Impact of membrane-anchored fluorescent probes on the mechanical properties of lipid bilayers

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ABSTRACT

Fluorescent probes are used in membrane biophysics studies to provide information about physical properties such as lipid packing, polarity and lipid diffusion or to visualize membrane domains. However, our understanding of the effects the dyes themselves may induce on the membrane structure and properties are sparse. As mechanical properties like bending elasticity were already shown to be highly sensitive to the addition of "impurities" into the membranes, we have investigated the impact of six different commonly used fluorescent membrane probes (LAURDAN, TR-DPPE, Rh-DPPE, DiIC18, Bodipy-PC and NBD-PC) on the bending elasticity of dye containing POPC GUVs as compared to single component POPC GUVs. Small changes in the membrane bending elasticity compared to single POPC bilayers are observed when 2 mol% of Rh-DPPE, Bodipy-PC or NBD-PC are added in POPC membranes. These binary membranes are showing non reproducible mechanical properties attributed to a photo-induced peroxidation processes that may be controlled by a reduction of the fluorescent dye concentration. For TR-DPPE, a measurable decrease of the bending elasticity is detected with reproducible bending elasticity measurements. This is a direct indication that this dye, when exposed to illumination by a microscope lamp and contrary to Rh-DPPE, does not induce chemical degradation. At last, LAURDAN and DiIC18 probes mixed with POPC do not significantly affect the bending elasticity of pure POPC bilayers, even at 2 mol%, suggesting these latter probes do not induce major perturbations on the structure of POPC bilayers.

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1. Introduction

Fluorescence spectroscopy and microscopy are widely used experimental techniques in biophysics that provide important information about structure and dynamics of membrane structure, mesoscale organization or dynamics [1]. In such studies, fluorescent probes are introduced in the membrane. Observations of fluorescence microscopy images of selected regions or isolated objects may be compared with results obtained *via* regular fluorescence spectroscopy. This spatial resolved information, that is lacking in fluorescence "cuvette" based experiments, is obtained by image acquisition of selected fluorescence parameters in specific regions of a model membrane system like giant unilamellar vesicles (GUVs) [1] or planar membranes fixed on solid supports [2–4]. Altogether, it is possible to provide a direct detection of membrane heterogeneity (e.g. domain shape and size) or to get local physical properties (e.g., fluidity, polarity, probe molecular diffusion) within the membrane [5–8].

Many different fluorescent dyes may be used depending on the specific properties to be studied. Some of them are closely related to phospholipid molecules while others do not resemble common membrane molecules. They may prefer selected membrane environments displaying different composition and lateral packing (i.e. fluid or gel), leading to a partitioning of the probe and the existence of rich and depleting regions within a given membrane. This is the case with the bicatenary fluorescent dyes DiIC18, TR-DPPE, Rh-DPPE, Bodipy-PC and NBD-PC, where the partition of these dyes is primarily depending on the chemical composition of a given lipid domain [1]. For example, Rh-DPPE shows a preferential partition into a liquid disordered phase in GUVs composed of DMPC/DSPC mixtures, whereas it is preferentially inserted into the gel phase in the case of DLPC/DPPC mixtures [9,10]. Other fluorescent dyes like pyrene, DPH, LAURDAN or PRODAN are named "environmental probes" because they sense changes in their microenvironment (pH, polarity, viscosity or temperature) when inserted into the membrane. The latter probes are not phospholipid-like molecules. They are generally displaying homogeneous membrane distribution, even in membranes displaying phase coexistence, therefore allowing simultaneous measurements from different regions of the membrane using a single probe [1].

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Generally speaking, it is admitted that low dye concentrations, i.e., smaller than about 1 mol%, do not affect membrane physical properties. This is somewhat more an assumption than an affirmation since only a few works quantified such membrane additive effects on bilayer or monolayer properties. Molecular dynamics simulations addressed this question by looking at the perturbation induced by the probes on the host lipid structure [11–15]. Calorimetry studies involving either bilayers [16–18] or monolayers [2,4,19] gave insights describing the above-mentioned perturbations induced by fluorescent probes on membrane organization. Phase separation was shown to occur when illuminating a multicomponent model membrane containing a fluorescent probe in relation to peroxide formation [20–22]. However, deciding which dye is harmless or, more precisely, which one will not induce large modifications in the host membrane is a crucial step. This information may prevent experimental artifacts and consequently erroneous data interpretation.

