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Photo-activated phase separation in giant vesicles made from different lipid mixtures

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ABSTRACT

Using giant unilamellar vesicles (GUVs) made from POPC, DPPC, cholesterol and a small amount of a porphyrin-based photosensitizer that we name PE-porph, we investigated the response of the lipid bilayer under visible light, focusing in the formation of domains during the lipid oxidation induced by singlet oxygen. This reactive species is generated by light excitation of PE-porf in the vicinity of the membrane, and thus promotes formation of hydroperoxides when unsaturated lipids and cholesterol are present. Using optical microscopy we determined the lipid compositions under which GUVs initially in the homogeneous phase displayed Lo–Ld phase separation following irradiation. Such an effect is attributed to the in situ formation of both hydroperoxized POPC and cholesterol. The boundary line separating homogeneous Lo phase and phase coexistence regions in the phase diagram is displaced vertically towards the higher cholesterol content in respect to ternary diagram of POPC:DPPC:cholesterol mixtures in the absence of oxidized species. Phase separated domains emerge from sub-micrometer initial sizes to evolve over hours into large Lo–Ld domains completely separated in the lipid membrane. This study provides not only a new tool to explore the kinetics of domain formation in mixtures of lipid membranes, but may also have implications in biological signaling of redox misbalance.

1. Introduction

Oxidation of biomolecules and the accumulation of these oxidized byproducts have a degenerative effect on one's health, and it can lead to disease or to the acceleration of the aging process [1]. Depending on the amount of oxidative misbalance impaired to the cells several outcomes are possible: cells can recover and survive, they enter in senescence, engage in apoptosis or die by uncontrolled necrosis. The ability to restrain and to control the effects of oxidation of biological molecules has, potentially, life saving benefits. From another perspective, controlled application of oxidative stress has been useful in the treatment of some diseases in a procedure that has been called photodynamic therapy (PDT). This therapeutic treatment involves damaging diseased tissues by applying the correct combination of a photosensitizer, visible light, and oxygen, which results in cell death and eradication of the disease [2–4].

The molecular mechanisms that transform an oxidative misbalance into a specific or generic damage, which in the end cause a cell to die, are still being studied. For that reason most PDT protocols are empirical and all of them are prone to fail in around 15% of the patients [5–7] indicating that further knowledge of the photochemical and photo-biological effects of these oxidative reactions is necessary.

It is believed that damaging membranes is one of the initial steps in the process of disturbing cell homeostasis [8,9]. Therefore, in order to improve our knowledge in the processes that cause changes in the cell viability under photo-induced oxidative misbalance, it is necessary to study details of the transformations that membranes face in the presence of an oxidative misbalance. The chemical changes have been thoroughly studied and are known in great detail in the beginning and in the end of the oxidative process; the intermediate species are not so well understood [10]. However, the effects that these transformations have on the physical properties of the membranes are still poorly understood. Recently, Kinnunen and co-works reported that oxidized phospholipids may facilitate the phospholipid flip-flop in liposomes [11]. Molecular dynamic (MD) simulations have shown that the oxidized tails can migrate toward the water phase, depending on their chemical structure modifications. This leads to an increase in the average area per lipid and concomitantly to a decrease of the bilayer thickness, causing membrane defects and increased permeability [12,13]. It has also been recently shown both experimentally [14] and by MD [15] that massive oxidation can cause membrane structure disruption.

We have been using Giant Unilamellar Vesicles (GUVs) to study the details of the physical transformation that a membrane suffers

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when the lipids are involved in photoinduced oxidative reactions. While studying GUVs suspended in methylene blue solutions, in order to follow the successive steps of the membrane destruction [14], we realized how important is to better control the localization of the singlet oxygen sources. We have thus synthesized a molecule (PE-porph, a derivative of protoporphirin bound to two PE molecules) that generates a known amount of singlet oxygen in the close vicinity of the membrane [16]. PE-porph allows the photo-oxidative reaction to induce *in situ* formation of lipid hydroperoxides. With this tool we were able to estimate the yield of the reaction between singlet oxygen and the double bond of POPC and quantitatively relate the change in membrane area with the formation of lipid hydroperoxides.

Lipid membranes do not act passively as a fluid matrix. Instead, the lipids are organized in different domains that play an important role in protein function. In biological membranes, the so-called lipid rafts exist as membrane micro-domains enriched in the liquid-ordered (Lo) phase, which results from packing of the long saturated alkyl chains of sphingolipids and cholesterol that segregate from the liquid-disordered (Ld) phase composed of unsaturated phospholipids [17–20]. Changes in the lipid composition of the rafts have been increasingly related with cell signaling processes [21]. For example, it has been shown that the organization of lipids in rafts is fundamental for the signaling involved in the intrinsic pathway of apoptosis [22]. Poly unsaturated fatty-acids, which are prone to be oxidized, seem to play important roles in the structure and formation of the membrane rafts [23]. Interestingly, Megli and co-workers have shown that phospholipid multilamellar vesicles made of linoleyl PC and DPPC containing certain percentages of oxidized phospholipids (typically from 5% to 15%), have two component EPR spectra at low temperatures (typically 12 °C or less), which are indicative of lipid phase separation [24]. Therefore, it is important to understand how an oxidative misbalance can alter the organization of the lipids in a membrane as well as the mechanisms of these changes.

