 s excitation light exposure show striking green-to-red photoconversion of BD-GM1. Green- and red-channel images of live cells: C) before, and D) immediately after 5 s exposure to light excitation confirm this effect in live cells. E) Spectral images of GUVs before and after the illumination. F) Quantification of spectral change confirms the photoconversion. (Images were taken with 488 nm then 543 nm laser excitations. Scale bars: 10 μm.)

mal lipid raft model of phase-separated synthetic vesicles. GUVs were prepared from a lipid mixture known to exhibit Lo/Ld phase coexistence at room temperature (DOPC/sphingomyelin (SM)/cholesterol (Chol), 40:40:20), and stained with BD-GM1. Fluorescence was observed in both phases (Figure 2A) with significant Ld enrichment (%Lo = 15; calculation details in Figure S4). After 5 s illumination of DOPC/SM/Chol GUVs, the green-channel intensity of the Ld phase decreased markedly, with only a slight decrease in Lo phase intensity (Figure 2B and C). Concomitantly, there was a dramatic increase in Ld phase intensity in the red channel, without any significant change in Lo phase red signal (Figure 2B and C). This result suggests phase-specific BD-GM1 photoconversion; however, the large difference in probe concentration in the two domains precluded unequivocal confirmation of this effect in the DOPC/SM/Chol membrane system.

To rule out the effect of concentration and to investigate phase dependence, photoconversion was tested in GUVs composed of Liver PC (LPC)/DPPC/Chol (35:35:30), where BD-GM1 has nearly uniform partitioning with a slight enrichment in the Lo phase (Figure 3A and B, Figure S5). If the domain-specific photoconversion observed in the Ld phase of DOPC/SM/Chol vesicles is a function only of higher analogue concentration in this phase, greater photoconversion would be expected in the Lo phase of LPC/DPPC/Chol GUVs. However, in these vesicles, as in DOPC/SM/Chol, photoconversion took place predominantly in the Ld phase (Figure 3C and D). Remarkably, the newly generated red fluorescent species did not show the same partitioning as the precursor green-fluorescent molecule. While the green signal was concentrated in the Lo phase after illumination, red fluorescence was highly Ld-enriched (Figure 3C). (Enrichment in the phases was due to different molecule concentration in two phases, not different quantum yield; Figure S6.) Even after 15 min of equilibration, the red signal remained concentrated in the Ld phase, thus suggesting that the moiety generated by photoconversion was highly Ld-preferring (Figure 3E). The Lo partitioning of the green signal, on the other hand, decreased remarkably (%Lo ≈ 35%) when equilibrium was reached (Figure 3E and F). Either a change in membrane lipid packing or conversion of BD-GM1 into a different green-fluorescent species (that prefers the Ld phase) could account for this result. To check whether a change in lipid packing of the membrane was caused by illumination or green-to-red photoconversion, we measured the diffusion of a far-red membrane molecule, Atto647N-PC, by fluorescence correlation spectroscopy, before and after photoconversion. We observed that the diffusion coefficient of Atto647N-PC did not change upon illumination or photoconversion (Figure S7). These data suggest that the repartitioning of the remaining green molecules after illumination was not due to any change in the membrane, but presumably was attributable to a structural change in the lipid analogue.

Finally, phase-specific photoconversion was confirmed by applying multispectral analysis of phase-separated GUVs: a dramatic increase in the red region of the spectrum in the Ld phase was observed after illumination with no spectral change in the Lo phase (Figure 3G).