Our work was motivated by the idea to study the influence of frequently used fluorescent probes, namely TR-DPPE, LAURDAN, Bodipy-PC, DiIC18, Rh-DPPE and NBD-PC, on membrane physical properties. We specifically determined how bending elasticity, a mechanical property characterizing how easily a membrane may deform at constant surface area, was changing as a function of the chemical nature of these fluorescent probes. Actually, bending elasticity strongly depends on the organization of the lipid bilayers. For example, it was shown to depend on the organization of the aliphatic region of the bilayer (the chain length of the saturated synthetic phosphatidylcholine, the temperature decrease when approaching the fluid-to-gel main phase transition [23] or the sterol content [24,25]) or the characteristics of the head region [26,27]. Consequently, this parameter is a good candidate to reflect any change in the organization of the main lipid component (here, POPC) induced by the addition of an externally added component like a fluorescent probe.

We demonstrated that the measured bending elasticity of the different binary POPC-dye systems are not strongly affected by the presence of 2 mol% of the probe when compared to pure POPC bilayers, a concentration much higher than what is usually used in fluorescent spectroscopy or microscopy. The main distinction between the different probe's effects will be noticed when comparing the reproducibility of the bending elasticity measurements, some of them showing an unusual poor reproducibility attributed to photo-induced peroxide lipid degradation. This phenomenon may be limited by a reduction of the fluorescent dye membrane concentration.

2. Materials and methods

2.1. Materials

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) was purchased from Avanti Polar Lipids Inc. (Birmingham, AL). 6-dodecanoyl-2-dimethylaminonaphthalene (LAURDAN), Texas Red 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (TR-DPPE), 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD C6-HPC named NBD-PC in this work), lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Rh-DPPE), 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiIC18(3) named DiIC18 in this work) and 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (β-BODIPY FL C5-HPC named Bodipy-PC in this work) were obtained from Invitrogen, Denmark or France. Fluorophore stock concentrations were quantified by absorption spectroscopy using published absorption coefficient. Buffer solutions at 1 mmol/L Tris (Trizma Base, Sigma–Aldrich Chimie, France) and 1 mmol/L EDTA (ethylenediaminetetraacetic

acid, Sigma-Aldrich Chimie, France) adjusted to pH 7.4 were used as the GUV suspension media.

2.2. GUV preparations

An organic solution containing POPC and a fluorescent dye at 2 mol% or 0.5 mol% probe/lipid molar ratio was prepared from stock organic solutions. The mixture was dried under vacuum using rotary evaporator, the obtained dry film being subsequently hydrated using pure water. Multilamellar vesicles were readily obtained by gentle agitation. Then, large unilamellar vesicles were produced according to standard procedures with a final lipid concentration of about 0.2 mg/mL and used directly for GUV electroformation in the Tris/EDTA buffer at room temperature, following the already published procedure referred to low salt concentration environment [28,29].

2.3. Flickering studies and bending elasticity measurements

Two experimental setups were used to observe POPC GUVs containing a fluorescent dye by phase contrast or fluorescence microscopy at room temperature ($T = 22$ °C).

Fluctuating vesicles containing one of the probes at 2 mol% were observed using an Axiovert 135 equipped with a ×63/0.75 phase contrast objective (Zeiss, Germany). Stroboscopic illumination by a xenon flash lamp (L7684 and its power supply, Hamamatsu, Japan) was used when studying GUV thermal fluctuations [30–32] while epi-illumination was chosen when observing GUV fluorescence. Video images were produced by a CCD C2400-77 (Hamamatsu, Japan) or a CCD DXC C33P (Sony, Japan) video camera and recorded on a DSR-20P DVCAM recorder (Sony, Japan).

Vesicles with dye concentration at 0.5 mol% were visualized using an Axiovert S100 equipped with a ×40/0.60 phase contrast objective (Zeiss, Germany). The pictures were captured by a CCD SSC-DC50AP video camera (Sony, Japan) at a rate of 25 frames per second with a video integration time of 4 ms and recorded on a computer hard disk.

Illumination power produced either by the xenon flash lamp or the more common halogen lamp (continuous illumination) was estimated to be about 10 W maximum. For both setups, the thermal fluctuations of every GUVs were analyzed for about 10 min and the contour extraction procedure was done using home-made software package, adapted from those already presented in Ref. [33].