Noteworthy, lipid hydroperoxides derived from unsaturated phospholipids, glycolipids, and cholesterol are prominent oxidative byproducts found in cellular and membrane mimetic systems [25]. They are known to alter the thermotropic phase properties of the bilayer [26] and the lipid packing [27]. It has also been shown that in some cases lipid hydroperoxides may stimulate signaling within the cell [10,28].

GUV has been extensively used to investigate the phase behavior of lipids and its relation to various aspects of lipid rafts. These studies have led to the understanding of the relationship between the molecular structure of a specific lipid and its role in the composition of lipid domains in a membrane bilayer. Phase diagrams have been shown for various model membranes using quasi-ternary mixtures, in which there is a tendency of the lipids to either be completely mixed or coexist in separate lipid phases. In fact, formation of lipid domains has been observed before by photo-stimulating fluorescent lipid probes in GUVs made of ternary mixtures of lipids. Several authors caution that lipid peroxidation can be a serious artifact in the study of GUV domains, suggesting ways in which this effect can be minimized, for instance, by using anti-oxidant agents [29–31]. Ayuyan and Cohen suggested that domains were generated due to the formation of peroxidation products, especially of sphingomelin (SM), by Haber–Weiss and Russel mechanisms of oxidation reactions [29]. Feigenson et al. suggested that large photoinduced domains arise from the coalescence of smaller pre-existent lipid clusters that grow under oxidation [30].

In this study, we investigated GUVs assembled from ternary mixtures of POPC, DPPC and cholesterol, with a small mole fraction of PE-porph, a modified lipid carrying one porphyrin, a moiety photosensitizer currently used in PDT. We monitored macroscopic changes of the membrane induced by lipid oxidation by means of optical microscopy. We explored significant parts of the ternary phase diagram, with cholesterol mole fractions up to 0.23. By controlling membrane composition we were able to identify the region in the POPC:DPPC: cholesterol phase diagram where Lo–Ld coexistence emerges from initially homogeneous mixtures due to the formation of both oxidized POPC and cholesterol species.

2. Materials and methods

2.1. Materials

The lipids 1,2-dipalmitoyl-sn-glycero-3-phosphocoline (DPPC), 1-palmiltoyl-2-oleoyl-sn-3-glycero-phosphocoline (POPC) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). The photosensitizer Dimethyl 8,13-Divinyl-3,7,12,17-Tetramethyl-21H, 23H-Porphine-2,18-Dipropyl-L-α-Dimyristoyl Phosphatidyl Ethanolamine, that we name PE-porph, was synthesized as previously described [16] and consisted of a porphyrin molecule attached to two phosphatidylethalonamines, schematically shown in Fig. 1. Similar to porphyrin incorporated in model membranes, PE-porph presents a maximum light absorption at *ca.* 400 nm and fluorescence emission above 600 nm. The quantum yield of singlet oxygen production is 0.5 [16]. Further, observation under the microscope in the fluorescence mode showed that PE-porph incorporated in the bilayer presents a fast photobleaching, with a decay time of 2 s under irradiation [16]. In some cases, a green-emitting egg-PC labeled with the fluorophore NBD, 1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine from Avanti Polar Lipids, was used to better visualize the presence of domains. The total molar fraction of the fluorescent probe was less than 0.1 mol% of the total lipid amount; low intensity illumination was used in the fluorescence microscopy to avoid artifacts due to light-induced domain formation by the NBD-probe [29–31].

2.2. Preparation of giant unilamellar vesicles

Giant unilamellar vesicles (GUVs) containing 1 mol % of PE-porph were grown by means of the electroformation technique [32]. Briefly, 8 μl of a 2 mg/ml lipid mixture in chloroform were spread as a thin film on the surfaces of two conductive glasses (coated with Fluor Tin Oxide). The glasses were placed in a desiccator for at least 2 h to remove any traces of chloroform from the lipid film. They were then mounted with their conductive sides facing each other and separated by a 2-mm-thick Teflon spacer. This electroswelling chamber was filled with a 0.2 M sucrose solution and placed inside an oven at ~60 °C. The glass plates were connected to a function generator and an alternating voltage of 2 V with a 10 Hz frequency was applied for 2 h. The vesicle solution was removed from the chamber and diluted into a 0.2 M glucose solution. This created a sugar asymmetry between the interior and the exterior of the vesicles. The osmolarity of both the glucose and sucrose solutions was measured with a cryoscopic osmometer Osmomat Gonotec 030 (Berlin, Germany) and carefully matched to avoid osmotic pressure effects. The solution was placed in an observation chamber. Due to the differences in density and refractive index between the sucrose and glucose solutions, the vesicles were stabilized by gravity at the bottom of the chamber and had better contrast when observed by phase contrast microscopy. The presence of PE-porph did not bring about any change to the vesicles in the darkness. The presence of NBD-probe in the membrane did not promote any visible change in lipid bilayer in the absence and presence of light.