A highly probable reason for the phase-specific fluorescent

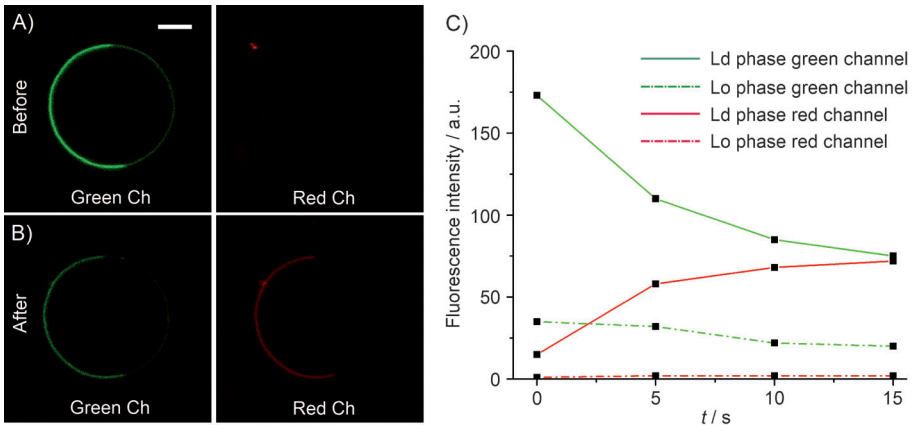

Figure 2. Domain-specific photoconversion of BD-GM1. Images A) before, and B) after 5 s blue illumination show that green signal decreases, while red signal increases, dramatically in the Ld phase in phase-separated DOPC/SM/Chol GUVs. C) These qualitative observations are confirmed by quantification of green- and red-channel fluorescence intensity in Lo and Ld phases during green light illumination. (Scale bar: 10 μm.)

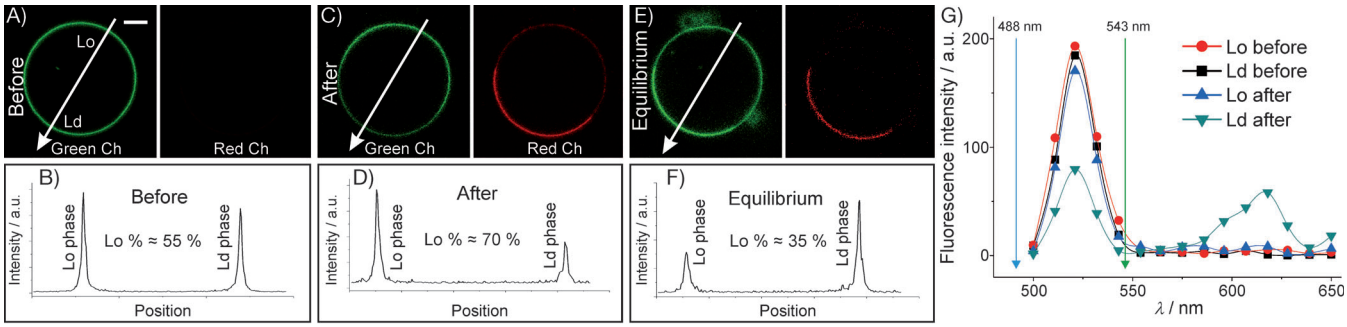

Figure 3. Green- and red-channel images (A, C, and E) and quantification (B, D, and F, calculated from the respective line scans; see Figure S4) of BD-GM1 partitioning in LPC/DPPC/Chol GUVs: A) and B) before, C) and D) immediately after 5 s excitation light exposure, and E) and F) after 15 min equilibration. These show that phase-specific photoconversion does not depend on the concentration of the probe in Ld phase and that the newly generated red molecule prefers the Ld phase. G) Phase-specific photoconversion is confirmed by spectral detection. (Scale bar: 10 μm.)

shift is photo-oxidation of unsaturated lipids like DOPC. In order to test this, we used DiPyhPC/DPPC/Chol (30:30:40), a lipid mixture that has been shown to be not prone to oxidation, as it consists of solely saturated phospholipids and cholesterol.[17] However, we observed the same fluorescence shift in these vesicles, even in the presence of antioxidants (Figure S8).

Having observed the striking lipid environment-dependent green-to-red photoconversion of BD-GM1 and its change in phase preference after equilibrium, we tested whether the same phenomenon occurs in the fluorophore when linked to an unrelated lipid moiety. To this end, we measured photoconversion of Bodipy (also called TopFluor (TF))-labeled cholesterol (TF-Chol), a reliable Lo-phase marker in phase-separated vesicles.[8,18,19] As previously observed,[8] TF-Chol partitioned into the Lo phase of DOPC/SM/Chol GUVs, with %Lo≈75% (Figure 4A). When TF-Chol was illuminated with blue wide-field light, no increase in the red channel signal was observed, thus showing that TF-Chol does not undergo the same green-to-red photoconversion as BD-GM1 (data not shown). This also suggests that illumination-induced membrane change is not the reason for the green-to-red fluorescence shift.