The data analysis procedure for bending elasticity measurements that was used herein differs analytically from the previously published technique that can be found in Ref. [24,34] and will be presented in details in an independent paper. Briefly, the distribution functions of the different fluctuating Fourier modes were analyzed over the recorded time following Gaussian statistics, bending elasticity and reduced membrane tension being the only fitting parameters [33–35]. The main point to notice for our present purpose is the large improvement of the quality of the bending elasticity measurements, allowing a better detection of the GUVs that do not follow the quasi-spherical expected behavior [33–35].

3. Results and discussion

In order to measure a possible influence of fluorescence dyes on membrane mechanical properties, we carried out bending elasticity measurements on pure POPC GUVs, as well as on POPC GUVs containing 2 mol% of a membrane marker dye. The six dyes studied herein are commonly used to monitor domain formation on GUVs [1]. Typical dye concentrations in such studies are around 0.2 to 0.4 mol% [36] and attain rarely 2 mol% [37]. Since we expected these dyes to have only a minor influence on bilayer bending elasticity, we chose a high dye content in order to distinguish the impact of the different dye molecules on the membrane mechanical properties. Fig. 1 shows the bending elasticity, k_c, of pure POPC bilayers (Fig. 1, dotted line), as
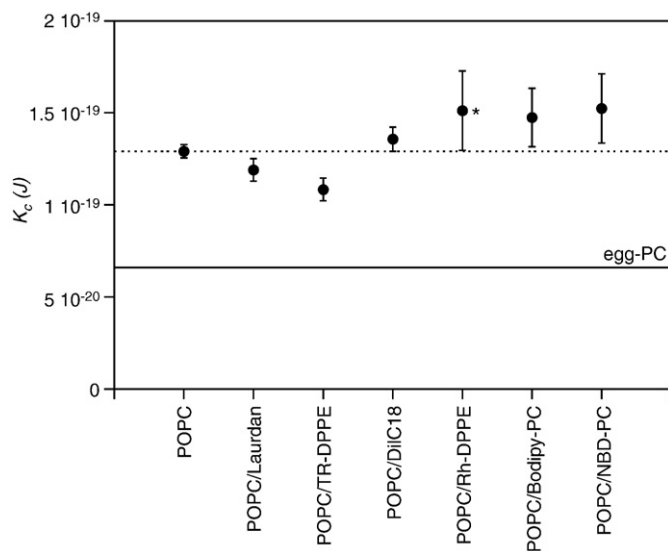
Fig. 1. Bending elasticities k_c and their associated standard deviations of POPC bilayers containing 2 mol% of one of the different fluorescent dyes studied in this work and comparison with bending elasticity of pure POPC (dotted line). The asterisk labeling POPC + Rh-DPPE value reminds that the corresponding measurements were made with selected giant vesicles that did not show facets during their observation (see text). For comparison, bending elasticity of egg-yolk phosphatidylcholine extracts is reported (full line).

measured by analyzing the thermal flickering of GUVs by video-microscopy, as well as bending elasticities obtained for POPC membranes containing 2 mol% of a membrane marker. For comparison, a published value of eggPC (Fig. 1, solid line) is also included [38]. As can be seen, all dyes modulate membrane bending elasticity. TR-DPPE, Rh-DPPE, Bodipy-PC and NBD-PC induce the most important variations of k_c, whereas LAURDAN and DiIC18 induced changes are very small. It can further be noticed that k_c values measured for individual GUVs containing Rh-DPPE, Bodipy-PC or NBD-PC are quite scattered (see error bars in Fig. 1) whereas the other dyes lead to smaller and more typical dispersions in the k_c measurements. Nevertheless, dye-induced modulations of k_c values can be considered as rather small when comparing the bending elasticity of the natural eggPC, equal to $(0.66 \pm 0.06) \times 10^{-19}$ J [38], with that of the synthetic model lipid POPC, equal to $(1.29 \pm 0.04) \times 10^{-19}$ J. In this context, it is worthwhile to remind that small variations in the length of the acyl chains result indeed in changes in k_c values that are larger [23] than those induced by the inclusion of 2 mol% fluorescence dyes.