Each vesicle containing a specific lipid composition was examined by at least three independent preparations.

2.3. Optical microscopy observation and irradiation

Observation of GUVs was performed under an inverted microscope, Axiovert 200 (Carl Zeiss, Oberkochen, Germany), equipped

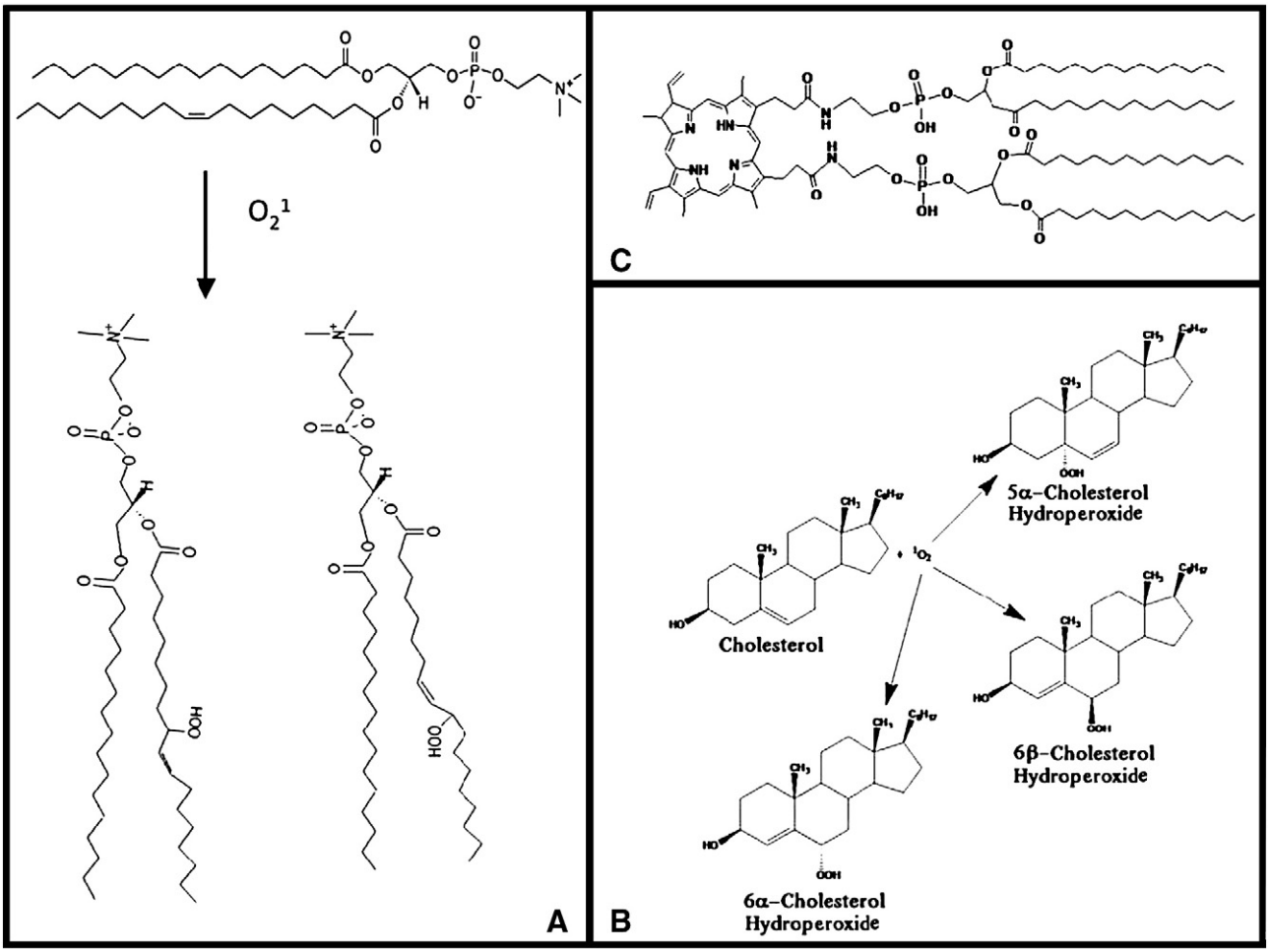

Fig. 1. Molecular structure of the hydroperoxides formed during the photo-oxidation reaction of POPC (A) and cholesterol (B) *via* singlet oxygen O_2^1. Chemical structure of the photosensitizer PE-porph, which consists of a porphyrin attached to the headgroups of two molecules of phosphatidylethanolamine (C).

with a Ph2 63× objective. Images were taken with an Axiocam HSm digital camera (Carl Zeiss). Irradiation of the PE-porph containing-samples was done using the HBO 130 W Hg lamp of the microscope using a 400-nm excitation filter. The power density of the irradiation was 5 W/cm^2, measured with a Powermeter (Coherent, Santa Clara, CA).