However, during blue light illumination (or 488 nm laser illumination), the intensity of the green fluorescence signal changed in both phases—decreasing much faster in the Lo than in the Ld phase (Figure 4B). Within 90 s of laser illumination (30–50 μW), the ratio of Lo/Ld fluorescence decreased from an initial value of ~3 to less than unity, indicative of Ld intensity surpassing Lo (Figure 4B–D). This effect cannot be attributed to domain-specific photobleaching alone, because while the Lo phase intensity decreased monotonically, the intensity in the Ld phase slightly increased just after the illumination (Figure 4D). We ascribe this effect to conversion of TF-Chol into another green-fluorescent molecule with preferential partitioning in the Ld phase. Thus, the fast decrease in Lo-phase intensity is likely due to a combination of conversion and bleaching. This hypothesis was validated by the observation that after 15 min equilibration, the Lo/Ld ratio of the fluorescent signal did not recover to its preillumination value (as would be expected if photobleaching was the only effect), but rather remained around unity, because of the combination of fluorescence from nonconverted, Lo-preferring TF-Chol and the newly generated Ld-preferring green-fluorescent species (Figure 4E). We repeated the experiment with DiPyhPC/DPPC/Chol vesicles, with and without antioxidants, to eliminate the effect of photo-oxidation; however, we observed the same conversion in both cases (Figure S9). This might also explain the Ld-preferring green signal of BD-GM1 after equilibration (Figure 3E and F), which most likely arose from a similar conversion. To test whether the same conversion occurs in other

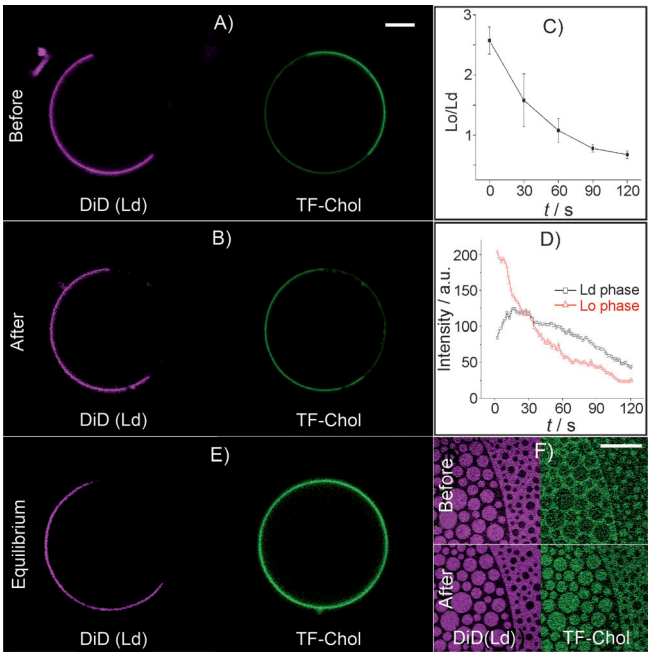

Figure 4. Photoconversion of TF-Chol. Partitioning of TF-Chol, A) before, and B) after time-series scan. No red-to-green photoconversion is detected, but rather there is light-induced conversion of TF-Chol to a green-fluorescent, but Ld-partitioning, species. C) Quantification of Lo/Ld intensity over time (average ±s.d. from five experiments), and D) intensity profiles in Lo and Ld phases in the green channel confirm this hypothesis by showing an increase in Ld fluorescence immediately after illumination ($n > 5$). E) Re-equilibrium of TF-Chol after 15 min shows that Lo/Ld intensity does not reach the pre-illumination value. F) The same conversion occurs in a phase-separated monolayer, thus demonstrating that this is not specific to the GUV system. (Scale bars: 10 μm.)

model systems, we employed phase-separated monolayers (DOPC/SM/Chol, 40:40:20). After a short period of illumination, the partitioning of the green signal changed quickly from Lo to Ld (Figure 4F), thus confirming that the conversion was not specific to the GUV system.

Fluorescent lipid analogues are important tools for probing the structure and function of biological and biomimetic membranes, as the separation of eukaryotic membranes into coexisting lipid-driven domains represents a central feature of their organization. Here we report some important photophysical properties of the most commonly used fluorescent raft lipid analogues, BD-GM1 and TF-Chol. We observed: photoconversion of BD-GM1 from green to red emission upon blue light illumination; a lipid-phase (lipid packing) dependence of this conversion; and, conversion of BD-GM1 and TF-Chol into green fluorescent moieties with different phase preferences.

Taken together, these findings prompt the conclusion that these photophysical phenomena can lead to lipid-environment-specific false positive signals in experimental techniques where spectral identity/separation is important (e.g., FRET and fluorescence cross-correlation spectroscopy). Conversely, properly controlled experimental designs can exploit these artifacts for the elucidation of membrane structure, by taking advantage of switchable fluorescence spectra and/or phase localization.

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Keywords: bodipy · lipids · photoconversion · photophysics · rafts

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