However, at a membrane concentration of 2 mol%, one of the studied dyes, Rh-DPPE, produced an unexpected result. Although it was possible to find, record and analyze quasi-spherical, fluctuating GUVs, we also noticed repeatedly GUVs exhibiting rigid looking facets. An example of such a vesicle observed by phase contrast as well as epifluorescence is shown in Fig. 2. Since it is impossible to analyze such vesicles in terms of bending elasticity, it should be underlined that the mean value given for this system (Fig. 1, asterisk) may be somewhat truncated. It should also be mentioned that facets exhibiting GUVs were never observed straight after electroformation, but only some time after the detachment of the GUVs from the breeding platinum electrodes [28,29]. Since electroformation using ITO-electrodes (in our case, we were using platinum electrodes [29]) has been shown to induce lipid peroxidation [20], electroformation of Rh-DPPE/POPC GUVs was made in parallel in two independent electroformation cells. Once electroformation finished, one cell was left in the dark, whereas the other was observed by optical microscopy for about 2 h. After some time, a substantial fraction of freely moving GUVs exhibited facets. At this point, we examined the light protected cell and found it was free of GUVs with facets. When
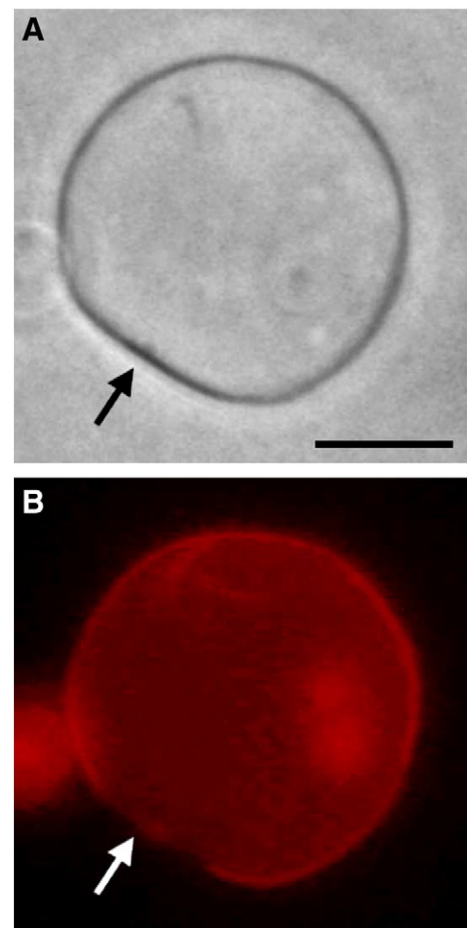
Fig. 2. Phase contrast (A) and fluorescence (B) images of the same GUV taken at about the same time. The arrows point to a large flat non-fluctuating area of the bilayer. The bar corresponds to 10 μm.

we illuminated further the light protected cell, we observed the apparition of facets onto GUVs. These results show that facet formation is not due to the electroformation conducted on Pt-electrodes but due to prolonged illumination.

Indeed, a similar dye, Rh-DOPE, has already been reported to be very sensitive to illumination [20]. Ayuyan and Cohen [20] have shown that excitation of this fluorescent probe using a regular fluorescence lamp during GUV microscope observation led to the generation of lipid peroxides, thereby promoting the formation of large rafts. In order to examine whether the rigid domains seen in the Rh-DPPE/POPC system are light-induced, individual, fluctuating GUVs were followed by normal phase contrast illumination for times longer than our typical observation times. We indeed observed spontaneous facet formation. Switching to epifluorescence mode, thereby increasing the illumination power, clearly accelerated this phenomenon. It can therefore be concluded that 2 mol% of Rh-DPPE result in light-induced domain formation in simple POPC vesicles. It may further be noticed in Fig. 2 that the rigid domain exhibited increased contrast when observed by phase contrast microscopy, while the fluorescence image indicated partial exclusion of the dye from these domains. This, together with the absence of thermal undulations of the membrane in these domains, is consistent with the interpretation that these light-induced domains are of higher order than the unperturbed bilayer.

To further verify that peroxides are at the origin of light-induced domains, we performed another experiment. 2 mol% of Rh-DPPE was incorporated into DPhC bilayers, a lipid known to be in a fluid L_α state at ambient temperature, yet without any double bonds, meaning that peroxide formation cannot occur. DPhC GUVs containing 2 mol%

of Rh-DPPE never showed any facets and bending elasticity was measured to be $(1.20 \pm 0.12) \times 10^{-19}$ J while, for pure DPhPC GUVs, k_c has been reported to be equal to $(1.17 \pm 0.10) \times 10^{-19}$ J [39]. This provides indeed evidence that the light-induced domains in the Rh-DPPE/POPC system are solely due to peroxide formation. The fact that Rh-DPPE does not influence the bending elasticity of DPhPC membranes also indicates that the seemingly dye-induced increase in k_c of POPC bilayers might be rather attributed to the apparition of peroxides than to the dye itself.