3. Results

In order to have a comprehensive understanding of how the *in situ* production of oxidized lipids impacts on POPC:DPPC:cholesterol membrane phase behavior, we investigated 25 different lipid compositions as indicated in the ternary diagram of Fig. 2. These compositions included some binary mixtures composed of either POPC and cholesterol, or DPPC and cholesterol, as well as DPPC and POPC, displayed in Fig. 2 by the symbols on the triangle sides. Note that, although not explicitly added in the diagram, the photosensitizer molecule, 1 mol% PE-porph, was always present, in such a way that the bottom left corner corresponds to a POPC/PE-porph vesicle whereas the bottom right corner represents a DPPC/PE-porph vesicle. The numbering on the right axis indicates the cholesterol mole fraction, X_{Ch}, in the bilayer. We mostly explored samples with fractions $X_{Ch} = 0.00$, 0.09, 0.16 and 0.23.

The lipid mixtures with POPC:DPPC ratio of 1:2 and 1:4, at zero cholesterol content, as well as the 1:4 ratio of POPC:DPPC with $X_{Ch} = 0.09$ (marked as open triangles in the ternary diagram, Fig. 2), display type II heterogeneities before irradiation [33]. This corresponds to optically visible domains due to phase separation between the gel and fluid phases, as revealed by NBD fluorescence (data not shown). For these samples, we did not study their responses to irradiation. All the other compositions led to GUVs that had initially homogeneous fluorescence, being therefore either type I mixtures with domains of nanoscopic dimensions [33,34], which could not be observed due to the optical resolution of our microscope, or truly homogeneous mixtures of the lipids. According to the ternary POPC:DPPC:cholesterol phase diagram described by Feigenson et al. [30], pure Lo mixtures of lipids were expected for cholesterol percentage above 20%.

In what follows, we first describe the photo-induced responses observed by phase contrast and fluorescence microscopy for GUVs made up of POPC:cholesterol, DPPC:cholesterol and POPC:DPPC; then we will consider the effect of the photo-oxidation on the ternary compositions.

3.1. Binary mixtures

3.1.1. POPC:cholesterol

We investigated several binary samples along the POPC-cholesterol binary side of the triangular diagram, shown as filled squares in Fig. 2 (enclosed in the region 1 in the phase diagram displayed on Fig. 3). According to previous observations [35,36], samples containing less than 10% of cholesterol (see Fig. 2) are in the Ld phase, while a Ld-Lo phase coexistence region should be expected for POPC-cholesterol samples with higher cholesterol fractions (see also Fig. 6). However, we did not notice any detectable difference in the morphology of the vesicles as observed with phase

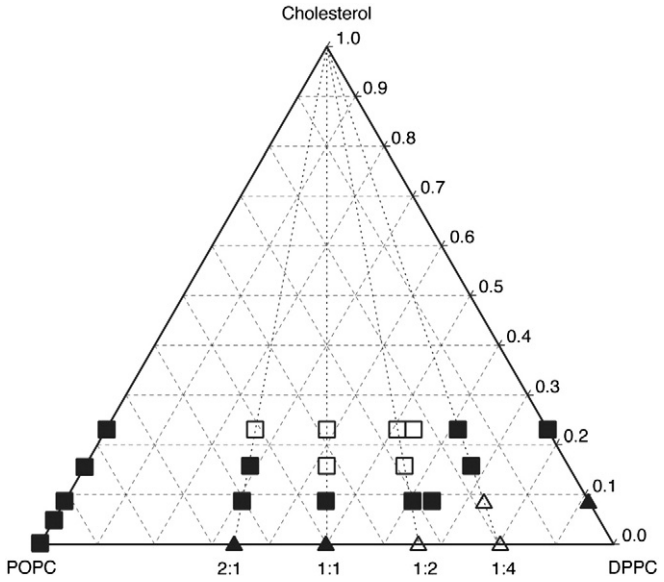

Fig. 2. Ternary diagram of samples composed of POPC:DPPC:cholesterol containing 1 mol% of PE-porph studied here. Square symbols correspond to vesicle samples that present round shapes and small undulations, typical of fluid-like membranes in the absence of light irradiation. They exhibit shape fluctuations and area increase under irradiation, except the sample of DPPC:cholesterol with $X_{ch} = 0.23$. Open symbols refer to phase separation: open triangles correspond to Ld-gel samples that phase separate following electroformation, while open squares correspond to samples evolving into Ld-Lo phase coexistence due to irradiation. Filled triangles correspond to samples that present round shapes without visible undulations before light exposition and concave deflections under irradiation.

contrast microscopy: all the GUVs were of a spherical shape, exhibiting usual small undulations, typical of fluid-like membrane features up to X_{Ch} of 0.23, in the absence of irradiation.

Under irradiation, we observed increased membrane fluctuations accompanied by morphological changes and apparent area increase similar to the effects previously reported on fluid bilayers made from mixtures of PE-porph and POPC [16]. The initial sugar asymmetry, seen by the presence of the phase contrast rings (Fig. 3), was maintained throughout the irradiation process. This fact revealed that no significant change in bilayer permeability to small molecules as sucrose and glucose occurred due to photoirradiation. In some cases, the increase in area led to the formation of macroscopic or microscopic membrane buds (Fig. 3). It has been previously shown that membrane area expansion depends on the PE-porph amount [16]. For 1 mol% of PE-porph into POPC membranes, the maximum area increased by 1% as evaluated by the changes from the spherical to prolate-shaped vesicles in the presence of an electric AC field [16]. Using the same amount of PE-porph and performing similar analysis, the maximum area increased by 1% and 1.3% in the membranes containing POPC and cholesterol with $X_{Ch} = 0.09$ and $X_{Ch} = 0.23$ (Fig. 2), respectively. Moreover, we observed that the PE-porph photobleaching became slower in the POPC:cholesterol membrane (data not shown).

The increase in area on POPC membranes was previously attributed to *in situ* formation of POPC hydroperoxide (Fig. 1A) due to the oxidative stress produced in the bilayer by the interaction between singlet oxygen and the POPC double bond [16]. Sodium azide controls were performed to make sure that the effect was in fact due to the reaction of singlet oxygen with double bonds of unsaturated lipids [14,16].

The observed increase in the PE-porph lifetime must be related to the fact that cholesterol in the phospholipid bilayers represents another target to singlet oxygen. As a consequence, cholesterol hydroperoxides (Fig. 1B) must be also formed concomitantly to POPC hydroperoxides.

3.1.2. DPPC:cholesterol

Unlike POPC, DPPC is a saturated lipid and thus it is not peroxidized by singlet oxygen. It is well known that pure DPPC at room temperature is in a gel phase [37]. In order to avoid vesicle deformation under cooling from the liquid to the gel state, after electroformation at 60 °C, we studied DPPC/PE-porph vesicles with a minimum amount of cholesterol of $X_{Ch} = 0.09$ (represented as a filled triangle in Fig. 2 and enclosed in the region sketched on the phase diagram of Fig. 4). Prior to irradiation, GUVs appeared to be of round shape, with no detectable membrane fluctuations. Under irradiation the membranes showed a qualitatively different shape evolution with respect to the fluctuating vesicles, as shown in Fig. 4. The average diameter of the vesicle changed by much less than 1% in response to irradiation; the membrane contour exhibited a systematic concave deflection with occasional flat regions (Fig. 4). For this DPPC:cholesterol binary mixture such photo-response must be related to the photo-oxidation of the cholesterol molecules (see Fig. 1B). The membrane apparent roughness is most likely related to its gel-like character [37].

As the concentration of cholesterol was increased in the lipid membrane to $X_{Ch} = 0.23$ (filled square in Fig. 2) near the Lo phase [37], the membrane again exhibited small undulations, typical of fluid-like membrane in the absence of irradiation. However, we did not detect any apparent area increase under light exposure. Therefore, this result demonstrated that hydroperoxized cholesterol

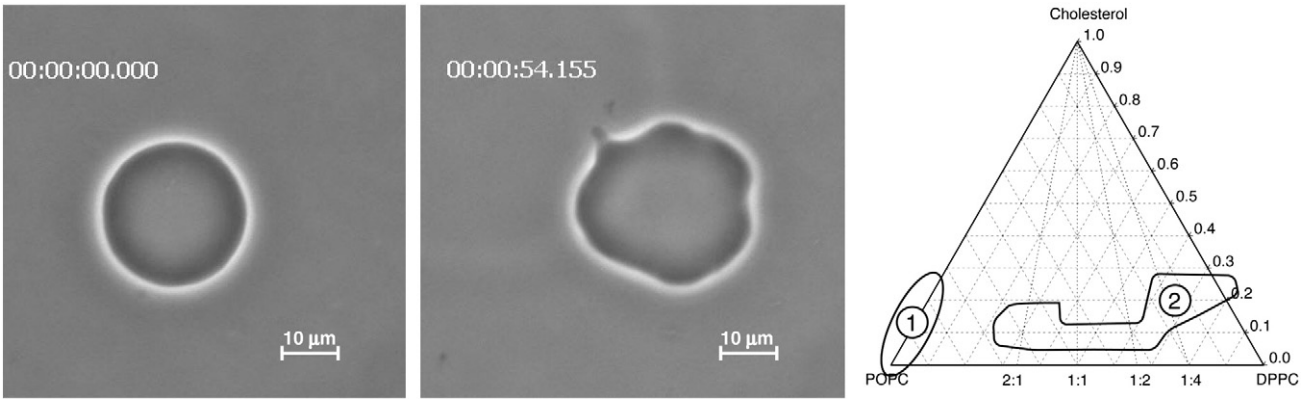

Fig. 3. GUV composed of POPC:cholesterol ($X_{Ch} = 0.09$) and 1 mol% PE-porph under irradiation. In the sequence of images, GUV responds to light exposure displaying increase in area and fluctuations in less than 1 min of irradiation, followed by emission of buds. Top left number on each image displays time in seconds. The beginning of irradiation is set at time 0. Triangle diagram on the right indicates the enclosed samples that do not phase separate under light irradiation: POPC:cholesterol samples are enclosed in region 1 whereas DPPC:cholesterol sample with $X_{Ch} = 0.23$ and part of ternary mixtures of POPC:DPPC:cholesterol (represented as filled squares in Fig. 2) are enclosed in region 2.