In this context it is interesting to examine a bit closer the k_c distributions of individual GUVs. Fig. 3 shows the measured k_c of individual GUVs for the DiIC18/POPC and the Rh-DPPE/POPC systems. Error bars of individual GUVs are overall very small for both systems, showing that k_c determination is accurate. In the case of the DiIC18 dye, k_c of the individual vesicles oscillates slightly around the mean value. The distribution is indeed small and not much larger than what is observed for pure POPC GUVs. This shows that the GUVs are homogeneous in composition. The mean value of measured individual k_c corresponds therefore to the bending elasticity of the system, which is a physical constant. A similar behavior is also seen for the LAURDAN or the TR-DPPE containing POPC GUVs. The situation is dramatically different for the Rh-DPPE dye containing GUVs. Individual GUVs exhibit extremely scattered k_c values ranging from about 1.2×10^{-19} J to about 2.0×10^{-19} J, which does not reflect the behavior of a physical constant of a homogeneous system, but indicates that individual GUVs vary in composition. Knowing that light-induced peroxide formation increases with increased illumination power or increased exposure length, we may speculate that such overlarge k_c distribution is due to GUVs with varying peroxide content. In this context, it is important to mention that illuminations of individual GUVs cannot be well controlled. More precisely, the process of finding, under the microscope, a vesicle in a cell (1 mm inner thickness [29]) whose diameter is larger than 15 μm and fulfilling the criteria imposed by the theory (quasi-sphericity) and by optical limitations (measurable fluctuations) is a difficult task. Such "perfect GUVs" are rare. Therefore the exploration time may be quite long and parts of the cell may be illuminated repeatedly, inducing possible radiation damage that might remained unnoticed by the observer. Therefore, when a new vesicle is selected for a new experiment it might be possible that this vesicle has been already illuminated for some time. It is therefore reasonable to attribute the k_c distribution in the Rh-DPPE/POPC systems as being due to varying peroxide content. Taking into account that the light-induced domains are of higher order than the fluctuating ones, k_c can be expected to increase as a function of peroxide content. This means that the lowest k_c values of individual GUVs correspond to the lowest

amount of peroxide or even peroxide-free bilayers. So, these lower k_c values reflect best the impact of the dye alone on the mechanical property. Taking only the 10 lowest k_c values lead to a mean of 1.35×10^{-19} J, which is very close to pure POPC.

Besides, it can be noted that the distribution of individual k_c values are not only huge in the case of the Rh-DPPE dye but also for Bodipy-PC and NBD-PC containing GUVs. Although neither Bodipy-PC nor NBD-PC containing GUVs showed any signs of light-induced domain formation, it might nevertheless be speculated that some lipid peroxidation occurs albeit not enough to induce large facets. Taking only the 10 lowest k_c values and using the same approach as made for Rh-DPPE, lead to an identical mean of 1.40×10^{-19} J for Bodipy-PC and NBD-PC. Again, this is very close to pure POPC.

In a recent work, Feigenson and co-workers [40] showed that dye-triggered light-induced domain formation occurs 50 times faster when the dye concentration is increased by a factor of 5 only. Therefore, we examined the influence of dye concentration for two dyes giving rise to the above-mentioned huge distributions. Rh-DPPE and Bodipy-PC membrane concentration was reduced from 2 mol% to 0.5 mol%.

For Bodipy-PC, the decrease of the dye molar ratio leads to a value of the bending elasticity very close to the one of pure POPC, that is $(1.32 \pm 0.11) \times 10^{-19}$ J. Values of individual GUVs were no more scattered but well within the typical dispersion of GUVs that are homogeneous in composition. This seems to indicate that at 2 mol% dye content, peroxides formation is not negligible and affects bending elasticity. In the case of Rh-DPPE, the decrease in dye concentration to 0.5 mol% reduced significantly the amount of GUVs with facets. Only one out of six independent GUV electroformation cells displayed vesicles with facets. Excluding this cell for k_c determination leads to a value of 1.27×10^{-19} J and a typical dispersion of 0.09×10^{-19} J. Again, this indicates that dye-induced alteration of membrane mechanical properties is weak whereas dye-triggered peroxide formation increases bending elasticity.