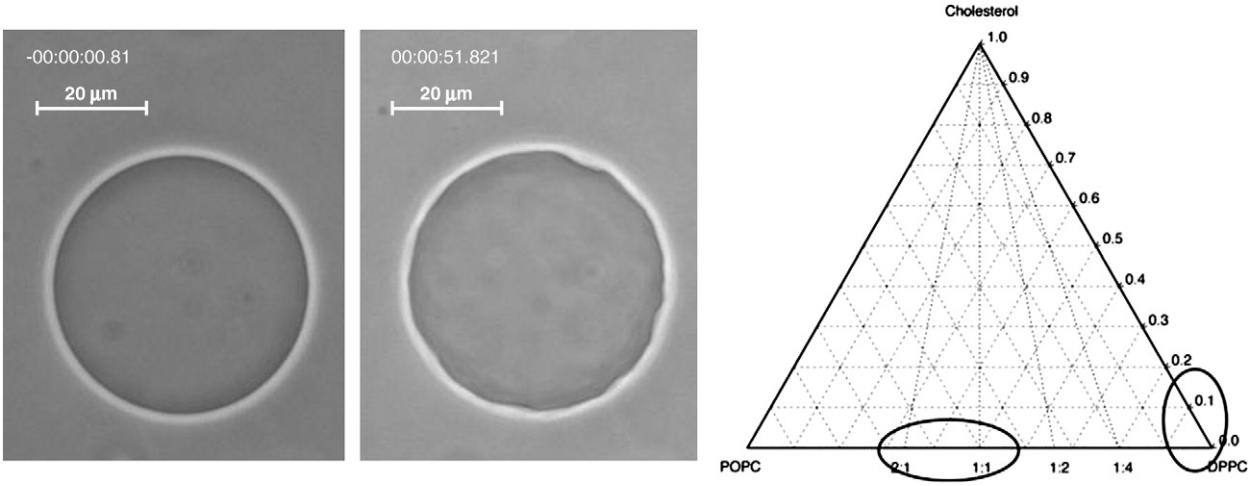

Fig. 4. GUV composed of DPPC:cholesterol (X_{Ch} = 0.09) and 1 mol % of PE-porph under irradiation. Left: before irradiation, GUV displayed round shapes with no detectable membrane fluctuations. Right: after irradiation the membrane contour exhibited concave deflections. Top left number on each image displays time in seconds. The beginning of irradiation is set at time 0. Samples composed of POPC:DPPC (2:1 and 1:1) in the enclosed regions on the phase triangle display qualitatively similar behavior.

does not cause changes in the membrane area as does POPC hydroperoxide.

3.1.3. POPC:DPPC

Along the binary line of POPC:DPPC vesicles (see Fig. 2), Ld-gel coexistence is expected [33] between 0.2 and 0.9 mole fraction of DPPC in the mixtures. GUVs made from 2:1 and 1:1 POPC:DPPC molar ratios (filled triangles in Fig. 2) exhibited an homogeneous structure prior to irradiation i.e. a spherical shape and a uniform distribution of NBD and PE-porph fluorescence. We observed little, or no membrane undulations, in these samples before illumination. Under irradiation, morphological changes presented characteristics similar to the vesicle responses described in Fig. 4 that were observed for GUVs composed of DPPC:cholesterol (X_{Ch} = 0.09). Samples displayed in the enclosed regions on the phase triangle of Fig. 4 exhibited, therefore, qualitatively similar behavior to photo-oxidation.

3.2. Ternary mixtures

We studied 13 samples with ternary compositions, as shown in Fig. 2, with X_{Ch} = 0.09, 0.16 and 0.23. All samples exhibited homogeneous fluorescence distribution under low light NBD. The effects of the irradiation on all three-component samples led initially to the response shown in Fig. 3, increasing both fluctuations and apparent surface area, with significant morphological changes for samples with greater amount of POPC. Samples represented as filled squares in Fig. 2 did not exhibit phase separation under irradiation. They are enclosed in the region 2 of the phase diagram in Fig. 3. On the other hand, vesicles with compositions shown as hollow squares in Fig. 2 displayed many small fluorophore-rich domains. These grew with time. Fig. 5 shows a typical example of such an evolution. We observed that the growing domains have a round shape, indicating Ld-Lo phase coexistence [31,38]. These phase-separated samples evolved over hours in the absence of light, and stabilized into a structure