However, one may try to classify the dyes studied herein into probes showing different effects. TR-DPPE is seemingly the dye that exhibits the most pronounced effect of bending elasticity. At 2 mol%, distributions are homogeneous for this probe and k_c is reduced, which is indicative of the absence of significant peroxide formation. At 2 mol%, LAURDAN and DiIC18, being both structurally very different, induce only very slight changes in k_c with distributions indicative of the absence of significant peroxide formation. This shows clearly that these dyes have only a negligible influence on membrane mechanical properties and may be used safely as membrane markers, especially at concentrations much lower than those used herein. Bodipy-PC and NBD-PC are, as such, probably not strong membrane perturbing agents, but peroxide formation may be a potential problem at high dye content and/or powerful illumination. From the viewpoint of our study, Rh-DPPE is clearly the most problematic membrane probe. The Rh-DPPE-triggered light-induced domain formation in simple POPC bilayers is indeed the most surprising finding of this work. Light-induced domain formation has already been reported in GUVs containing several lipids [20,40,41]. Yet, Feigenson and co-workers [40] pointed out that artifactual light-induced domain formation occurs more easily at compositions close to liquid-liquid phase boundaries and required longer illumination times for compositions farther away. To our knowledge, this is the first time such a light induced domain formation has been reported for simple POPC bilayers at, admittedly, rather high dye content. Illumination with the normal microscope (halogen) lamp or, even with the flash lamp, is clearly less powerful than typical fluorescence illumination. Yet, the searching and recording of GUVs in order to determine k_c is a slow and long task resulting in prolonged light-exposure, which may compensate for a lack of illumination power.

Finally, one may wonder why Rh-DPPE triggers domain formation in illuminated POPC GUVs, but not TR-DPPE, despite the fact that they are structurally extremely close. The simplest explanation is to
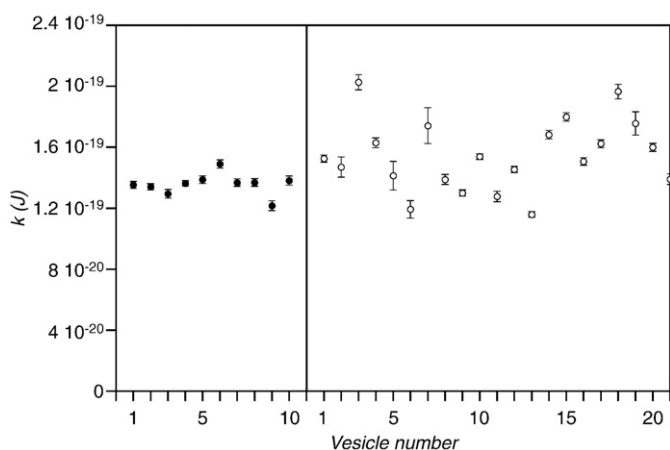

Fig. 3. Comparison of the bending elasticities measurements obtained with the POPC GUVs containing either 2 mol% DiIC18 (●) or 2 mol% Rh-DPPE (○) fluorescent dyes.

consider the quantum yield of these two probes. It is indeed well known that Texas Red has a much better quantum yield than lissamine (in ethanol, these quantum yields were found to be close to 100% and 50%, respectively [42]). Under the same illumination, the probe with the lower quantum yield will show more non-radiative intersystem crossing resulting in the reactive triplet state, thereby making the formation of oxygen radicals possible. So, lipid peroxide formation depends not only on illumination power or the amount of oxygen dissolved into the solution, but possibly quite directly on the probe quantum yield.

4. Conclusion

We showed that the measured bending elasticity of POPC membranes is only weakly affected by the incorporation of 2 mol% fluorescence probes. Since this dye concentration is much higher than what is usually used in fluorescent spectroscopy or microscopy, it can be safely assumed that, under more typical conditions, the influence of the probes themselves on membrane mechanical properties is negligible. The main problem that may arise with some dyes is related to photo-induced lipid peroxidation that may lead to domain formation even in a simple binary POPC/dye system. This phenomenon is associated with unusual scattering of bending elasticity values of individual GUVs. This demonstrates that bending elasticity measurements are a very sensitive tool to monitor membrane degradation.

Acknowledgments

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