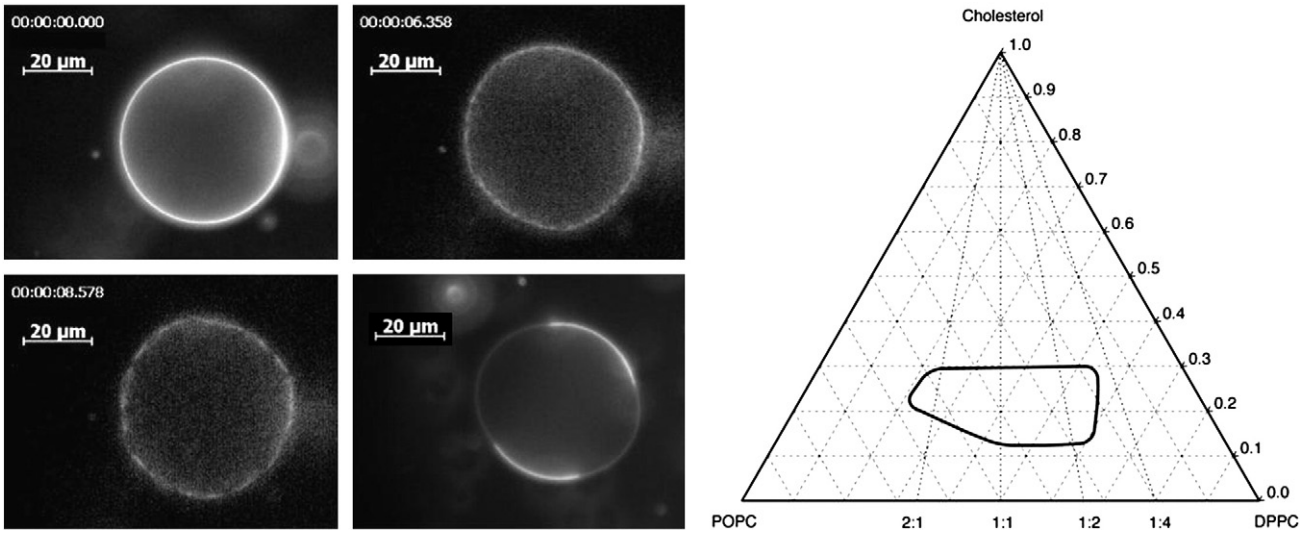

Fig. 5. Images obtained with fluorescence microscopy of the sample composed of POPC:DPPC 1:1 and X_{Ch} = 0.16 with 1 mol% of PE-porph under irradiation. Top left number on each image displays time. The beginning of irradiation is set at time 0. In the sequence of images taken from 0 to *ca* 9 s, GUV responds to light exposure displaying increase in area and fluctuations accompanied by Lo–Ld phase separation. Decreasing in fluorescence intensity with irradiation time is due to PE-porph photo-bleaching. The last snapshot corresponds to a NBD-containing membrane bilayer that evolved to two segregated fluid domains over hours after irradiation procedure. The ternary diagram displays the phase region where light-induced phase coexistence has been observed.

showing large fluid domains completely separated on the membrane (Fig. 5).

4. Discussion

Using POPC, DPPC, cholesterol and a derivative of the photosensitizer porphyrin, PE-porph, we investigated how photoinduced oxidative stresses change the phase behavior of the ternary mixture. The presence of the porphyrin photosensitizer anchored on the membrane led to singlet oxygen production under irradiation. Singlet oxygens are known to react with unsaturated lipids and cholesterol, producing lipid or cholesterol hydroperoxides.

Concerning the DPPC-cholesterol binary systems, the results showed that the membrane contour displays slightly concave deflections with $X_{Ch} = 0.09$, reflecting the cholesterol photo-oxidation, with no area increase. GUVs composed of 2:1 and 1:1 of POPC:DPPC displayed only a very small area increase with low associated fluctuations. Interestingly, for those mixtures that contained a certain amount of DPPC, the molecular changes imparted by POPC or cholesterol peroxidation resulted in moderate membrane buckling.

DPPC membranes containing a greater amount of cholesterol in Lo phase ($X_{Ch} = 0.23$) exhibited usual small undulations with undetectable area increase under irradiation. On the other hand, binary membranes composed of POPC:cholesterol displayed significant morphological changes similar to those observed from pure POPC membranes [16]. We have previously shown that POPC peroxidation, attributed to the formation of POPC hydroperoxide [16], resulted in the increase of the total membrane area. Such increase led to transitory shape fluctuations of the GUVs, and eventually to the formation of membrane buds. Taking into account the influence of cholesterol oxidation on DPPC and POPC fluid membranes observed in the current study, our results give support to conclude that the increase in membrane area from mixtures containing POPC and cholesterol is driven by POPC peroxidation up to $X_{Ch} = 0.23$. We can thus associate an area increase of 1.3% with the formation of 8.5% of POPC hydroperoxides according to Eq. 3 of ref. [16].

For the ternary mixtures made from POPC:DPPC:cholesterol, the photo-activated GUVs also exhibited increase in area and fluctuations related to POPC content. Some of the ternary compositions with the largest cholesterol content, shown as hollow squares in Fig. 2, that were initially in the homogeneous phase, displayed Lo–Ld phase separation following irradiation. This indicates a light induced phase shift of the ternary diagram phase boundaries. Fig. 6A displays a schematic phase diagram for this ternary system before irradiation, based on previous information of the phase diagrams of the binary mixtures [29,35–37] as well as on the 2-phase coexistence diagram of POPC/DPPC/Cholesterol ternary mixtures [30]. Note that in this diagram the coexistence gaps of the three binary mixtures are well established and accurately represented in the diagram. The phase boundaries

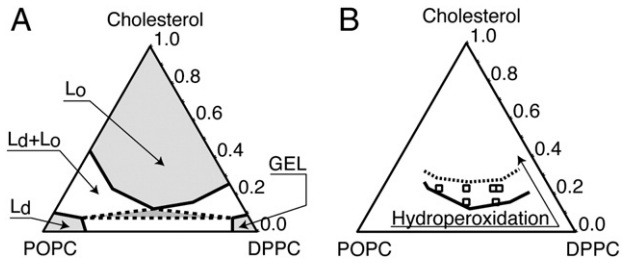

Fig. 6. A) Tentative boundaries of the two-phase (thick lines) and three-phase (dashed triangle lines) coexistence regions in the absence of photosensitizer, according to literature data. B) Sketch of the migration towards higher cholesterol content of the upper boundary in the ternary phase diagram following irradiation. Such boundary separates homogeneous and domain containing regions. Displacement of this boundary implies that samples represented as open squares (Fig. 2) originally in the homogeneous region, phase separate after lipid peroxidation.

lines however are not available from the literature; we represent here the most likely topology that is consistent with our data. We speculate that the three binary coexistence regions meet at a central ternary coexistence region. Data from Feigenson [33] are consistent with the lower POPC:DPPC two phase region, but neither the expected triangular three-phase region nor the two POPC:cholesterol and DPPC:cholesterol binary-phase regions have been experimentally determined. Other possible diagrams consistent with the three known gaps of the binary systems can be drawn, for instance by connecting two binary regions and assuming a closed third one. In any case, the molecular changes induced by hydroperoxidation transform the POPC:DPPC:cholesterol ternary diagram in the new phase diagram of the mixture POPC(POPC hydroperoxide): DPPC:cholesterol (cholesterol hydroperoxide). The combined effect of both POPC and cholesterol photo-oxidation in the phase diagram is still not clear. However, our results reveal that the boundary line separating homogeneous Lo phase and phase coexistence regions in the phase diagram is displaced vertically towards the higher cholesterol content in respect to ternary diagram of POPC:DPPC:cholesterol mixtures in the absence of oxidized species (Fig. 6).

Phase separation in binary systems has been reported with a modest addition of oxidized lipids (5–15 mol %) on lipid bilayers [23]. Such an effect was attributed to changes in lipid tail chemical structure [23] that may facilitate lipid lateral diffusion [13]. Here we show that the *in situ* generation of a small amount of hydroperoxized POPC combined with a certain amount to cholesterol (cholesterol hydroperoxide) induces Lo–Ld phase separation from initially macroscopically homogeneous phase. Molecular simulations suggest that the more hydrophilic peroxide group migrates towards the membrane surface, conferring a larger area per lipid [12,13,39]. Area increase per lipid could increase the incompatibility between the different phases and induce phase separation. Noteworthy, it has been theoretically shown that the insertion of a lipid with a more positive curvature in the Ld phase, as it could be the case of POPC hydroperoxide, increases the lateral tension and, hence, the line tension in the raft boundaries [40]. As a consequence, an ensemble of rafts initially existing in a membrane, dispersed in submicroscopic resolution sizes in the absence of lateral tension, could merge to form macroscopically observed rafts upon application of significant lateral tension [40].

Phase transitions in lipid mixtures with the same hydrophilic head are known to be driven by differences in the order state of lipid tails. Significantly, the presence of an unsaturation somewhere along the tail is enough to change the liquid–gel transition temperature and, therefore, the order state of the chain. Hydroperoxidation is most likely to have a similar effect, but no data are available to our knowledge on the liquid–gel transition of the hydroperoxidized form of POPC. This points out to the importance of establishing the phase diagrams of mixtures of controlled amount of hydroperoxidized forms of POPC with DPPC and cholesterol. Besides, information on the phase coexistence between phospholipids and oxidized forms of cholesterol is scarce in the literature. Thus, the influence of oxidation of cholesterol in the phase diagram is also an interesting challenge for future work.

Finally, it is interesting stressing that the phase separation response to oxidative stress opens a number of important pathways for biophysical studies. For instance, one could hypothesize that domain formation after oxidation might facilitate oxidized lipid detection by signaling for recovery or elimination by the cell.

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